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Application of Box-Behnken design in optimization for crude polysaccharides from fruits *of Tribulus terristris L.*

Guowei Shu*, Chunji Dai, He Chen and Xu Wang

College of Life Science and Engineering, Shaanxi University of Science and Technology, Xi'an, China

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ABSTRACT

In this work, Box-Behnken design (BBD) was used to study the effect of liquid: solid ratio, cellulase concentration and Reaction time on the extraction of Jili polysaccharide from fruits of Tribulus terrestris L. Regression analysis was performed on the data obtained. The most relevant variable was solid: liquid ratio. The coefficient determination (R^2) was good for the second-order model. Liquid: solid ratio of 24:1 (v/w), cellulase concentration of 2.0% and reaction time of 2.90h were found to be optimal for crude polysaccharides extraction from fruits of Tribulus terrestris L. By means of additional experiments, the adequacy of this model is confirmed.

Keywords: Box-Behnken design; extraction; *Tribulus terrestris L.*; polysaccharide

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INTRODUCTION

Tribulus terrestris L. is an annual herb that grows worldwide, especially in the subtropical area of Asia, Africa, Europe, America and Australia [1-3]. The fruits of *T. terrestris* known to the Chinese as "Jili" are allowed to use in functional food by the Ministry of Health of China and used in the folk medicine in India, China, Bulgaria and other countries against various diseases, including impotence tonic, cardiovascular diseases, anti-hypertensive urinary anti-infective, anti-inammatory and immunosuppressive activities [4-7]. The occurrence of saponins, polysaccharides, alkaloids, amides, flavonoids and cinammic acid has been reported in Jili [8-11].

Recently polysaccharides extracted from plants have drawn the attention of researchers and consumers due to their anti-tumor, antioxidant and immunomodulating agent [12-16]. However, few researches have been carried out extraction of polysaccharides from Jili.

The response surface methodology (RSM) is a collection of mathematical and statistical techniques for designing experiments, building models, evaluating the effects of several factors and obtaining optimum condition of factors for desirable responses [17-19], which provides the relationship between one or more measured dependent responses and a number of input factors. It has some advantages that include a less number of experiments; suitability for multiple variables can reveals possible interactions between variable, search for relativity between multiple variables and finding of the most suitable correlation and forecast response. In many cases, a second-order model is easy to estimate the parameters due to its flexibility and it works well in solving real response problems. Therefore, the second-order model is widely used in RSM. The most common designs, that is central composite design (CCD) [20] and Box-Behnken experimental design (BBD), of the principal response surface methodology have been widely used in various experiments. Box-Behnken design, a spherical and revolving design, has been applied in optimization of media, Extraction of natural active substances and other uses [21-28] because of its reasonable design and excellent outcomes.

In our previous research, we found that the hot water extracts from Jili can promote the growth of *L.acidophilus*, *L.bulgaricus, L. casei, L.reuteri, L.rhamnosus* [29-31]. The purpose of the present work was to optimize and to study the effect of liquid: solid ratio, cellulase concentration and reaction time on the production of Jili crude polysaccharides by BBD.

EXPERIMENTAL SECTION

2.1 Materials

Dried Jili were purchased from a local herb market (Xi'an, China). Cellulase(5ku/g) was purchased from Zhaodong Sun Shine Enzyme Co., Ltd (Zhaodong City Heilongjiang Province, China). All chemicals used were reagent grade unless otherwise specified.

2.2. Extraction of Jili crude polysaccharides

Dried Jili were ground in a high speed disintegrator (Model SF-2000, Chinese Traditional Medicine Machine Works, Shanghai, China) to obtain a fine powder, then was extracted with 95% ethanol at 50 for 6h and dried. The pretreated dry powder (25.0 g) extracted with distilled water (liquid: solid ratio (ml/g) ranging from 15:1 to 25:1) at pH 5.0 (adjusting the suspension pH by 0.1 mol/l NaOH or HCl), then cellulase powder (concentration (%, g/g pretreated dry powder of Jili) ranging from 1.5 to 2.5) was added to the mixture. while the temperature (55) of the water bath was kept steady, The extraction in a 1.0 l stainless steel boiler in the water bath was stirred with an electric mixing paddle for a given time (reaction time ranging from 1.5 to 3.5 h), The mixture was centrifuged (3000g, 15 min), then the supernatant was separated from insoluble residue with nylon cloth, The supernatant was collected for the determination of polysaccharides yield.

2.3. Determining content of glucose

The sugar content was determined by the reaction of sugars with phenol in the presence of sulfuric acid using glucose as a standard [32]. One milliliter of the supernatant for each treatment was accurately taken and filled into a 10 ml cuvette. Then 0.5 ml, 6% phenol was added into the cuvette and shaken-up. Then 2.5 ml sulfuric acid l was filled into the mixture in cuvette, shaken-up and stands for 30 min at room temperature. At last, absorbance values were recorded by a 722 spectrophotometer at the wavelength of 490 nm. At the same time, the wash solution was measured as blank control in an identical way.

2.4. Determination of polysaccharides yield

The percentage polysaccharides yield (%) is calculated as the Jili polysaccharides content of extraction divided by dried sample weight.

2.5. Experimental design

After determining the preliminary range of the extraction variables through single-factor test, a Box and Behnken experimental design, with three variables, was used to study the response pattern and to determine the optimum combination of variables. The effect of the variables X_1 (Liquid: solid ratio), X_2 (Cellulase concentration) and X_3 (Reaction time), at three variation levels (Table 1) in the extraction process [33], is shown in Table 2. Three replicates (runs 13-15) at the centre of the design were used to allow for estimation of a pure error sum of squares. Experiments were randomized in order to maximize the effects of unexplained variability in the observed responses due to extraneous factors.

Table 1 Variable value of the process and their corresponding levels

Variables	Symbol		Levels		
	Uncoded	Coded	-1	0	+1
Solid : liquid ratio (w/v)	X_1	x_1	15:1	20:1	25:1
Cellulase concentration(%,w/w)	X_2	x_2	1.5	2.0	2.5
Reaction time (h)	X_3	x_3	1.5	2.5	3.5

The variables were coded according to the following equation:

$$x_i=(X_i-X)/\Delta X_i,\ i=1,2,3 \tag{1}$$

Where x_i and X_i are the dimensionless and the actual values of the variable i, X the actual value of the variable i at the central point, andΔX_i the step change of X_i corresponding to a unit variation of the dimensionless value.

2.4. Statistical analyses

The experimental data were fitted in accordance to Eq. (2) as a second-order polynomial equation including the linear and interaction effects of each variable:

$$Y_i = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x_j^{\,2} + \sum \sum_{i<j} \beta_{ij} x_i x_j \quad (2)$$

Where Y_i= predicted response, β_0 = offset term, β_j = linear effect, β_{jj} = squared effect and β_{ij}=interaction effect. Regression coefficient, response surface and contour plots were analyzed and plotted by Design Expert 7.1 for windows version and Sigmaplot 11.0.

Table 2 Box-Behncken designs with experimental responses and predicted values for Jili crude polysaccharides yield

RUN	Variable levels			Experimental			Predicted (Y_i)	Y_0-Y_i
	x_1	x_2	x_3	I	II	Average (Y_0)		
1	-1	-1	0	1.49	1.43	1.46	1.44	0.02
2	1	-1	0	4.18	4.19	4.19	4.14	0.05
3	-1	1	0	2.5	2.61	2.56	2.61	-0.05
4	1	1	0	3.9	3.94	3.92	3.95	-0.03
5	-1	0	-1	1.83	1.75	1.79	1.70	0.09
6	1	0	-1	3.93	4.04	3.99	3.92	0.07
7	-1	0	1	2.11	2.2	2.16	2.23	-0.07
8	1	0	1	3.82	4.09	3.96	4.05	-0.09
9	0	-1	-1	3.13	3.02	3.08	3.19	-0.11
11	0	1	-1	3.61	3.48	3.55	3.59	-0.04
10	0	-1	1	3.49	3.44	3.47	3.43	0.04
12	0	1	1	4.13	4.11	4.12	4.01	0.11
13	0	0	0	3.68	3.74	3.71	3.74	-0.03
14	0	0	0	3.75	3.81	3.78	3.74	0.04
15	0	0	0	3.71	3.74	3.73	3.74	-0.01

R=0.9971; R^2=0.9943;Adj R^2 = 0.9842

The proportion of variance explained by the polynomial models obtained is given by the multiple coefficient of determination, R^2. The significance of each coefficient was determined using the F and p value. The behavior of the surface was investigated for the response function (Y_i) = g polysaccharide/100 g Jili, using the regression equation (3). Further, in order to deduce workable optimum conditions, a graphical technique was used by fixing one variable at a predetermined optimum condition. The optimum condition was verified by conducting experiments under these conditions. Responses were monitored and results compared with model predictions.

RESULTS AND DISCUSSION

3.1. Fitting the models

The application of BBD generated the following regression equation, which was an empirical relationship between Jili polysaccharides yield and the test variable in coded units, as given in the following equation:

$$Y_i=3.7400+1.0113x_1+0.2438x_2+0.1625x_3-0.3425x_1x_2-0.1000x_1x_3+0.045x_2x_3-0.6438x_1^2-0.0638x_2^2-0.1213x_3^2 \quad (3)$$

Each of the experimental values, Y_0, was compared with the predicted value, Y_i calculated from the model, as depicted in Fig.1. We could see that Y_0 accords with Y_i. The correlation measure for the estimation of the regression equation was the determination coefficient (R^2=0.9943). The determination coefficient,which was a measure of the goodness of fit of the model, indicated that only 0.57% of the total variations were not explained by the model.

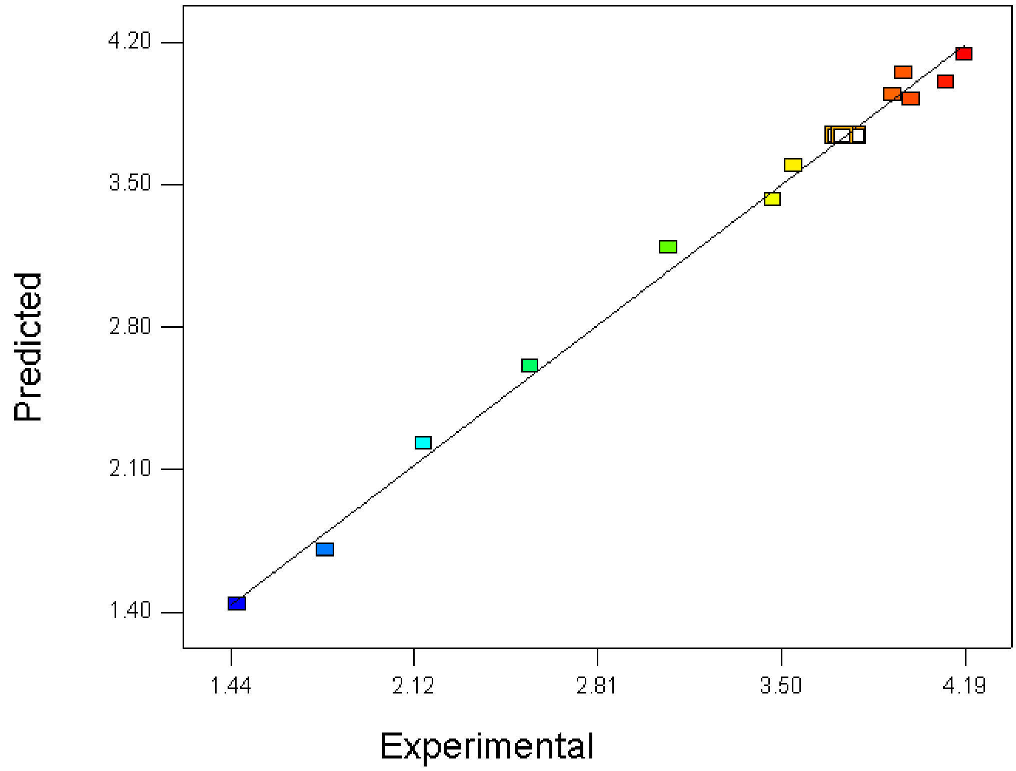


Fig.1. Comparison between predicted and experimental Jili polysaccharides yield

The regression coefficients for a polynomial model able to predict and quantify the Jili polysaccharides yield were estimated. The significance of each coefficient was determined using Fisher's F-test and p value in Table 3.The corresponding variables would be more significant if the absolute F value became larger and the p-value becomes smaller. The analysis of variance of the quadratic regression model (Table 3) demonstrated that this model was highly significant,as was evident from the Fisher *F*-test (F_{model}=97.7033) and from the very low probability value (P<0.0001). It could be seen that the variable with the largest effect was the linear term of liquid:solid ratio (x_1), followed by the quadratic of liquid:solid ratio (x_1^2), the linear term of cellulase concentrarion (x_2),and the interaction effect of liquid:solid ratio and cellulase concentration (x_1x_2). The variable F value (657.9021) and p value (p<0.0001) corresponds to x_1, while the F value for x_1^2, x_2 and x_1x_2 were smaller at 123.0514,38.2238 and 37.7342, respectively, but the p values were still significant at p = 0.0001,0.0016 and 0.0017.

Table 3 Significance of model and regression coefficient for Jili polysaccharide yield

Variables	Regression coefficient	Standard error	F value	Prob > F
Model			97.7033	<0.0001
Intercept	3.7400	0.0644	-	-
x_1	1.0113	0.0394	657.9021	<0.0001
x_2	0.2438	0.0394	38.2238	0.0016
x_3	0.1625	0.0394	16.9883	0.0092
x_1x_2	-0.3425	0.0558	37.7342	0.0017
x_1x_3	-0.1000	0.0558	3.2167	0.1329
x_2x_3	0.0450	0.0558	0.6514	0.4563
x_1^2	-0.6438	0.0580	123.0514	0.0001
x_2^2	-0.0638	0.0580	1.2067	0.3220
x_3^2	-0.1213	0.0580	4.3653	0.0910

3.2. Optimization of the process

The relationship between independent and dependent variables was illustrated by the 3-D representation of the response surfaces and the 2-D contours generated by the model. Two variables within the experimental range were depicted in 3-D surface plots when the third variable was kept constant at fixed level. The shapes of the contour plots, elliptical or circular, indicated whether the interactions between the corresponding variables were significant or not Circular contour plot indicated that the interactions between the corresponding variables were negligible, while elliptical contour plot indicated that the interactions between the corresponding variables were significant [34,35]. In the present study, three independent response surface plots and their respective contour plots were generated using Sigmaplot 11.0 as shown in Figs. 2-4. It was clear that the Jili polysaccharides yield was sensitive to minor alterations of the test variables (liquid: solid ratio, cellulase concentration and reaction time).

(b)

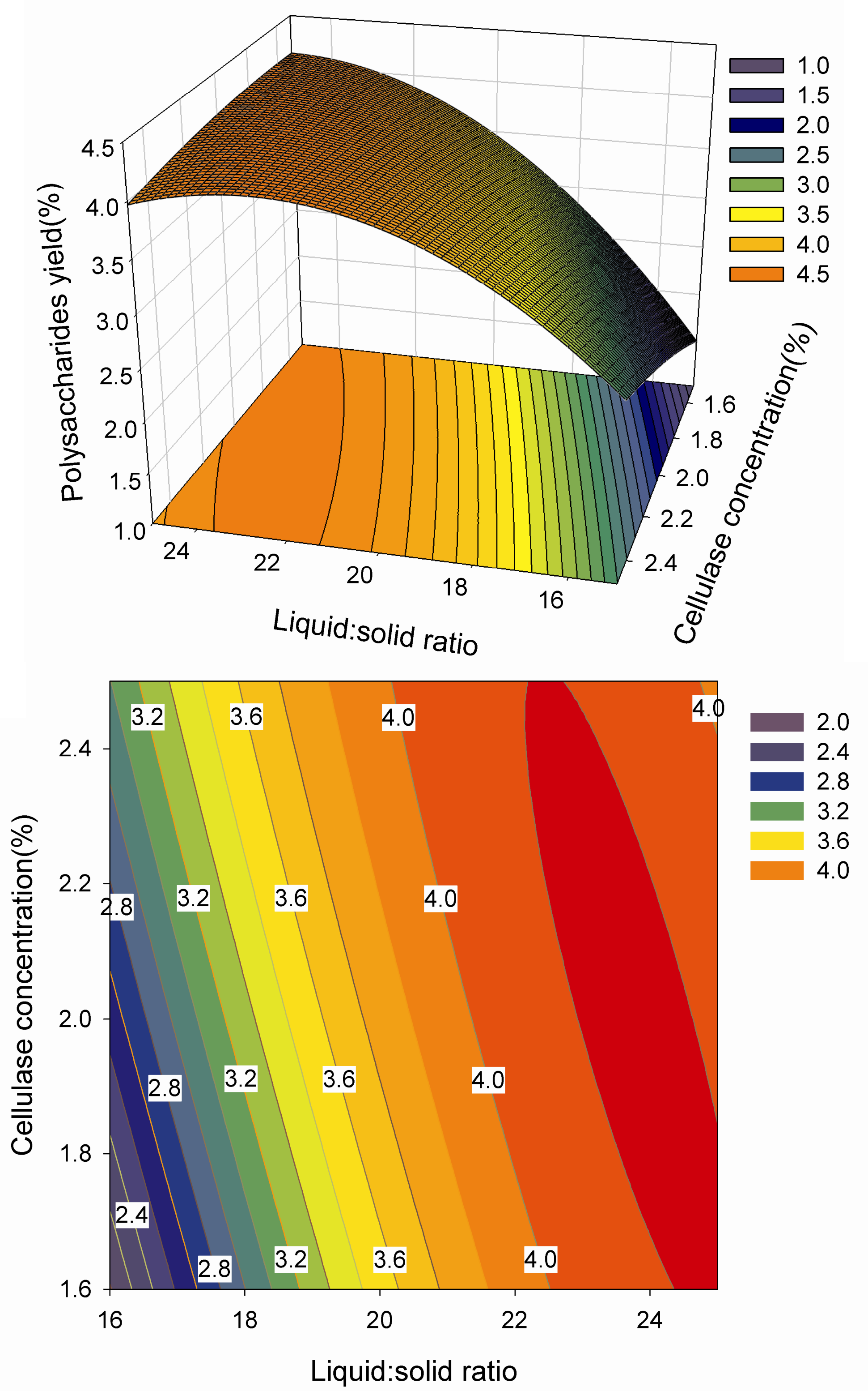
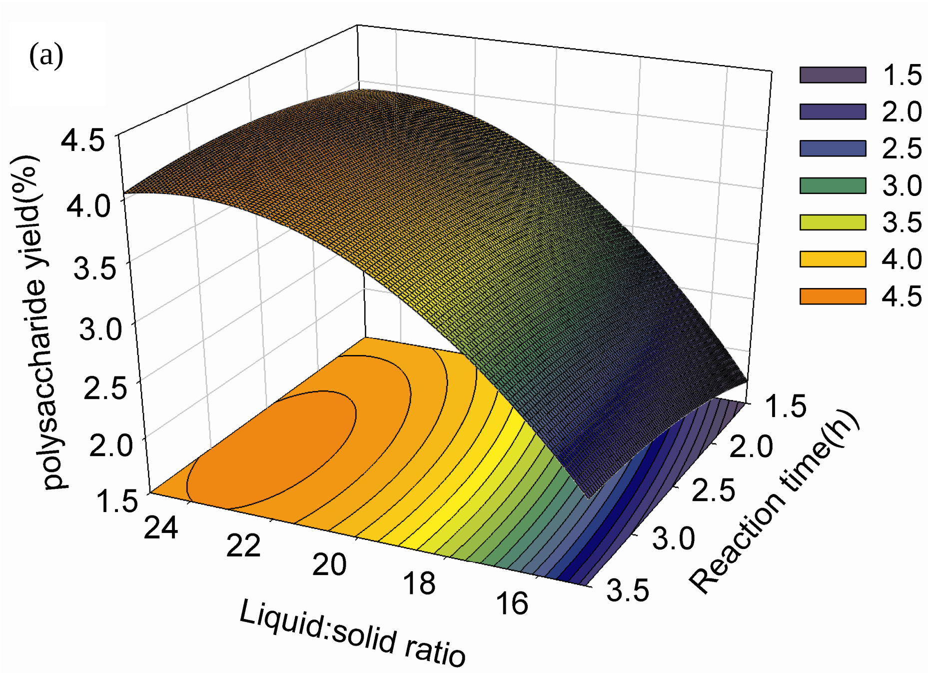


Fig.2 (a) 3-D plots and (b) contour plots of the response surface for the effect of liquid: solid ratio and cellulase concentration on Jili polysaccharides yield with reaction time 2.87h



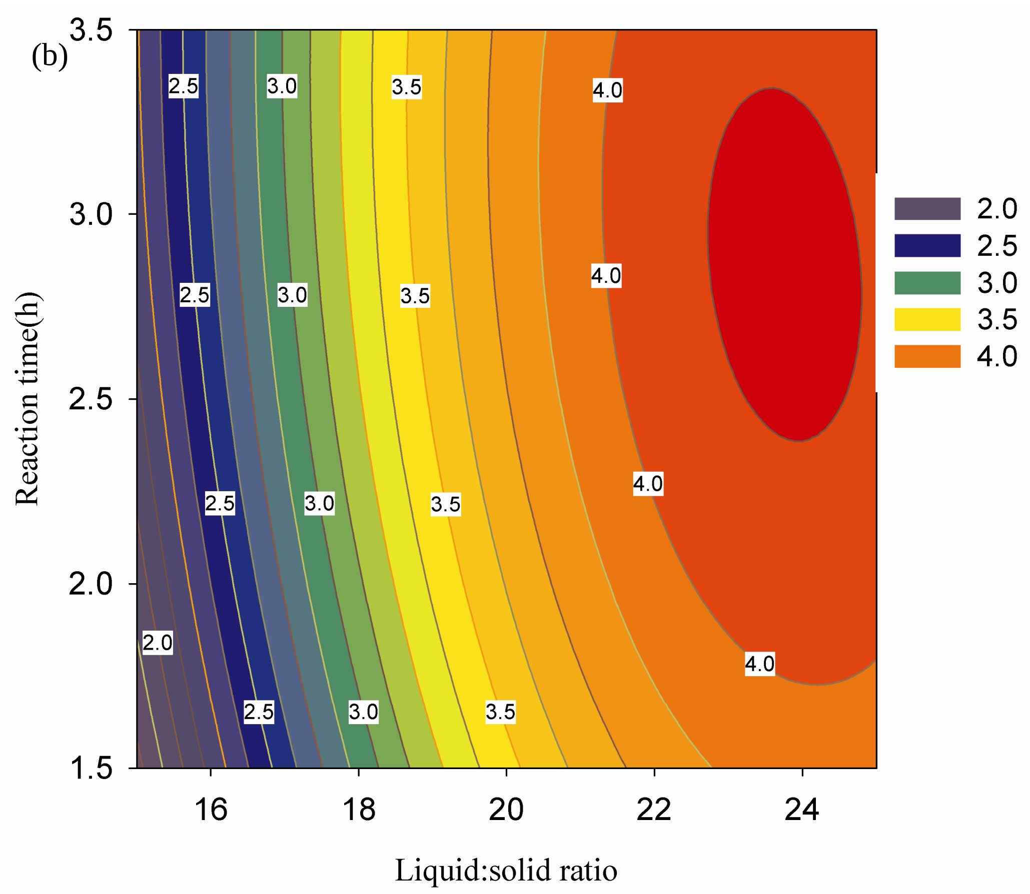


Fig. 3 (a) 3-D plots and (b) contour plots of the response surface for the effect of liquid: solid ratio and reaction time on Jili polysaccharides yield with cellulase concentration2.01%

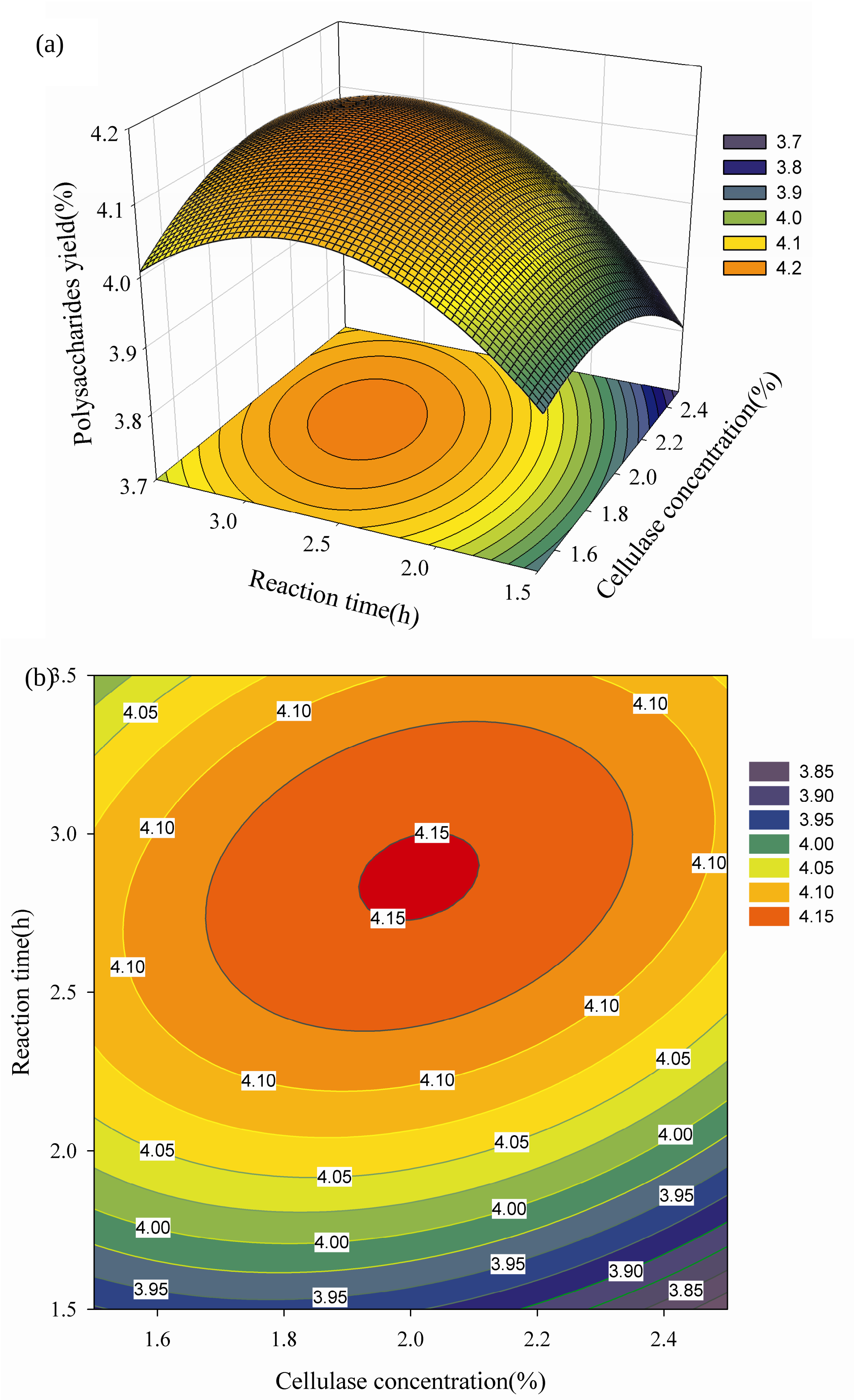


Fig. 4 (a) 3-D plots and (b) contour plots of the response surface for the effect of cellulase concentration and reaction time on Jili polysaccharides yield with liquid: solid ratio 23.75

Through these3-D plots and their respective contour plots, it was very easy and convenient to understand the interactions between two variables and to locate their optimum ranges. By analyzing the plots (Fig.2), the optimal values of the tested variables for obtaining Jili polysaccharides yield of approximate 4.15% lied in the following ranges: liquid: solid ratio 23.0-24.0,cellulase concentration 1.9-2.1% and reaction time 2.5-3.0h. From the above analysis, the maximum value of Jili polysaccharides yield occurred at a high liquid: solid ratio (Fig. 2), an appropriate cellulase concentration (Fig.3) and a long extracting reaction time (Fig.4).

If the partial derivative of Eq. (3) was zero, three equations could be constructed as follows:

$$1.0113-1.2875x_1-0.3425x_2-0.1000x_3=0 \quad (4)$$

$$0.2438-0.3425x_1-0.1275x_2+0.0450x_3=0 \quad (5)$$

$$0.1625-0.1000x_1+0.0450x_2-0.2425x_3=0 \quad (6)$$

Using Eq. (4) - (6) and Eq. (1), the following results could be obtained:

$x_1 = 0.75$, $x_2 = 0.026$, $x_3 = 0.37$, $X_1 = 23.75:1$, $X_2 = 2.01$, $X_3 = 2.87$.

3.3. Verification of results

The suitability of the model equation for predicting the optimum response values was tested using the optimum conditions mentioned above. The maximum predicted yield and experimental yield of Jili polysaccharides were given in Table 4. Additional experiments using the predicted optimum conditions for Jili polysaccharides extraction was carried out: Liquid: solid ratio 23.75, cellulase concentration 2.01%, reaction time 2.87h, and the model predicted a maximum response of 4.152%. To ensure the predicted result was not bias the practical value, experiment rechecking was performed using this modified optimal conditions: Liquid: solid ratio 24, cellulase concentration 2.0%, reaction time 2.9h. A mean value of 4.148±0.011 %(n=5) was gained, which was in agreement with the predicted value significantly ($p > 0.05$), obtained from real experiments, demonstrated the validation of the RSM model. The results of analysis confirmed that the response model was adequate for reflecting the expected optimization (Table 4), and the model of Eq. (3) was satisfactory and accurate.

Table 4 Optimum conditions and predicted and experimental value of response at the optimum condition

	Liquid:solid ratio	Cellulase concentration(%,w/v)	Reaction time(h)	Polysaccharide yield(%)
Optimum condition	23.75:1	2.01	2.87	4.152 (predicted)
Modified conditions	24:1	2.00	2.90	4.148±0.011[a] (Experimental)

[a] *Mean value of five experiments.*

CONCLUSION

The extraction of polysaccharides from Jili was optimized using Design Expert version7.1 software. The three variables involved in the optimization were liquid: solid ratio (x_1), cellulase concentration (x_2) and reaction time (x_3). The F and p value indicated that the variable with the largest effect was the liquid:solid ratio (x_1). This was followed by the quadratic effect of liquid: solid ratio (x_1^2), the cellulase concentration (x_2) and interaction effect of liquid: solid ratio and cellulase concentration (x_1x_2). From the BBD results, the meant Jili polysaccharides extraction yield was 4.148%, which corresponded well with the value that was predicted by the model.

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