Advance Journal of Food Science and Technology 10(1): 53-58, 2016
DOI: 10.19026/ajfst.10.1752
ISSN: 2042-4868; e-ISSN: 2042-4876

Submitted: April 27, 2015 Accepted: May 10, 2015 Published: January 05, 2016

Research Article

Optimize the Extract of Polysaccharides from *Chlorella pyrenoidosa* Based on Inhibitory Effects of Saccharase

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Abstract: In this study, response surface methodology was employed to optimize the extraction process of Crude Polysaccharides from *Chlorella pyrenoidosa* (CCPS) on the activity of saccharase with maximum inhibition. Three independent and main variables, including extraction time (h), temperature (°C) and ratio of liquid to raw material (mL/g), which were of significance for the saccharase inhibition rate of CCPS were studied and the quadratic regression rotary combination design was based on the results of a single-factors test. The experimental data were fitted to a second-order polynomial equation using multiple regression analysis and also examined using the appropriate statistical methods. The best extraction conditions are as follows: extraction time 100 min, temperature 85°C, ratio of liquid to raw material 50 mL/g. Under the optimization conditions, the experimental saccharase inhibition rate of CCPS was 42.27%, which was well matched with the predictive inhibition rate of 41.78% and the yield of polysaccharide was 4.23%.

Keywords: *Chlorella pyrenoidosa*, polysaccharides, response surface methodology, saccharase

INTRODUCTION

Chlorella pyrenoidosa is an edible unicellular green microalgae that is widely distributed and cultivated freshwater microalgae can be widely applied as an important ingredient for food industry (Robledo and Pelegrin, 1997; Kay, 1991). Significant attention has recently been drawn to the use of microalgae for developing functional food, *Chlorella pyrenoidosa* has been named green healthy food by FAO, rich in nutrients, such as protein (dry weight 50-65%), total lipid (5-10%), total carbohydrate (10-20%) and the antioxidants, vitamin C (200-500 mg/kg) and vitamin E (120-300 mg/kg) (Sheng *et al*., 2007).

In the past several years, many reports focus on the extraction, preparation, purification, identification and biological activities of the immunostimulatory, hypoglycemic action and antitumor extracted from *Chlorella pyrenoidosa* (Sheng *et al*., 2007; Shi *et al*., 2007; Suárez *et al*., 2005; Miyazawa *et al*., 1988; Suárez *et al*., 2010; Yang *et al*., 2006; Cherng and Shih, 2006; Senthilkumar and Ashokkumar, 2012). Whereas, little attention was devoted to the inhibitory effects of saccharase of the crude polysaccharides of *Chlorella pyrenoidosa*. Saccharase is a key enzyme of carbohydrate digestion and absorption, which belongs to the alpha-glucosidase. Saccharase can catalyze the hydrolysis of glycosidic bond α-1, 4- and it is an oligosaccharide hydrolase in the small intestine. The body's absorption to sucrose depends on saccharase activity located small intestinal brush border. Alpha-glucosidase inhibitors can inhibit alpha-glucosidase activity on intestinal brush border and reduce glucose production and absorption and then adjust the blood sugar level.

Therefore, we reported the optimization of extracting parameters based on the inhibitory effects of saccharase for the production of Crude Polysaccharides (CPS) from *Chlorella pyrenoidosa*. Response Surface Methodology (RSM) is an effective statistical technique for optimizing complex processes and it was widely used in optimizing the natural active ingredient extraction process variables (Zhu and Liu, 2013). In this paper, RSM was firstly employed for the extraction process of PPS based on the inhibitory effects of saccharase. The aim of this research was to develop an approach that would bring a better understanding of the combined inhibitory effects of the key processing variables (extraction time, extraction temperature and ratio of liquid to raw material) on the desired response (saccharase inhibition rate of CCPS), as well as to seek optimum conditions of the crude polysaccharides with the activity of maximum inhibition extraction from *Chlorella pyrenoidosa*.

MATERIALS AND METHODS

Materials: The unicellular green algae *Chlorella pyrenoidesa* was obtained from Hua Dan Agricultural

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Products Limited Company in Hangzhou, China. Saccharase, BR, 200 u/mg, Sigma-Aldrich, USA. All other chemicals and solvents used were of analytical grade and obtained from Hangzhou, Zhejiang province, China.

Extraction of CCPS: Dried ground *Chlorella pyrenoidesa* samples (10 g) was mixed with 50 mL NaOH (3%) in a 250 mL beaker and interrupted oscillation for 3 h in 4°C, then was performed using a 20 kHz ultrasonic device (VCX 400, Sonics and Materials, USA and 0-400 W) for 20 min (power 125 W) and adjusted the solution to the neutral by 1 M hydrochloric acid. Next, The resulting sample solution was extracted further with distilled water (ratio of liquid to raw material (mL/g) ranging from 10:1 to 100:1), while the temperature of the water bath was kept steady for a given temperature (within±1.0°C, extraction temperature ranging from 50 to 100°C). The water-material slurry in a 2.0 L stainless steel boiler in the water bath was stirred with an electric mixing paddle for a given time (extraction time ranging from 30 to 130 min) during the entire extraction process. The extracted slurry was centrifuged at 2000×g for 10 min to collect the supernatant and the insoluble residue was treated again as mentioned above.

The supernatant was incorporated and treated with polyamide static adsorption and decolorization and then the supernatant was concentrated to one-fifth of initial volume using a rotary evaporator (RE-52AA, Yarong Technology and Science Inc., Shanghai, China) at 60°C under vacuum. The resulting solution was mixed with four volumes of dehydrated ethanol (ethanol final concentration, 80%) and kept overnight at 4°C. Then the solution was centrifuged at 2000×g for 10 min, washed three times with dehydrated ethanol and the precipitate was collected as the crude extract, which was freeze dried at -40°C under vacuum and ground to powder. The percentage yield of CCPS (Y) was calculated by the following equation:

$$\text{CCPS yield (\%)} = m_0/M \times 100$$

where,
w_0 = The weight of the dried CCPS weight
W = The weight of the dried raw material weight

Experimental design: Response Surface Methodology (RSM) with Central Composite Design (CCD) was

Table 1: Independent variables and their levels used in the response surface design

	Levels				
Independent variables	-1.68	-1	0	1	1.68
Extraction temperature (X_1) (°C)	60	70	80	90	100
Extraction time (X_2) (min)	50	70	90	110	130
Ratio of liquid to raw material (X_3) (mL/g)	10:1	20:1	40:1	60:1	80:1

Table 2: The detection method of the saccharase inhibition rate

	Sample tube	Control tube 1	Control tube 2
0.2 mol/L acetate buffer (mL)	0.5	0.8	0.7
5% sucrose solution (mL)	0.5	0.5	0.5
Deionized water (mL)	0.5	0.5	0.5
Saccharase (mg)	0.2	0.2	0.0
CCPS (mg/mL)	0.3	0.0	0.3

Table 3: Experimental design and responses of the variables to the extract parameters

Run	X_1/extraction temperature (°C)	X_2/extraction time (min)	X_3/ratio of liquid to raw material (mL/g)	Polysaccharide on the inhibition rate of saccharase (%)
1	-1	1.00	1.00	34.12
2	-1	-1.00	1.00	30.23
3	0	0.00	0.00	39.35
4	0	0.00	0.00	39.50
5	0	0.00	-1.68	34.18
6	1	-1.00	-1.00	30.81
7	1.68	0.00	0.00	40.21
8	0	0.00	0.00	40.25
9	-1	1.00	-1.00	28.13
10	0	1.68	0.00	35.33
11	0	-1.68	0.00	27.30
12	1	-1.00	1.00	29.57
13	1	1.00	1.00	38.81
14	0	0.00	0.00	41.23
15	0	0.00	1.68	38.33
16	0	0.00	0.00	42.23
17	-1	-1.00	-1.00	28.87
18	1	1.00	-1.00	34.23
19	0	0.00	0.00	38.22
20	-1.68	0.00	0.00	26.24

Table 4: Estimated regression model of relationship between response variable and independent variables (X_1, X_2 and X_3)

Variables	d_f	Parameter estimation	Prob.>F
X_1	1	2.60	0.0007
X_2	1	2.15	0.0027
X_3	1	1.29	0.0383
X_1^2	1	-2.73	0.0004
X_2^2	1	-3.40	<0.0001
X_3^2	1	-1.66	0.0105
X_1X_2	1	1.19	0.1242
X_1X_3	1	-0.50	0.4960
X_2X_3	1	1.30	0.0957

employed to investigate the optimal inhibitory ratio parameters of CCPS. Three independent parameters namely, extraction temperature, extraction time and ratio of liquid to raw material at five different levels each, were employed. The parameters were chosen after determining the preliminary range of extraction variables through single-factor test and their levels were based on the results of a single-factors test. Table 1 gives the range of variables employed. The actual set of experiments performed (experimental runs 1-20) and the inhibitory ratio of CCPS obtained were shown in Table 1 to 4. All the experiments were carried out at random in order to minimize the effect of unexplained variability in the observed responses due to systematic errors. A second-order polynomial equation was developed to study the effects of variables on the inhibitory ratio. The equation indicates the effect of variables in terms of linear, quadratic and cross-product terms:

$$Y = \beta_0 \pm \sum\beta_i X_i \pm \sum\beta_{ii} X^2_i \pm \sum\beta_{ij} X_i X_j$$

where,

- Y = The inhibitory ratio of CCPS (%)
- X_i and X_j = The levels of variables
- β_0 = The constant term
- β_i = The coefficient of the linear terms
- β_{ii} = The coefficient of the quadratic terms
- β_{ij} = The coefficient of the cross-product terms

All the experimental data were statistically analyzed by the MATLAB statistical package (version 7.0, Math Works, Natick, MA, USA). Subsequently, three additional confirmation experiments were conducted to verify the validity of the statistical experimental strategies.

Determination of saccharase inhibition rate: Method for the determination of CCPS to saccharase inhibition rate was shown in Table 2.

DNS (1.5 mL) was added to each tube and each tube was boiling water bath for 5 min, then added deionized water to 25 mL after cooling. The absorption value was measured in the A_{520}. Sucrose enzyme inhibition rate calculation formula is as follows:

$$\text{Inhibition rate (\%)} = [A_{control1} - (A_{sample} - A_{control2})] / A_{control1}$$

RESULTS AND DISCUSSION

Optimization of the extraction parameters of CCPS: Statistical analysis and the model fitting: On single factor analysis, process variables and their ranges were selected and independent variables were coded at five levels between -1.68 and 1.68. The experimental conditions and the corresponding response values from the experimental design are given in Table 3.

There were a total of 20 runs for optimizing the three individual parameters. The following polynomial equation was derived to represent the saccharase inhibition rate of CCPS as a function of the independent variables tested. Where Y is the predicted inhibition rate of CCPS and X_1, X_2 and X_3 are the coded values for extraction temperature, extraction time and ratio of liquid to raw material, respectively. Table 4 shows that runs 3, 4, 7, 8, 13, 14, 15 and 16 showed high levels of the saccharase inhibition rate of CCPS at 39.35, 39.50, 40.21, 40.25, 38.81, 41.23, 38.33 and 42.23%, respectively. The maximum inhibition rate was observed in run number 16, while the minimum inhibition rate was achieved in run number 20. The values of the coefficients in the second-order polynomial equation are presented in Table 4.

By applying multiple regression analysis on the experimental data, the response variable and the test variables are related by the following second-order polynomial equation:

$$Y = \min\{-[40.17 + 2.60\times X_1 + 2.15\times X_2 + 1.29\times X_3 - 2.73\times X_1^2 - 3.40\times X_2^2 - 1.66\times X_3^2 + 1.19\times X_1X_2 - 0.5\times X_1X_3 + 1.3\times X_2X_3]\} \quad (1)$$

The p-values were used as a tool to check the significance of each coefficient. It can be seen from this table that, among the test variables used in this study, the linear coefficients X_1, X_2 and X_3, a quadratic term coefficients X_1^2, X_2^2 and X_3^2 were significant, with very small p-values ($p<0.05$). The other term coefficients were not significant ($p>0.05$). For the model fitted, the coefficient of determination (R^2), which can check the goodness of a model, was 0.9218. This indicated that the sample variation of 92.18% for the saccharase inhibitory ratio of CCPS was attributed to the independent variables and only 7.82% of the total variation cannot be explained by the model.

Optimization of extraction conditions: Three factors at five level central composite rotatable response surface design was used to investigate the influence of process variables on the saccharase inhibitory ratio of CCPS. To understand the interaction between the independent variables and estimate the saccharase inhibitory ratio of CCPS over independent variables, Three Dimensional (3D) response surfaces and the

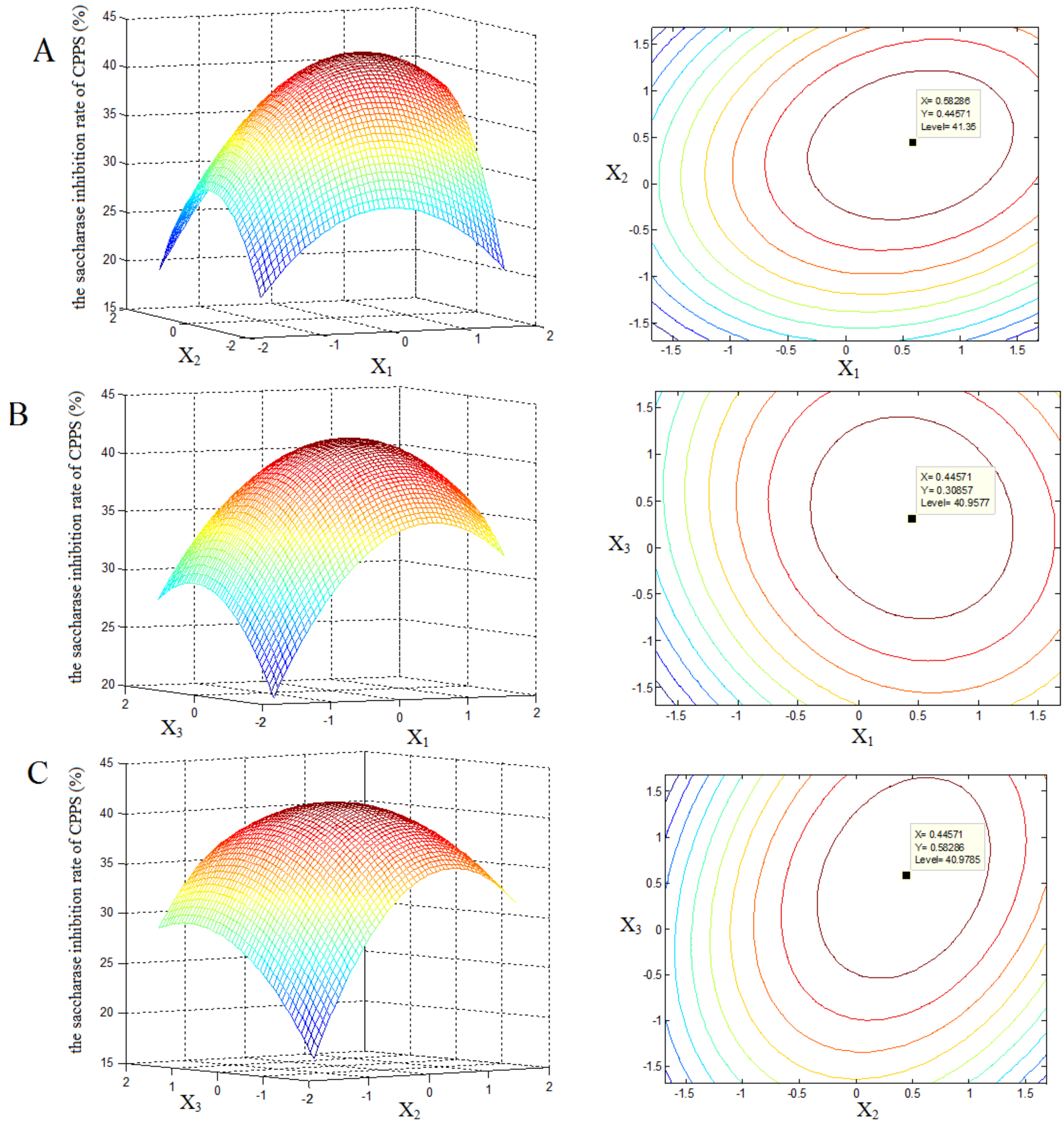

Fig. 1: Response surface plots and contour plots showing the effect of the extraction temperature, extraction time and ratio of liquid to raw material on the response inhibitory ratio

contour plots were obtained by Design-Expert. In this study, two factors were varied in their range in order to understand their main and interactive effects on the dependent variables, while the other one variable was fixed constant at its zero level (center value of the testing ranges).

The results of the saccharase inhibitory ratio of CCPS affected by the three factors are presented in Fig. 1A to C. In the three figures, the maximum predicted value indicated by the surface was confined in the smallest ellipse in the contour diagram. The independent variables and maximum predicted values from the figures corresponded with the optimum values of the dependent variables obtained by the equations.

The 3-D plot and the contour plot based on independent variables extraction temperature and time were shown in Fig. 1A, while the ratio of liquid to raw material was kept at a zero level. Figure 1A showed the effects of the extraction temperature and time on the saccharase inhibitory ratio of CCPS and the maximal inhibitory ratio (44.57%) was obtained when a temperature of 86°C and a time of 102 min were used.

Table 5: Predicted and experimental values of the response at optimum and modified conditions

	Extraction temperature (°C)	Extraction time (min)	Ratio of liquid to raw material (mL/g)	Inhibitory ratio of CCPS (%)
Optimum conditions	85.40	101.02	50.12	41.78% (predicted)
Modified conditions	85.00	100.00	50.00	42.27±0.076% (experimental)

From Fig. 1A, it can be seen that increasing the extraction temperature or time within the particular ranges tested in this study led to an increase in the inhibitory ratio of CCPS, while at the temperature and the time greater than 86°C and 102 min separately, the inhibitory ratio of CCPS decreased.

The 3-D plot and the contour plot in Fig. 1B, which given the extraction time (0 level), the maximum inhibitory ratio (30.86%) of CCPS was achieved when the extraction temperature 84°C and ratio of liquid to raw material 49 were used. Meanwhile, it showed that the inhibitory ratio of CCPS increased evidently with increasing of extraction temperature from 60 to 84°C and the ratio of liquid to raw material from 10 to 49, but beyond 84°C and 49, the inhibitory ratio of CCPS decreased slowly.

As shown the 3-D plot and the contour plot in Fig. 1C, when a fixed extraction temperature (0 level) was used, the maximal inhibitory ratio (58.29%) of CCPS was obtained when the extraction time and the ratio of liquid to raw material was 99 min and 49 separately, while at the extraction time and the concentration of the ratio greater than 99 min and 49 separately, the inhibitory ratio of CCPS slowly decreased. All these findings confirmed that there is an optimal extraction temperature, extraction time and quantity of water for the effective extraction of CCPS.

According to Fig. 1 and single parameter study, it can be concluded that optimal extraction condition of CCPS were extraction temperature 85.40°C, extraction time 101.02 min, ratio of liquid to raw material 50.12. Among the three extraction parameters studied, the extraction temperature was the most significant factor to affect the inhibitory ratio of CCPS, followed by extraction time and ratio of liquid to raw material according to the regression coefficients significance of the quadratic polynomial model (Table 5).

Verification of the models: The suitability of the model equation for predicting the optimum response values was tested by using the selected optimal conditions. The maximum predicted inhibitory ratio and experimental inhibitory ratio of CCPS were given in Table 5. The model predicted a maximum response of 41.78% under the optimum conditions. To ensure the predicted result was not biased toward the practical value, experiment rechecking was performed by using these modified optimal conditions: extraction temperature 85°C, extraction time 100 min, ratio of liquid to raw material 50. A mean value of 42.27±0.076% (n = 3) was gained, obtained from real experiments, demonstrated the validation of the RSM model. The extraction yield of CCPS under this optimal extraction condition was 4.23%. The results of analysis confirmed that the response model was adequate for reflecting the expected optimization (Table 5) and the model of equation was satisfactory and accurate.

CONCLUSION

In this study, the nutritional components of *Chlorella* powder were rich in carbohydrates, protein and minerals and *Chlorella* can be higher total carbohydrate content and rarely reducing sugar content. RSM was proved to be useful for optimization of technology of CCPS extraction based on the saccharase inhibition rate of CCPS. The statistical analysis based on a CCD design showed that the optimum conditions were found to be extraction temperature of 85.40°C, extraction time of 101.02 min and ratio of liquid to raw material of 50.12 mL/g. Under the most suitable conditions, the experimental inhibition rate of CCPS was 42.27±0.076%, which was closed with the predicted inhibition rate 41.78%.

ACKNOWLEDGMENT

This study was supported by the financial support of program for the “Twelfth Five-Year” National Science and technology project in rural areas (No. 2013BAD10B02) and granted from key laboratory of Marine Food quality and hazard controlling technology of Zhejiang province.

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