Orthogonal Experimental Study on Preparation of Chitosan Oligosaccharide by Chitooligase

Feng Xu¹, Zhaotao You¹, Haiwu Rong² and Xin Chen¹, *

¹School of Environment and Chemical Engineering, Foshan University, Foshan, Guangdong, China;
²School of Mathematics and Big Data, Foshan University, Foshan, Guangdong, China

*Corresponding author e-mail: 670511263@qq.com

Abstract. Chitosan oligosaccharides were prepared by enzymatic hydrolysis of chitosan with papain, and the chitosan oligosaccharides were accumulated, and the reducing sugar concentration and reducing sugar rate in the enzymatic solution were used as evaluation indexes. The results showed that with the increase of pH and reaction temperature, the concentration of reducing sugar in the enzymatic hydrolysate increased first and then decreased, and the appropriate reaction time, pH, reaction temperature, enzyme base ratio and substrate concentration were 1.0 h, 5.0, 45 ℃, 1:10 and 1.75%, respectively. In the process of degradation of chitosan by papain, the pH value had the greatest influence on the experiment, while the influence of the other four factors was not obvious, and the order of influence of the five factors was as follows: pH value > enzyme base ratio > substrate concentration > time > temperature.

1. Introduction
Enzymatic degradation of chitosan can be divided into specific enzyme and non-specific enzyme degradation. Chitosanase, n-acetylglucosamines and chitinase are specific enzymes, which can hydrolyze chitosan [1] with good degradation effect, producing chitosan oligosaccharides with high degree of polymerization. The study showed that the activity time of chitosan immobilized could be greatly increased. Chitosan solution and immobilized enzyme were continuously reacted for 10 times at 60 ℃, the enzyme activity remained basically unchanged, and the activity loss was less than 10%. Although the degradation effect and rate of the specific enzyme are not comparable to that of the non-specific enzyme, the specific enzyme cannot be industrialized and its cost is relatively high compared with the non-specific enzyme. Nonspecific enzymes include lysozyme, lipase, cellulase and papain. Under suitable reaction conditions, papain degrades chitosan the fastest, followed by cellulase. Besides, papain has low cost, good stability, and significant degradation effect on chitosan [2-3].

Therefore, this study used a non-specific enzyme (papayas’) to prepare chitosan oligosaccharide, which explored the effects of reaction temperature, time, pH, enzyme base ratio and substrate concentration on the preparation of chitosan oligosaccharide, and explored the optimal combination of conditions, improving the yield of chitosan oligosaccharide, and providing basic research data for the preparation of chitosan oligosaccharide by industrial enzymatic method. In addition, the semi-wet grinding method [4] was used to accumulate the prepared chitosan oligosaccharides, the high accumulation density chitosan oligosaccharides with the accumulation density greater than 0.50 g/mL were prepared, which provided experimental reference for the preparation of high accumulation...
density chitosan oligosaccharides, and proposed its application prospect in the field of cosmetics and health care products.

2. Experimental parts

2.1. Experimental design

2.1.1. Preparation of solution. Glucosamine hydrochloride solution: 1 g glucosamine hydrochloride was weighed and the volume was fixed to 1000 mL with distilled water.

DNS reagent: weigh 182 g potassium sodium tartrate, dissolve in 500 mL distilled water, heat, add 6.3g 3, 5-dinitrosalicylic acid, 21 g NaOH and 5 g phenol successively in the hot solution, stir to dissolve, cool with distilled water to 1000 mL, store in brown bottle at room temperature;

Acetic acid-sodium acetate buffer: 0.2 mol/L acetic acid solution: 7 mL glacial acetic acid, plus distilled water to 1000 mL; 0.2 mol/L sodium acetate solution: 27.2 g sodium acetate was weighed, dissolved in distilled water, constant volume to 1000 mL. The buffer solution with pH of 3.6, 4.0, 4.4, 5.0, 5.4 and 5.8 was proportionally configured.

2.1.2. Degradation of chitosan. A certain amount of chitosan was measured and dissolved in 20 mL acetic acid-sodium acetate buffer solution, which was shaken in an ultrasonic cleaning machine for 5 min. The corresponding proportion of papain was added and placed in a thermostatic water bath for degradation reaction. After the reaction, the enzyme was inactivated by boiling water bath heating for 5 min, and the papain was removed. The filtrate was poured into the dialysis bag for dialysis, the dialysate was retained, 3-5 times of ethanol was added into the dialysate for precipitation, the ethanol was evaporated and dried, and the oven was dried to obtain the degraded oligochitosan.

2.1.3. Determination of reducing sugar content in enzymatic hydrolysis solution. The content of reducing sugar in enzymatic hydrolysate was determined by DNS.

2.2. Experimental content

2.2.1. Determination of molecular weight. The molecular weight (Mw) of chitosan oligosaccharide was determined by high performance liquid chromatography (HPLC) according to the method described in literature [5]. Specific analysis conditions are as follows, chromatographic column: TSK g 3000-pwxl; Mobile phase: 0.2 m acetic acid /0.1m sodium acetate solution; Flow rate: 0.8 mL/min; Column temperature: 30 ℃; Detector: differential detector. The sample concentration was 0.4% (w/v). The gels were calibrated with different molecular weights of dextran 80,000, 50,000, 25,000, 12,000, 5000 and 1000 Da.

2.2.2. L25(55) Orthogonal experiment. On the basis of single factor experiment, the optimum influence range of reaction time, temperature, pH value, enzyme base ratio and substrate concentration on chitosan oligosaccharide degradation reaction was found. Time, temperature, pH value, enzyme base ratio and substrate concentration were used to make the orthogonal experiment of five factors and five levels, so as to obtain the best combination of these five factors.

2.2.3. Verification experiment. According to the best combination conditions obtained from the orthogonal experiment, the correctness of the experimental results is verified.

3. Results and discussion

3.1 Results and analysis of orthogonal experiment data
According to Table 1 and Table 2, 25 experiments were conducted and the following results were obtained.
Table 1. Orthogonal factors of chitosan enzymatic hydrolysis reaction.

| Standard | Factor | A Temperature (℃) | B pH value | C Enzymatic hydrolysis time (min) | D Substrate concentration (%) | E: S |
|----------|--------|-------------------|------------|----------------------------------|-------------------------------|------|
| 1        |        | 30                | 3.4        | 30                               | 1.00                          | 1:10 |
| 2        |        | 35                | 3.8        | 45                               | 1.25                          | 1:15 |
| 3        |        | 40                | 4.2        | 60                               | 1.50                          | 1:20 |
| 4        |        | 45                | 4.6        | 75                               | 1.75                          | 1:25 |
| 5        |        | 50                | 5.0        | 90                               | 2.00                          | 1:30 |

Table 2. Orthogonal experiments and results of chitosan enzymatic hydrolysis reaction.

| Temperature (℃) | pH | Time (min) | Substrate concentration (%) | E: S | Reduction sugar yield (%) |
|-----------------|----|------------|----------------------------|------|---------------------------|
| 30              | 3.4| 30         | 1.00                       | 1:10 | 0.18                      |
| 30              | 3.8| 45         | 1.25                       | 1:15 | 0.61                      |
| 30              | 4.2| 60         | 1.50                       | 1:20 | 0.23                      |
| 30              | 4.6| 75         | 1.75                       | 1:25 | 7.36                      |
| 30              | 5.0| 90         | 2.00                       | 1:30 | 5.39                      |
| 35              | 3.4| 45         | 1.50                       | 1:25 | 1.72                      |
| 35              | 3.8| 60         | 1.75                       | 1:30 | 2.31                      |
| 35              | 4.2| 75         | 2.00                       | 1:10 | 0.78                      |
| 35              | 4.6| 90         | 1.00                       | 1:15 | 10.10                     |
| 35              | 5.0| 30         | 1.25                       | 1:20 | 7.95                      |
| 40              | 3.4| 60         | 2.00                       | 1:15 | 0.44                      |
| 40              | 3.8| 75         | 1.00                       | 1:20 | 1.65                      |
| 40              | 4.2| 90         | 1.25                       | 1:25 | 4.46                      |
| 40              | 4.6| 30         | 1.50                       | 1:30 | 3.75                      |
| 40              | 5.0| 45         | 1.75                       | 1:10 | 20.81                     |
| 45              | 3.4| 75         | 1.25                       | 1:30 | 1.36                      |
| 45              | 3.8| 90         | 1.50                       | 1:10 | 3.56                      |
| 45              | 4.2| 30         | 1.75                       | 1:15 | 4.27                      |
| 45              | 4.6| 45         | 2.00                       | 1:20 | 0.36                      |
| 45              | 5.0| 60         | 1.00                       | 1:25 | 16.63                     |
| 50              | 3.4| 90         | 1.75                       | 1:20 | 0.96                      |
| 50              | 3.8| 30         | 2.00                       | 1:25 | 0.72                      |
| 50              | 4.2| 45         | 1.00                       | 1:30 | 1.91                      |
| 50              | 4.6| 60         | 1.25                       | 1:10 | 18.93                     |
| 50              | 5.0| 75         | 1.50                       | 1:15 | 12.85                     |

Table 3. Analysis of orthogonal experiment results.

| Number | Factors | A | B | C | D | E | Reduction sugar yield (%) |
|--------|---------|---|---|---|---|---|---------------------------|
| 1      |         | 1 | 1 | 1 | 1 | 1 | 0.18                      |
As shown in Table 3, according to the range R analysis, the primary and secondary relationship of the five factors on the yield of chitosan oligosaccharide was B > E > D > C > A. The optimal combination of enzymatic preparation of chitosan oligosaccharide was A5B5C3D4E1, that is, the temperature was 50 ℃, pH 5.0, the reaction time was 60 min, the substrate concentration was 1.75% and the enzyme substrate ratio was 1:10.

3.2 Verify experimental data and analysis
According to Table 3, the temperature of 50 ℃, the pH value of 5.0, the time of 60 min, and the optimal combination of substrate concentration of 1.75% and enzyme base ratio of 1:10 was used for the verification experiment. The results showed that the concentration of chitosan oligosaccharide in the enzymatic solution was 4.33 mg/mL, and the yield of chitosan oligosaccharide was 24.73%. The yield is higher than that of previous experiments.
3.3 Determination of molecular weight results and analysis

The result is shown in Figure 1.

![Figure 1. High performance liquid chromatography.](image)

The molecular weight (Mw) of chitosan oligosaccharide samples was analyzed under the following conditions. Mobile phase: 0.2m acetic acid /0.1m sodium acetate solution; Flow rate: 0.8 mL/min; Column temperature: 30℃; Detector: differential detector. The sample concentration was 0.4% (w/v). The gels were calibrated with different molecular weights of dextran 80,000, 50,000, 25,000, 12,000, 5000 and 1000 Da. As shown in Figure 1, the molecular weight of the product is 1600.

3.4 Results of accumulation density determination of chitosan oligosaccharide

| Number | Sifted for chitosan oligosaccharide (g) | Volume (mL) | Stacking density (g/mL) |
|--------|----------------------------------------|-------------|------------------------|
| 1      | 0.83                                   | 1.6         | 0.51                   |
| 2      | 0.75                                   | 1.4         | 0.53                   |
| 3      | 0.78                                   | 1.6         | 0.49                   |

As can be seen from Table 4, the results of three measurements of the accumulation density of chitosan oligosaccharides were 0.51 g/mL, 0.53 g/mL and 0.49 g/mL, respectively, all belonging to the range of high accumulation density. Besides, the average accumulation density was 0.51 g/mL.

4. Conclusions

(1) With the increase of pH value and reaction temperature, the concentration of reducing sugar in the enzymatic hydrolysate increased first and then decreased. The appropriate reaction time, pH, reaction temperature, enzyme base ratio and substrate concentration were 1.0 h, 5.0, 45 ℃, 1:10 and 1.75%, respectively.

(2) In the process of degradation of chitosan by papain, the pH value had the greatest influence on the experiment, while the influence of the other four factors was not obvious, and the order of influence of the five factors was as follows: pH value > enzyme base ratio > substrate concentration > time > temperature.

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