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Optimization of enzymolysis-ultrasonic assisted extraction of polysaccharides from *Momordica charabtia* L. by response surface methodology

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ABSTRACT

An efficient enzymolysis-ultrasonic assisted extraction (EUAE) was developed and optimized for the extraction of polysaccharide from *Momordica charabtia* L. The single factor experiments and orthogonal experiments were used for the key experimental factors and their test range. Based on the preliminary experimental results, the response surface methodology (RSM) and Box–Behnken design (BBD) were applied for the optimization of EUAE conditions. Using the multiple regression analysis and analysis of variance (ANOVA), the experimental data were fitted to a second-order polynomial equation and were used to generate the mathematical model of optimization experiments. The optimal extraction conditions were as follows: a pH of 4.38, a extraction temperature of 52.02 °C and a extraction time of 36.87 min. Under the optimal extraction conditions, the extraction yield of *Momordica charabtia* L. polysaccharides (MCP) was 29.75 ± 0.48%, which was well matched with the predicted value (29.80%) of the BBD model.

1. Introduction

Momordica charantia L., a favorable vegetable crop, belongs to the Cucurbitaceae family. The crop Called "kugua" in Chinese is well known as bitter gourd, bitter melon, balsam pear and Karela in English (Gupta et al., 2010). It has been widely planted in different regions including Amazon, East Africa, Asia, and the Caribbean (Grover & Yadav, 2004). In addition to one kind of nourishing food, *Momordica charantia* L. has been also used as folk medicine to prevent and treat fever polydipsia, diarrhea, colic and diabetes in China (Shih, Lin, & Lin, 2008). Modern researches have demonstrated that its anti-diabetic efficacy derives from its polysaccharides, which can effectively reduce the glucose levels of blood (Zhou et al., 2013).

Due to this, more and more attentions have been paid to the extraction of polysaccharides from plants. Considering the good water solubility of polysaccharides, its extraction method always follows the hot water extraction (Yin & Dang, 2008). However, this extraction method has some obvious shortcomings such as high extraction temperature, large energy consumption, long extraction time, and low extraction yield (Yin, You, & Jiang, 2011). Subsequently, ultrasonic-assisted extraction and microwave-assisted extraction were used as the extraction method alternative to the extraction of polysaccharides (Yan et al., 2011; Wang et al., 2010), which can greatly accelerate the extraction speed. Recently, there appears another extraction method termed enzyme-assisted extraction. In this extraction procedure, enzymes can effectively catalyze the degradation of cell walls to cause the release of polysaccharides contained inside plant cells (Zhu et al., 2014). Therefore, enzyme-assisted extraction has the advantage of being environment-friendly, highly efficient, and easily operated over the ordinary extraction methods (Zhang et al., 2013).

RSM has been widely used as a collection of statistical and mathematical techniques for the test of process factors and their reciprocal interaction (Chandra, Barata, Ferreira-Dias, Malfeito-Ferreira, & Loureiro, 2014; Yolmeh, Najafi, & Farhoosh, 2014; Elksibi, Haddar, Ticha, Gharbi, & Mhenni, 2014). By means of the empirical statistics analysis, RSM not only greatly reduces the number of experimental trials needed to optimize the extraction conditions, but also generates a mathematical model to explain the reciprocal interaction of experimental factors and obtain the optimal values (Wang, Dong, & Tong, 2013). BBD, one type of RSM, is more efficient and easier to arrange and interpret the optimization experiments compared with other methods (Wu et al., 2013). It has been widely used to optimize various biochemical processes.

A few researches have been reported for the enzyme-assisted extraction of polysaccharides from *Momordica charantia* L. (Zhang et al., 2010; Wu, Kan, Chen, & Li, 2005). However, these extraction procedures only use cellulose and trypsin, which can not effectively

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Table 1
Results and analysis of orthogonal experiments.

No.	Enzyme concentration (%)	pH	Extraction temperature(°C)	Extraction time (min)	Yield of MCP (%)
1	1.5	3.0	40	30	25.79
2	1.5	4.0	50	40	28.51
3	1.5	5.0	60	50	27.64
4	2.0	3.0	50	50	27.18
5	2.0	4.0	60	30	28.25
6	2.0	5.0	40	40	26.73
7	2.5	3.0	60	40	26.56
8	2.5	4.0	40	50	26.21
9	2.5	5.0	50	30	28.92
K_1	27.31	26.51	26.24	27.65	
K_2	27.39	27.66	28.84	27.77	
K_3	27.23	27.76	28.20	27.01	
R^*	0.16	1.25	1.96	0.76	

* *R* refers to the result of extreme analysis.

destroy the pectin of cell wall to release the MCP. The objective of this study was to investigate the simultaneous application of cellulose, pectinase and trypsin in the extraction of MCP with the ultrasonic wave technology. For this aim, single factor experiment, orthogonal test design and BBD were used for the optimization of the EUAE procedure.

2. Materials and methods

2.1. Materials

The fruit of *Momordica charantia* L. was purchased from the local market (Shunyu vegetable market, Jinan, China). The cellulose, pectinase and trypsin were bought from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), whose product batch numbers were 64001132, 64007033 and 64008860, respectively. All solvents and chemicals were at least of analytical grade and obtained from Tianjin Kermel Chemical reagent Co., Ltd. (Tianjin, China). Doubly-distilled water was used throughout the whole experiment.

2.2. Optimization of EUAE of MCP

2.2.1. EUAE procedure

Fresh fruit of *Momordica charantia* L. was dried, smashed and homogenized to prepare the experimental sample. The sample power was defatted with petroleum ether (boiling point: 60–90 °C) and then pretreated with 80% ethanol twice to remove some colored materials, monosaccharides, oligosaccharides, and some small molecule materials. The purified sample power was then filtrated and dried for the following EUAE.

5.0 g pretreated sample was extracted with 150 mL complex enzyme solution (the ratio of cellulose: pectase: trypsin was 2:2:1) in a SB-3200DT ultrasounic cleaner (Ningbo Scientz Biotechnology Co., Ltd, Zhejiang, China). The extraction temperature and extraction time of ultrasound cleaner were set according to the corresponding experimental design. After three replications, the extracts were incorporated and concentrated to one-fifth of the initial volume using a rotary evaporator at 65 °C under vacuum. The concentrate was precipitated with the addition of anhydrous ethanol to a final concentration of 80% (v/v) and incubated for 12 h at 4 °C in refrigerator. The precipitant was collected and dried to obtain the crude MCP. The content of MCP was measured by the common phenol-sulfuric acid method (Masuko et al., 2005), which used D-glucose as the standard substance to construct a standard curve. The extraction yield of MCP (%) was calculated as follows:

$$\text{MCP yields} \quad (\%) = \frac{\text{weight of MCP(g)}}{\text{weight of sample(g)}} \times 100\% \tag{1}$$

2.2.2. Hot water extraction procedure

Hot water extraction of CMP was also done to serve as the reference extraction method. The pretreated sample power was immersed in 150 mL doubly-distilled water and extracted in boiling water for 3 h. After three replications, the extracts were treated as the operations mentioned above.

2.2.3. Single factor experimental design

Single factor experiments were carried out to study the effect of complex enzyme concentration, pH, extraction temperature and extraction time on the EUAE yield of MCP. During the optimization of experimental factors, one factor was changed while the other factors were kept constant in each experiment. All the experiments were repeated three times.

2.2.4. Orthogonal test design

The preliminary study was used for the selection of key experimental factors and test ranges values. An orthogonal L_9 (3^4) test design with 4 factors and 3 levels was established on the basis of single factor experiments. As shown in Table 1, nine experiments were conducted according to Section 2.2.1, and each experiment was performed in triplicate.

2.2.5. BBD and statistical analysis

Based on the preliminary orthogonal test design, BBD with three factors and three levels was used for the further optimization of EUAE conditions. Experimental factors including pH, extraction temperature and extraction time were chosen as three independent variables and their range values were based on the results of preliminary experiments. The independent variables were designated as X_1, X_2, and X_3, respectively. The complete quadratic equation reflecting the effect of three independent variables on the extraction yield of MCP was shown as follows:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i X_i + \sum_{i=1}^{3}\beta_{ii} X_i^2 + \sum_{i=1}^{3}\sum_{j=i+1}^{3}\beta_{ij} X_i X_j \tag{2}$$

where Y is the response variable; β_0, β_i, β_{ii}, and β_{ij} are the regression coefficients for intercept, linearity, square, and interaction, respectively; X_i and X_j are the different independent variables ($i \neq j$).

To deduce the quadratic Eq. (2), seventeen factorial points and five replicates of the central points was designed by BBD and shown in Table 2. All trials were performed in triplicate. The statistical analyses of data were also carried out using BBD to establish the mathematical model, interpret the interaction and estimate the response of the independent variables. Subsequently, three additional confirmation experiments were carried out to verify the validity of the statistical experimental strategies.

Table 2
BBD matrix and response values for the extraction yield of MCP.

No.	pH	Extraction temperature (°C)	Extraction time (min)	Yield of MCP (%)
1	3.00	40.00	40.00	25.87
2	3.00	60.00	40.00	26.48
3	3.00	50.00	30.00	27.21
4	3.00	50.00	50.00	27.06
5	5.00	40.00	40.00	26.69
6	5.00	60.00	40.00	28.41
7	5.00	50.00	30.00	28.89
8	5.00	50.00	50.00	27.53
9	4.00	40.00	30.00	26.81
10	4.00	60.00	30.00	28.05
11	4.00	40.00	50.00	26.13
12	4.00	60.00	50.00	26.90
13	4.00	50.00	40.00	29.58
14	4.00	50.00	40.00	29.63
15	4.00	50.00	40.00	29.44
16	4.00	50.00	40.00	29.53
17	4.00	50.00	40.00	29.62

3. Results and discussion

3.1. Single factor experimental analysis of EUAE

3.1.1. Effect of enzyme concentration on the extraction yield of MCP

The amount of enzymes is an important factor influencing the extraction yield. Seen from Fig. 1(A), the initial increase of enzyme concentration resulted in an obvious extraction yield increase of MCP while the further increase of enzyme concentration almost had no obvious effect on the extraction yield change of MCP. This indicated that the complex enzyme amount of 2% (w/v) was sufficient to obtain high polysaccharides yield. Taking the consumption of enzyme and the extraction yield of MCP, the enzyme concentration of 2.0% was high enough for the EUAE.

3.1.2. Effect of pH on the extraction yield of MCP

As enzyme belongs to bioactive substances, its conformation and activity seriously depend on the pH of enzyme solution (Zhu et al., 2014). Due to this, it is important to select a suitable pH to ensure its enzyme activity. As shown in Fig. 1(B), the extraction yield of MCP firstly increased with the increase of pH and then decreased with the further increase of pH. The extraction yield decrease of MCP is due to low enzyme activities resulted from the inappropriate pH value. Thus the pH about 4.0 was the best choice in the present work.

3.1.3. Effect of extraction temperature on the extraction yield of MCP

The enzyme activity not only depends on the pH, but also relies on the extraction temperature. The effect of extraction temperature on the extraction yield of MCP was studied and the results were shown in Fig. 1(C). The extraction yield of MCP increased over the extraction temperature range of 30–50 °C. When the extraction temperature was higher than 50 °C, the extraction yields of MCP kept on decreasing, which can be explained by the fact that high extraction temperature always results in the increase of enzyme activity (Yin et al., 2011). Therefore, the extraction temperature of 50 °C was chosen for the EUAE.

3.1.4. Effect of enzymatic extraction time on the extraction yield of MCP

Generally speaking, a long extraction time is in favor of the yield of polysaccharides (Chen, Tang, Chen, Wang, & Li, 2009). The effect of extraction time on extraction yield of MCP was showed

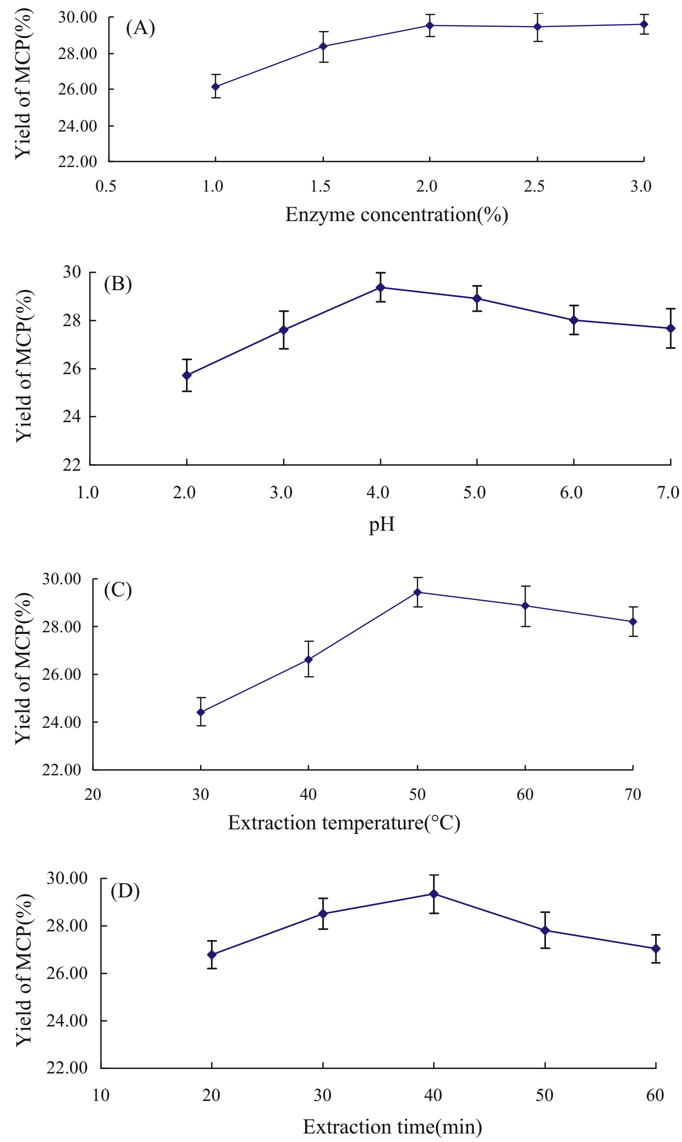

Fig. 1. Effect of different extraction factors ((A) enzyme concentration, %; (B) pH; (C) extraction temperature, °C, and (D) extraction time, min) on extraction yield of MCP. The effects of enzyme concentration, pH, extraction temperature, and extraction time were first studied by a single-factor design 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 MCP.

in Fig. 1(D). The first increase of extraction time resulted in an extraction yield increase of MCP and then the further increase of extraction time led to an extraction yield decrease of MCP. This phenomenon can be attributed to the polysaccharide hydrolysis under high temperature and long extraction time (Liu, Miao, Wen, & Sun, 2009). As a result of this, 40 min was an appropriate extraction time for the EUAE.

According to the single factor study, the following conditions could be used for the following experiments: a complex enzyme concentration of 1.5–2.5% (w/v), a pH of 3.0–5.0, an extraction temperature of 40–60 °C, and an extraction time of 30–50 min.

3.2. Orthogonal analysis of enzyme-assisted extraction

The extraction yields of MCP obtained from orthogonal experiments were listed in Table 2. The maximum extraction yield of 28.92 ± 0.65% was achieved at the extraction conditions including an enzyme concentration of 2.5%, a pH of 5.0, a temperature of

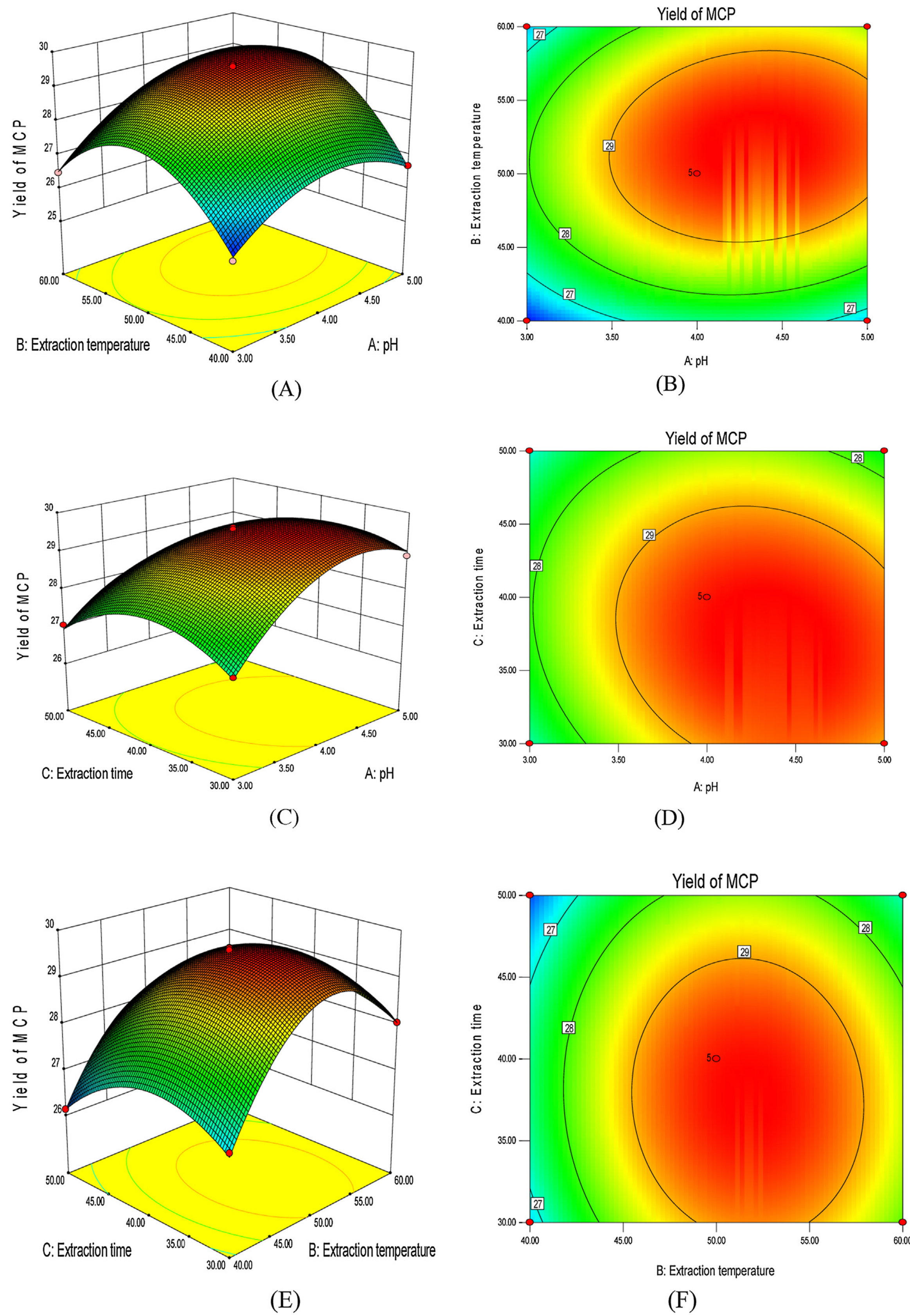

Fig. 2. Response surface plots (A, C, and E) and contour plots (B, D, and F) showing the interactive effects of pH (X_1), extraction temperature (X_2), and extraction time (X_3) on the extraction yield of MCP.

Table 3
Analysis of the variance (ANOVA) for the second-order polynomial model.

Source	Sum of squares	df	Mean square	F-value	Prob > F
Model	29.20	9	3.24	239.55	<0.0001
X_1	3.00	1	3.00	221.61	<0.0001
X_2	2.35	1	2.35	173.85	<0.0001
X_3	1.39	1	1.39	102.97	<0.0001
X_1X_2	0.31	1	0.31	22.74	0.0020
X_1X_3	0.37	1	0.37	27.03	0.0013
X_2X_3	0.055	1	0.055	4.08	0.0832
X_1^2	4.20	1	4.20	310.13	<0.0001
X_2^2	12.15	1	12.15	897.19	<0.0001
X_3^2	3.33	1	3.33	245.58	<0.0001
Residual	0.095	7	0.014		
Lack of fit	0.071	3	0.024	3.89	0.1112
Pure error	0.024	4	6.050E − 003		
Cor total	29.29	16			
	R^2 = 0.9968	$R_{Adj}{}^2$ = 0.9926	C.V.% = 0.43		

50 °C and a time of 30 min. That is, the extraction condition was the combination of experimental factors termed $A_3B_3C_2D_1$ (1–3 are the levels of experimental factors). By means of the comparison of R values obtained from the extreme difference analysis, the effect order of experimental factors on the extraction yields was C > B > D > A (extraction temperature > pH > extraction time > enzyme concentration). As the effect of enzyme concentration on the extraction yield of MCP was the smallest, three experimental factors (B, C and D) were investigated in the following BBD while the enzyme concentration was 2.5% (w/v).

3.3. BBD and analysis

3.3.1. Statistical analysis and the model fitting

BBD with three factors and three levels were carried out to optimize the mutual effect of three independent variables (pH, extraction temperature, and extraction time) on the extraction yield of MCP. Table 2 showed the design matrix and the extraction yields of MCP. After these data were deal with the BBD, a second-order polynomial equation reflecting the empirical relationship between response variable and the independent variables was presented as follows:

$$Y = 29.56 + 0.61X_1 + 0.54X_2 - 0.42X_3 + 0.28X_1X_2 - 0.30X_1X_3 - 0.12X_2X_3 - 1.00X_1^2 - 1.70X_2^2 - 0.89X_3^2 \quad (3)$$

The analysis of variance, goodness-of-fit and the adequacy of the regression model were summarized in Table 3. The model F-value was 239.55 and the model p-value was smaller than 0.0001, meaning that the model was highly statistically significant. The F-value and p-value of the lack of fit were 3.89 and 0.1112, which confirmed the goodness-of-fit and suitability of regression model. The determination coefficient (R^2 = 0.9968) indicated that only 0.32% of the total variation was not explained by the model. The adjusted determination coefficient ($R_{Adj}{}^2$ = 0.9926) further testified the minute difference between the experimental values and predicted values. The low coefficient variation value (C.V.% = 0.42) clearly revealed that the experimental values of regression model was precise and reliable. Table 3 also demonstrated that the linear coefficients (X_1, X_2 and X_3), quadratic term coefficients (X_1^2, X_2^2 and X_3^2) and cross product coefficients (X_1X_2 and X_1X_3) were significant ($p < 0.05$) while the other terms' coefficients were not significant ($p > 0.05$).

3.3.2. Optimization of the EUAE procedure

The 3D response surface and 2D contour plots drawn by Design BBD were shown in Fig. 2. Fig. 2 described the regression Eq. (2) by means of graphical approach, which provided a method to visualize the mutual effect of test variables at different levels on the extraction yield and the reciprocal interactions between test variables. That is, 3D response surface plot illustrated the mutual influences on the extraction yield of MCP while 2D contour plot interpreted the reciprocal interactions between test variables. The circular contour plots indicate the negligible interactions between the corresponding variables, while an elliptical contour plots indicate the significant interactions between the corresponding variables (Guo, Zou, & Sun, 2010). Fig. 2(A) and (B) showed the pH (X_1) and extraction temperature (X_2) both imposed significant effects on the extraction yield. Fig. 2(C) and (D) showed that pH (X_1) exhibited a significant effect whereas extraction time (X_3) exerted a weaker effect on the polysaccharides yield. Similar to Fig. 2(C) and (D), extraction temperature (X_2) was significant to polysaccharides yield while extraction time (X_3) was insignificant to the polysaccharides yield, which was shown in Fig. 2(E) and (F).

3.3.3. Verification of predictive model

According to Fig. 2, it could be deduced that the optimal EUAE conditions are as follows: pH (X_1) 4.38, extraction temperature (X_2) 52.02 °C, and extraction time (X_3) 36.87 min. Under these optimal EUAE conditions, a maximum response of 29.80% was obtained by the mathematical prediction of the model. To confirm the suitability of the model equations, triplicate confirmatory experiments were carried out under the optimal EUAE conditions. The average yield of MCP was 29.75 ± 0.48%, which was higher than that of hot water extraction yield of 22.32% (33.29% enhanced). Therefore, the model was suitable for the optimization of EUAE process.

4. Conclusion

The EUAE was performed for the extraction of polysaccharides from *Momordica charabtia* L. Based on the single factor experiments and orthogonal experiments, BBD was used to further optimize the EUAE conditions including pH, temperature and extraction time. The optimal conditions were as the following: a pH of 3.87, an extraction temperature of 52.02 °C, and an extraction time of 36.87 min. Under these extraction conditions, the experimental yield of polysaccharides was 29.75 ± 0.48%, which was close with the predicted yield value of 29.80%. Therefore, the model established by BBD was suitable for the optimization of the EUAE conditions.

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