Preparation of environmentally friendly chitooligosaccharide via enzymatic hydrolysis using response surface methodology

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Abstract. In this paper, chitooligosaccharide was prepared via the enzymatic hydrolysis of papain with chitosan as a raw material. The effects of pH, reaction time, temperature, and mass ratio of chitosan and enzyme on the product yield were analyzed. Single-factor and orthogonal experiments were used to determine the main factors affecting the reaction, and response surface methodology was used to optimize chitooligosaccharide preparation conditions. Optimum conditions were achieved at pH 4.50, with an enzymolysis time of 77.4 min, an enzymolysis temperature of 45 °C, a substrate concentration of 1.75% and an enzyme to chitosan weight ratio of 8.85. Under these conditions, the chitooligosaccharide yield reached 37.78%.

1. Introduction

Chitosan, β-(1,4)-N-acetylamino-2-deoxy-D-glucan, is the only homogeneous polysaccharide-containing amino group in nature. It is mainly derived from crustaceans (such as shrimps, crabs, and insects), fungi, the cell walls of certain plants, and organs of mollusks. The amount of chitosan found in nature is less than the amount of cellulose.⁠¹⁻⁴ Chitooligosaccharide, β-1,4-oligosaccharide-glucosamine, is a type of oligosaccharide, with a polymerization degree between 2 and 20, which is derived from chitosan by enzymatic hydrolysis, chemical degradation or microwave ultrasonic degradation technology.⁠⁵ The molecular weight of chitooligosaccharide is generally between 1000 and 3000 Daltons. Compared with the macromolecular chitosan, chitooligosaccharide has unique and superior biochemical properties. Chitosan has a tight crystal structure, and its macromolecular chain structure contains a large number of -NH₂ and -OH groups. Chitosan does not dissolve in water, and is only soluble in weak acids.⁶ When chitosan degrades to chitooligosaccharide, the strong polar groups of -OH and -NH₂ increase rapidly, improving water solubility. Thus, chitooligosaccharide is an excellent natural flocculant.⁷

Methods for the preparation of chitooligosaccharide include physical degradation, chemical degradation, hydrogen peroxide oxidation and biodegradation (enzymatic hydrolysis).⁸⁻¹⁰ With enzymatic hydrolysis, enzymes are used effectively to cut off β-1,4 glycosidic bonds to obtain the specific molecular weight of chitosan. The enzymatic hydrolysis method benefits from mild reaction conditions, an easily controlled degradation process, a high yield of chitosan oligosaccharides, and a moderate degree of polymerization. Therefore, enzymatic hydrolysis is an ideal degradation method and has attracted widespread attention.
Response surface methodology (RSM) was introduced and gradually improved by Box and his collaborators in the 1950s. Using a regression equation to estimate the function, RSM can describe the relationship between factors and experimental results in multiple-factor experiments via polynomial fitting. By establishing the functional relationship between factors and experimental results, it is possible to analyze the function surface, and to determine the correlation among factors, and between factors and response values. Furthermore, RSM can provide the optimal combination of factors and the optimal response values over the entire region.[11]

RSM can be used to determine the effects of various factors and their interactions with non-independent variables during processing, and to accurately represent the relationship between factors and response values. At present, RSM research mainly focuses on the optimization of reaction process conditions under enzymatic hydrolysis. For example, Wang et al.[12] optimized process conditions for ultrasonic extraction of alfalfa polysaccharides using RSM. Yao et al.[13] optimized enzymolysis technology using RSM in a single factor experiment, taking the yield of sugar as a response value after enzymatic hydrolysis of fiber. However, there are few studies aimed at optimizing the enzymatic hydrolysis extraction of chitooligosaccharide based on RSM.

In this study, chitosan was used as a raw material to investigate the chitooligosaccharide yield, including factors such as temperature, pH, enzymatic hydrolysis time, mass ratio of chitosan and enzyme and substrate concentration. Chitooligosaccharides with a molecular weight less than 3000 were prepared, and the main factors influencing reaction yield were investigated using a single factor orthogonal experiment. A response surface rotation regression design was used to determine the optimum process conditions for the preparation of chitooligosaccharide via enzymatic hydrolysis of chitosan. This research can provide basic data for the industrial application of papain to prepare chitooligosaccharide via enzymatic hydrolysis of chitosan.

2. Experimental section

2.1 Chemicals and materials

Reagents: chitosan (deacetylation degree 95%, pharmaceutical grade; Zhejiang Golden Shell Pharmaceuticals); papain (800,000 U/g, food grade; Guangxi Nanning Pangbo Biological Engineering Co., Ltd); glacial acetic acid (AR grade; Tianjin Fuyu); sodium acetate (AR grade; Tianjin Damao); ethanol (AR grade; Tianjin Yongda); 3,5-dinitrosalicylic acid (AR grade; Shanghai Runjie); sodium hydroxide (AR grade; Tianjin Damao); sodium potassium tartrate (AR grade; Guangzhou chemical reagent); sodium sulfite (AR grade; Guangzhou chemical reagent); re-steamed phenol (AR grade; Tianjin Baishi); D-glucosamine hydrochloride (AR grade; Guangzhou Donghong)

2.2 Preparation of chitooligosaccharide

Chitosan was weighed accurately and dissolved in a pH-adjusted HAc-NaAc buffer solution. Enzymatic hydrolysis was carried out for 1 h at a constant temperature of 40 °C. Then, 1 mL of the enzymatic hydrolysate and 1 mL of DNS reagent were extracted in a 10 mL colorimetric test tube. The tube was covered with a stopper and shaken. The enzyme was inactivated by heating for 5 min at 100 °C. The tube was cooled to room temperature; then, distilled water was added up to the 10 mL scale line. The tube was shaken and centrifuged for 3 min, and the supernatant was extracted and the absorbency measured at 540 nm.

The range of values of each factor was determined, and the three main factors were ascertained. Based on these data and the principle of quadratic orthogonal rotation, a regression model was established of the effects of the three main factors on the preparation of chitooligosaccharide. The relationship between yield and various factors can be predicted by a ternary quadratic polynomial:

\[ Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_4X_1^2 + a_5X_2^2 + a_6X_3^2 + a_7X_1X_2 + a_8X_1X_3 + a_9X_2X_3 \]  

(1)

Where X1, X2 and X3 are independent variables.
2.2.1 Analysis and characterization of chitooligosaccharide yield. One mL of enzymatic hydrolysate and 1 mL of DNS reagent were placed into a 10 mL colorimetric test tube. The tube was covered with a stopper, shaken, and heated in boiling water for 5 min. After cooling to room temperature, distilled water was added to the colorimetric test tube up to the 10 mL scale line, and the tube was shaken and centrifuged for 3 min. The absorbency of the solution was measured at 540 nm to obtain the concentration of glucosamine hydrochloride in the solution. The formula for calculating the yield of chitooligosaccharide in the enzymatic hydrolysate is shown in Equation (2):

\[ y(\%) = \frac{X \times C \times 20 \times 100}{M \times 1000} \]  

(2)

Where, \( X \) is the concentration of glucosamine hydrochloride in the solution (mg/mL); \( C \) is the dilution factor of the sample solution during the measurement (10); and \( M \) is the mass of the substrate chitosan (g).

2.2.2 Determination of the average molecular weight of chitooligosaccharide

The chitooligosaccharide was completely hydrolyzed into a sugar unit, using DNS reagent as a chromogenic reagent. The factor by which the reduction end group of the chitooligosaccharide increased after hydrolysis is the average polymerization degree of the chitooligosaccharide (n). According to this principle, the mean relative molecular mass of chitooligosaccharide (Mr) was determined.

\[ M_r = \frac{10A_1}{A_0} \times 179 \times (\frac{10A_1}{A_0} - 1) \times 18 \]  

(3)

Where \( A_0 \) is the absorbency of 1 mg/mL solution of chitooligosaccharide; and \( A_1 \) is the absorbance value of 1 mg/mL solution of chitooligosaccharide after hydrolysis. The hydrolysis method was as follows: 3 mL HCl solution (6 mol/L) was added to 1 mL chitooligosaccharide solution (1 mg/mL), with a moderate amount of NaOH solution (6 mol/L) used to neutralize the acid, in a water bath at 100°C for 2 h; this was followed by dilution with distilled water up to 10 mL.

3. Results and Discussion

3.1. Single factor experiments

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3.1.1. Effects of pH on chitooligosaccharide yield. The chitooligosaccharide yield at varying pH values is shown in Fig.1. As the pH increases, the chitooligosaccharide yield initially increases and then decreases. The chitooligosaccharide yield peaks at pH 4.5 because the activity of papain gradually decreases with increasing pH value. When the pH exceeds 5.5, chitosan precipitates. Therefore, the pH range will be set between 3 and 5 in the orthogonal experiment.
3.1.2. Effects of enzymolysis time on chitooligosaccharide yield. The chitooligosaccharide yield at different enzymatic hydrolysis times is shown in Fig. 2. The chitooligosaccharide yield increases rapidly as the enzymatic hydrolysis time increases up to 60 min. After 60 min, the reaction slows down and eventually stops, and the chitooligosaccharide yield tapers off. The activity of the enzyme is inhibited, and even inactivated, as the substrate (chitosan) decreases and the product (chitooligosaccharide) accumulates. Therefore, most of the chitosan has degraded after 60 min of enzymatic hydrolysis. As such, the enzymatic hydrolysis time range was set at 30-90 min for the orthogonal experiment.

3.1.3. Effects of enzymolysis temperature on chitooligosaccharide yield. Low temperatures inhibit the activity of the enzyme, while high temperatures deactivate the enzyme. The chitooligosaccharide yield at different temperatures is shown in Fig. 3. The chitooligosaccharide prepared via papain enzymatic hydrolysis of chitosan has the highest degree of enzymatic hydrolysis at 45°C. Therefore, the enzymatic hydrolysis temperature will range from 35 to 55°C in the orthogonal experiment.

3.1.4. Effects of mass ratio of chitosan and enzyme on chitooligosaccharide yield. The chitooligosaccharide yield at different mass ratios of chitosan and enzyme is shown in Fig. 4. As the mass ratios of chitosan and enzyme ranged from 30:1 to 10:1, the yield of chitooligosaccharide increased rapidly. When the mass ratios of chitosan and enzyme exceeded 10:1, the yield of chitooligosaccharide gradually became balanced. Although the chitooligosaccharide yield is higher as the mass ratios of chitosan and enzyme further increased to 5:1, considering the dosage and cost of the enzyme, the optimal mass ratios of chitosan and enzyme is selected as 10:1.

Figure 3. Yield of chitooligosaccharide under different enzymolysis temperatures.

Figure 4. Yield of chitooligosaccharide under different mass ratios of chitosan and enzyme.

Figure 5. Yield of chitooligosaccharide under different concentrations of substrate chitosan.
3.1.5. Effects of substrate concentration on chitooligosaccharide yield. The chitooligosaccharide yield at different substrate concentrations is shown in Fig.5. As the substrate concentration increases, so too does the yield of chitooligosaccharide. Not all enzyme molecules can combine with the substrate when the substrate concentration is low. As the substrate concentration increases, more enzyme molecules are able to bind to the substrate, leading to an increase in the chitooligosaccharide yield. However, as the substrate concentration continues to increase beyond the optimum point, the chitooligosaccharide yield tends to taper. This is thought to occur because the substrate is saturated, or because the increase in substrate concentration increases the viscosity, which limits wide-range contact between the enzyme and the substrate. Based on these results, the optimum substrate concentration for papain degradation of chitosan is 1.75%.

3.2. Orthogonal experiments

Given the pre-determined ranges for each factor, which were based on the results of the single factor experiments, an orthogonal experiment was designed with five factors and five levels, L25(55). Twenty-five groups of experiments were carried out, as shown in Table 1, to obtain the chitooligosaccharide yield for each group. As shown in Table 1, the highest yield was achieved with Group 15. The related experimental conditions were: temperature of 45 °C, pH of 5.0, enzymatic hydrolysis time of 45 min, substrate concentration of 1.75%, and mass ratio of chitosan and enzyme of 10:1.

| NO. | Temperature (°C) | pH | Time (min) | Substrate concentration (%) | Mass ratio of chitosan and enzyme | Yield (%) |
|-----|-----------------|----|------------|-----------------------------|----------------------------------|-----------|
| 1   | 35              | 3.0| 30         | 1.00                        | 10/1                             | 0.41      |
| 2   | 35              | 3.5| 45         | 1.25                        | 15/1                             | 0.83      |
| 3   | 35              | 4.0| 60         | 1.50                        | 20/1                             | 7.56      |
| 4   | 35              | 4.5| 75         | 1.75                        | 25/1                             | 10.06     |
| 5   | 35              | 5.0| 90         | 2.00                        | 30/1                             | 5.94      |
| 6   | 40              | 3.0| 45         | 1.50                        | 25/1                             | 0.41      |
| 7   | 40              | 3.5| 60         | 1.75                        | 30/1                             | 1.30      |
| 8   | 40              | 4.0| 75         | 2.00                        | 10/1                             | 16.78     |
| 9   | 40              | 4.5| 90         | 1.00                        | 15/1                             | 15.50     |
| 10  | 40              | 5.0| 30         | 1.25                        | 20/1                             | 5.08      |
| 11  | 45              | 3.0| 60         | 2.00                        | 15/1                             | 0.51      |
| 12  | 45              | 3.5| 75         | 1.00                        | 20/1                             | 2.80      |
| 13  | 45              | 4.0| 90         | 1.25                        | 25/1                             | 5.34      |
| 14  | 45              | 4.5| 30         | 1.50                        | 30/1                             | 7.90      |
| 15  | 45              | 5.0| 45         | 1.75                        | 10/1                             | 25.53     |
| 16  | 50              | 3.0| 75         | 1.25                        | 30/1                             | 0.76      |
| 17  | 50              | 3.5| 90         | 1.50                        | 10/1                             | 0.82      |
| 18  | 50              | 4.0| 30         | 1.75                        | 15/1                             | 0.78      |
| 19  | 50              | 4.5| 45         | 2.00                        | 20/1                             | 10.59     |
| 20  | 50              | 5.0| 60         | 1.00                        | 25/1                             | 6.77      |
| 21  | 55              | 3.0| 90         | 1.75                        | 20/1                             | 0.37      |
| 22  | 55              | 3.5| 30         | 2.00                        | 25/1                             | 0.23      |
| 23  | 55              | 4.0| 45         | 1.00                        | 30/1                             | 0.059     |
| 24  | 55              | 4.5| 60         | 1.25                        | 10/1                             | 22.36     |
| 25  | 55              | 5.0| 75         | 1.50                        | 15/1                             | 15.42     |
Table 2 shows the extreme difference between factors in the orthogonal experiments. The relationship among factors in terms of extreme difference was: pH value > mass ratio of chitosan and enzyme > enzymolysis time > enzymolysis temperature > substrate concentration. Therefore, in terms of chitooligosaccharide yield, the pH level is the fundamental factor, mass ratio of chitosan and enzyme is the second most important factor, and enzymatic hydrolysis time is the third most important factor. RSM was carried out with these three factors, with the enzymolysis temperature fixed at 45°C and the substrate concentration fixed at 1.75%.

| Factors               | X1  | X2    | X3    | X4         | X5                                      |
|-----------------------|-----|-------|-------|------------|-----------------------------------------|
| Temperature (°C)      | K1  | K2    | K3    | K4         | K5                                      |
| pH                   | 24.7999 | 39.0849 | 39.0879 | 19.7276    | 38.4351                                 |
| Enzymolysis time (min)| 2.4577   | 5.9807    | 30.5267 | 66.4198    | 55.7514                                 |
| Substrate concentration (%) | 14.4034 | 34.4361 | 38.5053 | 45.8197 | 34.0521                                 |
| Mass ratio of chitosan and enzyme | 25.5405 | 34.3785 | 32.1119 | 35.0524 | 15.9592                                 |

Table 2. Analysis of extreme difference in orthogonal experiment.

3.3. Results and analysis of the response surface methodology data

3.3.1. Establishment of the regression equation. Based on the results of the orthogonal experiments, the 45°C enzymatic hydrolysis temperature, 1.75% substrate concentration, and chitooligosaccharide yield (%) were taken as the response values to investigate the interaction among the three experimental factors: pH value (X1), mass ratio of chitosan and enzyme (X2) and enzymolysis time (X3). Using quadratic orthogonal rotational combination design, the experimental design was constructed as shown in Table 3, to obtain the chitooligosaccharide yield under the corresponding conditions. As shown in Table 3, the value ranges of X1, X2, and X3 are not of the same order of magnitude. Therefore, to facilitate data processing, the value ranges were represented as follows: 0.8 ≤ X1 ≤ 1.0, 0.67 ≤ X2 ≤ 1.00, and 0.33 ≤ X3 ≤ 1.00. SAS software (ver. 9.1.3; SAS Institute) was used to obtain the regression equation for the relationship between the factors and the yield of chitooligosaccharide. The regression equation is as follow:

\[
Y_1 = -806.68 + 1739.27X_1 - 49.50X_2 + 179.88X_3 - 957.14X_1^2 - 28.25X_2^2 - 75.89X_3^2 + 70.66X_1X_2 - 69.62X_1X_3 + 22.38X_2X_3
\]  

(4)
Table 3. Scheme and results of rotational quadratic orthogonal experiments.

| NO | X₁  | X₂ (S: E) | X₃ (min) | Yield (%) |
|----|-----|-----------|----------|-----------|
| 1  | 4.00| 5.0       | 60       | 26.30     |
| 2  | 4.00| 7.5       | 67.5     | 25.41     |
| 3  | 4.00| 10.0      | 75.0     | 28.85     |
| 4  | 4.00| 12.5      | 82.5     | 27.15     |
| 5  | 4.00| 15.0      | 90.0     | 21.44     |
| 6  | 4.25| 7.5       | 60.0     | 30.87     |
| 7  | 4.25| 10.0      | 67.5     | 34.98     |
| 8  | 4.25| 12.5      | 75.0     | 31.16     |
| 9  | 4.25| 15.0      | 82.5     | 27.34     |
| 10 | 4.25| 5.0       | 90.0     | 33.00     |
| 11 | 4.50| 10.0      | 60.0     | 38.71     |
| 12 | 4.50| 12.5      | 67.5     | 33.85     |
| 13 | 4.50| 15.0      | 75.0     | 35.29     |
| 14 | 4.50| 5.0       | 82.5     | 34.58     |
| 15 | 4.50| 7.5       | 90.0     | 36.12     |
| 16 | 4.75| 12.5      | 60.0     | 30.08     |
| 17 | 4.75| 15.0      | 67.5     | 31.18     |
| 18 | 4.75| 5.0       | 75.0     | 35.18     |
| 19 | 4.75| 7.5       | 82.5     | 30.69     |
| 20 | 4.75| 10.0      | 90.0     | 35.92     |
| 21 | 5.00| 15.0      | 60.0     | 22.79     |
| 22 | 5.00| 5.0       | 67.5     | 26.13     |
| 23 | 5.00| 7.5       | 75.0     | 23.24     |
| 24 | 5.00| 10.0      | 82.5     | 32.59     |
| 25 | 5.00| 12.5      | 90.0     | 24.55     |
| 26 | 4.50| 10.0      | 60.0     | 33.02     |
| 27 | 4.50| 10.0      | 67.5     | 35.49     |
| 28 | 4.50| 10.0      | 75.0     | 38.97     |
| 29 | 4.50| 10.0      | 82.5     | 37.09     |
| 30 | 4.50| 10.0      | 90.0     | 36.30     |

Table 4 shows the response surface quadratic model analysis of variance. The value of F is 10.63 (P < 0.0001), indicating that the effect of all factors in the model is significant, and R² = 0.9271. Therefore, the regression equation obtained via quadratic orthogonal rotational combination design has a good fit.
Table 4. Variance analysis of regression equation.

| Sources of variation | Freedom degree | Sum of squares | Mean square | F-value | P-value | R²       |
|----------------------|---------------|----------------|-------------|---------|---------|----------|
| Regression model     | 9             | 588.3          | 65.36       | 10.63   | <0.0001 |          |
| Error                | 20            | 122.9          | 6.147       |         |         |          |
| Sum                  | 29            | 711.2          |             |         |         | 0.921    |

Table 5 shows the parameters of the quadratic regression model. The coefficient of $X_3$ is negative. The cross terms in the equation have little effect on the experimental results, so they can be ignored. Thus, another simulation equation can be obtained as follows:

$$Y_2 = -744.73 + 1648.26X_1 + 28.97X_2 + 70.30X_3 - 914.37X_1^2 - 24.78X_2^2 - 40.56X_3^2 \quad (5)$$

Table 5. Parameters of quadratic regression model.

| Source of variance | DF | Parameter Estimate | Standard Error | T-Value | P-Value | Significance |
|--------------------|----|--------------------|----------------|---------|---------|--------------|
| Constant term      | 1  | -806.7             | 172.8          | -4.67   | 0.0001  | *            |
| $X_1$              | 1  | 1739               | 308.2          | 5.64    | <0.0001 | *            |
| $X_2$              | 1  | -49.5              | 98.53          | -0.5    | 0.6209  |              |
| $X_3$              | 1  | 179.88             | 185.5          | 0.97    | 0.3439  |              |
| $X_2^2$            | 1  | -957.1             | 141.5          | -6.76   | <0.0001 | *            |
| $X_1^2$            | 1  | -28.25             | 12.74          | -2.22   | 0.0383  |              |
| $X_1X_2$           | 1  | -75.89             | 54.96          | -1.38   | 0.1826  |              |
| $X_1X_3$           | 1  | 70.66              | 77.54          | 0.91    | 0.373   |              |
| $X_2X_3$           | 1  | -69.62             | 154.5          | -0.45   | 0.6571  |              |
| $X_2X_3$           | 1  | 22.38              | 46.36          | 0.48    | 0.6346  |              |

The equation can be solved by completing the square to obtain a set of optimal solutions. The F value was 16.89 ($P < 0.0001$), indicating that the effect of all factors in the model is significant, and $R^2 = 0.9150$. Therefore, the regression effect of the equation is significant.

3.3.2. Analysis of the response surface. Based on the regression equation, a related analysis graph of different factors can be made. The stereogram of the response surface analysis was drawn by Design Expert software (ver. 8.0.6; Stat-Ease Inc.), as shown in Fig.6.

Fig.6(a) shows the effects of pH and the substrate to enzyme ratio (S:E) on the chitooligosaccharide yield. When the enzymatic hydrolysis time is fixed at 75 min, the yield of chitooligosaccharide increases with the growth of pH. When the pH is 4.5, the chitooligosaccharide yield is highest; with the further increase in pH, the chitooligosaccharide yield decreases gradually. Excessive pH reduces the activity of papain and affects the binding of the enzyme to the substrate. When S:E increases from 5:1 to 15:1, the chitooligosaccharide yield increases initially and then decreases. High enzyme concentration increases the enzymatic efficiency, but when the enzyme concentration reaches the limit that the system can accommodate, any further increase of the enzyme concentration will hinder the movement of the enzyme molecule, thereby reducing the yield of chitooligosaccharide. When the S:E is about 8.7, the chitooligosaccharide yield is highest.

Fig.6(b) shows the effect of S:E and enzymatic hydrolysis time on the yield of chitooligosaccharide. When the pH is fixed at 4.5 and S:E is increased from 5:1 to 15:1, the yield of chitooligosaccharide reaches a maximum when S:E is 8.85. During the process of enzymatic hydrolysis, from 60 to 90 min, the chitooligosaccharide yield reaches a maximum at 77.4 min.
Fig. 6(c) shows the effect of enzymatic hydrolysis time and temperature on the chitooligosaccharide yield. It can be seen that the chitooligosaccharides yield is highest when S:E is 10:1, pH is 4.01 and enzymatic hydrolysis time is 77.5 min.

Figure 6. The three-dimensional (3D) response surface graph (left) and two-dimensional contour (right) plots of chitooligosaccharide yield as the function of (a) pH and mass ratio of chitosan and enzyme (S:E); (b) S:E and enzymolysis time; (c) pH and enzymolysis time.

3.3.3. Determination and verification of optimal process parameters of enzymolysis. From the rotational quadratic orthogonal experiment, it was found that when X1 = 0.9, X2 = 0.67, and X3 =
0.83, the chitooligosaccharide yield was optimized. To maximize $Y$, the first-order partial derivative and second-order partial derivative of $Y$ with respect to $X_1$, $X_2$, and $X_3$ should be zero. The first-order partial derivative of Equation 4 is obtained as follows:

$$\frac{\partial Y}{\partial X_i} = 0$$ (6), substitute $X_1 = 0.9$ and $X_2 = 0.67$ into Equation 6, then $X_1 = 0.87$;

$$\frac{\partial Y}{\partial X_i} = 0$$ (7), substitute $X_1 = 0.9$ and $X_2 = 0.83$ into Equation 7, then $X_2 = 0.58$;

$$\frac{\partial Y}{\partial X_i} = 0$$ (8), substitute $X_2 = 0.67$ and $X_3 = 0.83$ into Equation 8, then $X_3 = 0.903$.

Taking the second partial derivative, the solution of the Equation 4 is:

$$\frac{\partial Y}{\partial X_i X_j} = 0$$ (9), substitute $X_1 = 0.9$ into Equation 9, then $X_2 = 0.59$ and $X_3 = 0.86$;

$$\frac{\partial Y}{\partial X_i X_j} = 0$$ (10), substitute $X_2 = 0.67$ into Equation 10, then $X_1 = 0.90$ and $X_3 = 0.86$;

$$\frac{\partial Y}{\partial X_i X_j} = 0$$ (11), substitute $X_3 = 0.83$ into Equation 11, then $X_1 = 0.90$ and $X_2 = 0.59$.

Another a set of optimal solutions, $X_1 = 0.90$, $X_2 = 0.59$, and $X_3 = 0.86$, can be obtained from Equation 5 by completing the square. In addition, the optimal solution $X_1 = 0.90$, $X_2 = 0.59$, and $X_3 = 0.86$, which is obtained from the rotational quadratic orthogonal experiment, was taken into account, so there are eight optimal solutions in all. The experimental and theoretical values of the chitooligosaccharide yield, and the optimal enzymatic hydrolysis conditions, are shown in Table 6. The relative error between the calculated value of the regression model and the measured value of the experiment is less than 1%, which reflects the feasibility of the model in terms of relative error.

### Table 6. The experimental and theoretical values of the chitooligosaccharide yield and the optimal enzymatic hydrolysis conditions.

| No. | pH | mass ratio of chitosan and enzyme | Time (min) | Experimental value (%) | Theoretical value (%) | Relative error |
|-----|----|----------------------------------|-----------|------------------------|-----------------------|----------------|
| 1   | 4.5| 8.70                             | 74.7      | 37.70                  | 37.84                 | 0.37          |
| 2   | 4.5| 8.70                             | 74.7      | 37.71                  | 37.84                 | 0.34          |
| 3   | 4.5| 8.85                             | 77.4      | 37.78                  | 37.90                 | 0.32          |
| 4   | 4.5| 10.05                            | 74.7      | 37.47                  | 37.60                 | 0.35          |
| 5   | 4.5| 10.05                            | 78.3      | 37.66                  | 37.73                 | 0.19          |
| 6   | 4.5| 8.78                             | 78.0      | 37.75                  | 37.89                 | 0.37          |
| 7   | 4.5| 10.05                            | 77.5      | 37.56                  | 37.72                 | 0.42          |
| 8   | 4.5| 10.05                            | 74.7      | 37.33                  | 37.61                 | 0.74          |

#### 3.4. Average relative molecular weight of chitooligosaccharide

The average relative molecular weight of chitooligosaccharide was analyzed by reducing the end group DNS. Under the above optimal solution conditions, the pH was 4.50, the mass ratio of chitosan and enzyme was 8.85:1, the enzymatic hydrolysis time was 77.4 min, the enzymatic hydrolysis temperature was 45°C, and the substrate concentration was 1.75%. The average relative molecular weight of chitooligosaccharide was 1483 ($A_0 = 0.111, A_1 = 0.101$).

#### 4. Conclusions

Chitooligosaccharide was prepared via the enzymatic hydrolysis of chitosan with papain. Several factors, including enzymatic hydrolysis time, enzymatic hydrolysis temperature, mass ratio of chitosan and enzyme and substrate concentration, were studied. Response surface methodology was used to optimize the parameters for the preparation of chitooligosaccharide. Finally, under the optimal
conditions, namely pH of 4.50, mass ratio of chitosan and enzyme (S:E) of 8.85:1, and enzymatic hydrolysis time of 77.4 min, the enzymatic hydrolysis temperature was 45 °C, the substrate concentration was 1.75%, and the chitooligosaccharide yield was 37.78%. The actual yield of 37.78% was very close to the predicted value of the surface model, 37.90%, so the response surface model is suitable model to optimize the enzymatic hydrolysis conditions. In addition, the molecular weight of chitooligosaccharide prepared under the optimal conditions was found to be 1,483, which is lower than 3,000. These results provide basic data for the extraction of chitooligosaccharide via enzymatic hydrolysis of papain with chitosan as a raw material.

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