Supporting information

Understanding mechanism of adsorption in the decolorization of aqueous methyl violet (6B) solution by okra polysaccharides: experiment and theory

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1. Extraction of polysaccharide from okra

1.1 Materials

The okra pods were purchased from Nanjing Agricultural Cooperatives. The chemicals bought from Sinopharm Chemical Reagent Co. Ltd. are as follows: sodium hydroxide, hydrated citric acid, phenol, concentrated sulfuric acid, anhydrous ethanol, glucose and methyl violet (6B). Papain was obtained from Nanjing O'donnie Biotechnology Co. Ltd. and ferric sulfate was purchased from Shanghai Macklin Biochemical Co. Ltd. All these chemicals were of analytical reagent grade.

1.2 Extraction process

The okra fruit were first dried, then pulverized using a mechanical disintegrator and sifted through a 50-mesh sieve. The resulting powder was collected for extraction. After the extraction temperature and time were set, 1 g of okra powder was extracted in an ultrasonic cleaning bath (KH3200DB, Kushan Ultrasonic Instrument Co. Ltd.), using certain volume of distilled water. The extracted solution was centrifuged at 8000r/min for 5 min, and the supernatant obtained was the crude aqueous polysaccharide solution, which was precipitated with anhydrous ethanol, then centrifuged again (same rpm and time). The sediment was collected and freeze-dried to obtain the okra polysaccharide.

1.3 Calculation of extraction yield
Using D-glucose as a standard, polysaccharide concentrations were measured based on the phenol-sulfuric acid method, described as follows. A calibration curve was constructed for determination of the extraction yield. 0.2 mg/mL glucose solution was prepared as the stock solution. Then 0.4, 0.8, 1.2, 1.6, 2.0 mL of the glucose stock were added into five different 10mL volumetric flasks. Different amount of distilled water was added into each flask to reach a total volume of the solution equal to 2.0mL. A blank solution was made by adding 2mL distilled water only in a separate flask. Into each of the 6 flasks described above, 1mL phenol solution with a mass fraction of 5% was added, followed by 5mL concentrated sulfuric acid (98%). All solutions were shaken for 30 min before absorbance measurements. Absorbance values of the 6 solutions at 490 nm were obtained using a Shimadzu UV-2450 spectrophotometer. The data were used to make a calibration curve of absorbance versus glucose concentration.

1.4 Single-factor experiments

Three factors were considered, with the following preliminary ranges: A. extraction time (10-60min), B. liquid-solid ratio (10:1-70:1) and C. extraction temperature (40-70 °C).

1.5 Optimization of extraction

On the basis of single factor experiment, the preliminary ranges of these factors affecting the yield of polysaccharide were determined. Polysaccharide yield under various sets of the three factors within the preliminary ranges were determined. Using the above data, Box-Benhken design with three independent variables (extraction time-A, liquid-solid ratio-B, extraction temperature-C) at three levels were performed by Design-Expert 8.0.6 software. The
experimental design was carried out to determine the optimal extraction process parameters.

2. Characterization and physicochemical analysis

2.1 FT-IR spectroscopy

The dried powder of okra polysaccharide was mixed thoroughly with KBr powder and pressed into pellets for FT-IR measurement, using Vertex 80V (Brock Co., Germany) IR spectrometer. Absorption spectra were obtained with a wavenumber range of 4000 to 400 cm\(^{-1}\).

2.2 Molecular weight

The molecular weight of the polysaccharide was determined by gel permeation chromatography with a refractive index detector. The chromatographic conditions were as follows, stationary phase: TSK gel G4000 gel column; column temperature: 40°C; mobile phase: re-distilled water; flow rate: 0.6 mL/min; injection volume: 20 \(\mu\)L. With a standard curve established using D-glucose, the molecular weight of okra polysaccharide was calculated.

2.3 Monosaccharide composition

The monosaccharide components in okra polysaccharides were determined by ion chromatography (Dionex ICS-3000 Ion Chromatography with a pulsed amperometric detector, ANASTAR chromatography workstation). Conditions were as follows, sugar guard column: Carbopac PA10 (2 \(\times\) 50 mm); analytical column: Carbopac PA10 (2 \(\times\) 250 mm); column temperature: 30°C; injection volume: 20 \(\mu\)L; mobile phase: 20 mmol/L NaOH; flow rate: 0.2 mL/min. 5.0 mg of polysaccharide sample was added into a hydrolysis tube, with 5 mL of 2 mol/L trifluoroacetic acid. The tube was filled with nitrogen for hydrolysis under 120°C, then
cooled down to room temperature. After filtering with a 0.45 μm microporous membrane, the sample solution was quantitatively diluted for future determination.

2.4 Uronic acid content

The hydrolysate of okra polysaccharide could react with carbazole reagent under strong acid environment. Therefore, the content of uronic acid was determined by carbazole-sulfate method. 9.9 mg of dried galacturonic acid was weighed and dissolved in distilled water to reach the concentration of 99 μg/mL, as the stock solution. 0.478 g sodium tetraborate was dissolved in 100 mL concentrated sulfuric acid with the assistance of ultrasound, thus sodium tetraborate -sulfuric acid solution was obtained. The carbazole solution was prepared by 10 mg carbazole dissolving into 10 mL anhydrous ethanol at the same time.

After 0.2, 0.3, 0.4, 0.5 mL of the stock solution was drawn into separate test tubes, the volume of each solution was brought to 1.0 mL with distilled water. In ice water bath, 6 mL sodium tetraborate solution was added into each of the test tubes and mixed using a vortex mixer blender. After being heated in a boiling water bath for 5 min, these tubes were taken out immediately and cooled down to room temperature. 0.1% carbazole solution was added into each solution, followed by shaking and boiling for 5 min, and cooling down to room temperature. A calibration curve was constructed using absorbance values of the treated galacturonic acid standards at 530 nm, measured on the UV-2450 spectrometer. The okra polysaccharide sample solution (1 mg/mL) was treated through the same steps as described above, then the absorbance of the treated sample was measured. This absorbance value was used to obtain the uronic content
in the sample, based on the calibration curve.

2.5 Measurement of zeta potential

The polysaccharide of okra was prepared into a series of aqueous solutions with concentrations of 1, 5, 10, 25, 50, 75 and 100 mg/L. The zeta potential value of each solution was measured by a Malvern laser particle size analyzer (Zetasizer Nano-ZS, Malvern Co.).

3. Result and discussion

3.1 Single-factor experimental analysis

3.1.1 Effect of extraction time

As shown in Fig. S1 (a), with the increase of extraction time, the yield increased rapidly and the maximum value appeared at 30min, but the yield decreased gradually after exceeding 30min. Increasing extraction time was beneficial to the dissolution of soluble polysaccharides in okra and the extraction rate of polysaccharides increased, but too long extraction time could cause enhanced hydrolysis of polysaccharide, leading to decrease of the yield of polysaccharides.² The durations of sonication were controlled between 20 and 40min based on the results presented in Figure 1(a).

3.1.2 Effect of liquid-solid ratio

Figure S1 (b) shows the effect of liquid-solid ratio (mL/g) on the yield of polysaccharide extracted from okra. The yield reached maxima when the ratio was 60:1, beyond which the yield start to decline slightly.

Considering that larger volume of extraction liquid will lead to greater difficulty of subsequent
concentration and alcohol precipitation processes, and hence higher cost, we kept the liquid-solid ratios within 50:1 ~ 70:1 (mL/g).

3.1.3 Effect of extraction temperature

As shown in Figure S1(c), with the increase of extraction temperature, the extraction rate increased and peaked at 60°C and decreased after that. The explanation is that at low temperature, the polysaccharide in cell wall was not completely dissolved, as the temperature increased, the viscosity of the solvent reduced and the mobility of molecules increased, accelerating the dissolution of polysaccharides from the cell wall. However, if the temperature was too high, the polysaccharides underwent partial degradation, leading to deepened color of the sample. Considering all these effects, ultrasonic temperature should be controlled within 50 °C ~ 70 °C.
3.2 Response surface method (RSM) and data analysis

3.2.1 Range and level of factors

Based on preliminary experiment results, the range of independent variables and their levels were presented in Table S1.

| Independent variables | Factor level | -1 | 0  | 1  |
|-----------------------|--------------|----|----|----|
| A. Extraction time    |              | 20 | 30 | 40 |
| B. Liquid-solid ratio |              | 50 | 60 | 70 |
| C. Extraction temperature |          | 50 | 60 | 70 |

3.2.2 Box-Bonhken design

The response of interest was the extraction yield of polysaccharides. The results of 17 runs using Box-Bonhken design were presented in Table S2, in a random order.

Table S2 Experimental data for response surface analysis
### 3.2.3 Analysis of variance

Table S3 shows the results of variance analysis, including its adequacy, variance and fitness. The response function (Y) was the extraction yield of polysaccharide (%). This value was related to test variables by a second-order polynomial equation as follows:

\[
Y = 22.04 + 1.13A + 1.25B + 0.75C - 0.47AB + 0.78AC - 0.56BC - 2.32A^2 - 4.42B^2 - 1.30C^2
\]  \hspace{1cm} (1)

| Run | A/min | B/(mL/g) | C/^oC | Yield/% |
|-----|-------|----------|-------|---------|
| 1   | -1    | -1       | 0     | 11.99   |
| 2   | 1     | -1       | 0     | 15.62   |
| 3   | -1    | 1        | 0     | 15.91   |
| 4   | 1     | 1        | 0     | 17.67   |
| 5   | -1    | 0        | -1    | 17.60   |
| 6   | 1     | 0        | -1    | 17.86   |
| 7   | -1    | 0        | 1     | 17.42   |
| 8   | 1     | 0        | 1     | 20.79   |
| 9   | 0     | -1       | -1    | 13.95   |
| 10  | 0     | 1        | -1    | 17.07   |
| 11  | 0     | -1       | 1     | 16.69   |
| 12  | 0     | 1        | 1     | 17.56   |
| 13  | 0     | 0        | 0     | 22.12   |
| 14  | 0     | 0        | 0     | 21.77   |
| 15  | 0     | 0        | 0     | 22.06   |
| 16  | 0     | 0        | 0     | 22.31   |
| 17  | 0     | 0        | 0     | 21.94   |

#### Table S3 Summary of variance analysis

| Source | Sum of square | Degree of freedom | Mean square | F-value | P-value |
|--------|---------------|-------------------|-------------|---------|---------|
| Model  | 153.34        | 9                 | 17.04       | 111.48  | <0.0001 |
| A      | 10.13         | 1                 | 10.13       | 66.25   | <0.0001 |
| B      | 12.45         | 1                 | 12.45       | 81.46   | <0.0001 |
| C      | 4.47          | 1                 | 4.47        | 29.25   | 0.001   |
| AB     | 0.87          | 1                 | 0.87        | 5.72    | 0.0481  |
| AC     | 2.42          | 1                 | 2.42        | 15.82   | 0.0053  |
|     | 1   | 1.27 | 8.28 | 0.0237 |
|-----|-----|------|------|--------|
| BC  | 1.27| 1    | 1.27 | 8.28   |
| A²  | 22.69| 1   | 22.69| 148.44 |
| B²  | 82.31| 1   | 82.31| 538.52 |
| C²  | 7.13 | 1   | 7.13 | 46.65  |
| Residual | 1.07 | 7   | 0.15 |        |
| Lack of fit | 0.91 | 3   | 0.30 | 7.44   |
| Pure error | 0.16 | 4   | 0.041|        |
| Total | 154.41| 16   |      |        |

Note: *p<0.05; **p<0.01 and ***p<0.001

The regression coefficient values of the equation were listed in Table S3. The P-value was used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interactions between the variables. The smaller the value of P, the more significant the corresponding coefficient.⁵

According to analysis of variance of the quadratic regression model, the P-value of the model was smaller than 0.0001, which indicated this regression equation model was extremely significant and this method is quite reliable to optimize the extraction process. The $R^2$ value of the equation was 0.9842>0.9, revealing that the predicted value of the equation had a good correlation with the measured value, and the experimental error was very small. Therefore, the regression equation can be used to analyze and predict the test results. In addition, AC, AB and BC all had significant effects on the yield of polysaccharides, indicating that all these three factors had certain influence on the yield and their interactions were significant.

The magnitude of the absolute value of the coefficient of the primary term in the regression equation indicated the degree of influence of each factor on the response value.⁶ According to this information, the order of the three factors affecting the yield of polysaccharide was:
Liquid-solid ratio > Extraction time > Extraction temperature.

3.2.4 Interactions analysis

The response surface and the contour plots, representing the regression equation (1), was obtained using Design-Expert 8.0.6 software. Based on this, thus we could analyze the influence and interactions of those three factors. The steepness of the slope of the response surface shows how the response values changes with the change of the factors. The color on the contour plot reflects impacts of the factors on the yield. The shape of ellipse means the interaction between factors is obvious, while circular shape means the opposite.7

In Figure S2, it is clear that the steep shape of each response surface indicated the obvious interaction of combined influence from those factors. And the density of axial contour revealed the extent of influence from each factor. With the information shown in Figure S2, we could conclude that the influence of liquid-solid ratio was the greatest, followed by extraction time extraction temperature has the least influence on the yield.

![Response surface plot and contour plot](image)

Figure S2 (a) Response surface plot and contour plot of extraction time and liquid-solid ratio and their impacts on yield
3.2.5 Prediction and verification of optimal conditions

According to the data processed by Design-Expert 8.0.6, the predicted optimal test variables providing an extraction yield of 22.35% were: extraction time: 31.42min, liquid-solid ratio: 51.25:1 (mL/g), and extraction temperature: 62.74°C. These conditions were slightly modified for practical purpose, which include extraction time: 31min, liquid-solid ratio: 51:1 (mL/g), and extraction temperature: 63°C. Under such conditions, three practical parallel tests were subsequently carried out, and the actual average extraction yield obtained was only 0.54% lower from the theoretical value, showing strong agreement with the predicted value. This finding
indicated that the regression model was accurate and feasible to optimize the extraction process of polysaccharide from okra.

3.3 Physicochemical analysis

3.3.1 FT-IR spectroscopy

FT-IR spectra of polysaccharide from okra in the range of 4000-400 cm\(^{-1}\) are shown in Figure S3. Absorption bands were obvious at approximately 3400 cm\(^{-1}\), 2938 cm\(^{-1}\) and 1000-1200 cm\(^{-1}\), which are common to all polysaccharides.\(^7\) The intense broad adsorption peak around 3400 cm\(^{-1}\) can be attributed to O-H stretching vibration caused by hydrogen bonding in and between the molecules of saccharides. The less prominent band at 2938 cm\(^{-1}\) suggests C-H asymmetric vibration of saccharides.\(^8\) Both absorptions bands are characteristic of polysaccharide.\(^9\) The characteristic peak at 1740 cm\(^{-1}\) can be assigned to C=O stretching vibration of carbonyl ester.\(^10\) The signal at 1638 cm\(^{-1}\) is related to C=O asymmetric stretching vibration of carboxylic group. The absorption peaks appeared between 1300-1000 cm\(^{-1}\) are caused by C-O stretching vibrations on the sugar ring.
3.3.2 Gel permeation chromatography

As shown in Figure S4, the distribution of molecular weight of okra polysaccharide was relatively wide. Based on the retention time of okra polysaccharide sample and the standard curve of D-glucose (\( \lg M_w = -0.08988t + 8.15719, R^2 = 0.9992 \)), we calculated the average molecular weight of okra polysaccharide, which was 68010 kDa.

Figure S3 FT-IR spectroscopy of polysaccharide from okra
3.3.3 Monosaccharide composition

The composition of OPS samples was determined using ion chromatography, by comparing the relevant peaks to those of a standard made of three monosaccharides (rhamnose; galactose; glucose). The peaks of the three monosaccharides were shown in Figure S5. The molar ratio of three monosaccharides in okra polysaccharide was rhamnose: galactose: glucose=1:0.56:0.13. Further analysis of other components indicated by other peaks in the IC chromatogram was not performed in this work.
3.3.4 Uronic acid content

Figure S6 was the standard curve of galacturonic acid. Its linear regression fitting equation is $Y=0.01519X+0.08402$ ($R^2=0.9976$). Based on this equation, the uronic acid content in okra polysaccharide was found to be 10.71%
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