Orthogonal experimental study on preparation of chitosan oligosaccharide based on cellulase hydrolysis

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Abstract. The conditions of enzymatic hydrolysis of chitosan by cellulase were studied by orthogonal experiments. The effects of temperature, pH, reaction time and enzyme dosage on the decomposition efficiency of chitosan were investigated and the following conclusions were obtained by experiments: the major and minor relationship between the factors affecting the conversion of chitosan oligosaccharide prepared by cellulase hydrolysis is temperature > pH > time. The optimum conditions are as follows: at the reaction temperature of 50 °C, the pH of 4.8 and the hydrolysis time of 3.5 h. Under these reaction conditions, the content of reducing sugar is the highest, which is 1.59 mg·mL⁻¹, and the economic benefit is the best.

1. Introduction

Chitosan is grayish white or white with pearl glossy flake or powdered solid, which decomposes at about the temperature of 185 °C. The relative molecular weight varies from hundreds of thousands to millions due to different preparation methods and raw materials [1]. Insoluble in water and alkaline solution, it can be dissolved in some organic acids and dilute inorganic acids, such as hydrochloric acid, salicylic acid, nitric acid, acetic acid, etc., but insoluble in dilute sulfuric acid, phosphate, etc. [2]. In acidic aqueous solution, hydroxyl, amino and other polar groups in chitosan molecules are hydrated with water molecules to form transparent and viscous chitosan colloidal solution. When immersed in acidic solution for a long time, because of the existence of its semi-acetal structure, it is easy to be degraded in acidic solution, and the viscosity of the solution decreases. Chitosan contains a large number of amino groups and hydroxyl groups, which can react with the molecules of other substances to prepare chitosan derivatives. Chitosan is a large number of natural alkaline polysaccharides found so far, which has unique physiological functions and characteristics, such as moisture retention, film formation, adsorption, antibacterial, viscosity and biocompatibility. Chitosan has the biological function of cellulose in higher plant tissue and collagen in higher animal tissue, and has the characteristics of oral non-toxicity and biodegradability. It is called the sixth essential element of human life by researchers in the fields of materials science and pharmacy [3].

Chitosan adsorbs many substances. Amino and hydroxyl groups in molecules can form stable chelates with many metal ions (such as Hg²⁺, Cu²⁺, Ag⁺, Pb⁺², Cd²⁺, etc.), which can be used for purifying tap water, treating heavy metal wastewater and separating of metal ions in hydrometallurgy. At present, the recovery of copper from industrial wastewater by chitosan has been industrialized, and the extraction of radioactive element U from seawater by chitosan is being studied [4]. There are free...
amino groups in chitosan molecules, which are protonized in dilute acid solution, so that chitosan molecular chain is positively charged and becomes cationic flocculant, and the general pollutants have negative electricity. Therefore, chitosan can cementify and precipitate substances with opposite charges. Chitosan has a strong flocculation effect on activated sludge, and ammonia matrix, amino acid, nucleic acid, halogen, enzyme, dye, etc. can be adsorbed by it by complexation and adsorption, so it can be used in the treatment of printing and dyeing wastewater, food industry wastewater and dye wastewater. Chitosan can flocculate and precipitate the effective components in wastewater, and it is non-toxic and harmless, so the precipitate can be recovered and purified and reused as raw materials or secondary materials, and the flocculant can also be reused after regeneration \[5\].

The purpose of this study was to explore the major and minor relationship between the factors affecting the conversion of chitosan oligosaccharide prepared by hydrolysis of Cellini.

2. Orthogonal experimental condition control

2.1. Orthogonal experiment of L25

The orthogonal experiment of three factors and five levels were conducted by time, temperature and pH, so as to find the best combination of the three factors.

2.2. Verification test

The best results of orthogonal test were verified.

3. Data analysis and Conclusion

3.1. Analysis and Conclusion of Orthogonal Experimental Data

The orthogonal experimental data and analysis of L25 (35) is shown in Table 1.

| Test number | Temperature (°C) | Time (h) | pH  | Experimental results |
|-------------|------------------|----------|-----|----------------------|
| 1           | 40               | 2.5      | 3.6 | 0.653                |
| 2           | 40               | 3.5      | 5.4 | 0.692                |
| 3           | 40               | 4        | 4   | 1.123                |
| 4           | 40               | 4.5      | 5   | 0.796                |
| 5           | 45               | 2.5      | 5.4 | 0.697                |
| 6           | 45               | 3        | 4   | 1.239                |
| 7           | 45               | 3.5      | 5   | 1.126                |
| 8           | 45               | 4        | 3.6 | 1.059                |
| 9           | 45               | 4.5      | 4.6 | 1.436                |
| 10          | 50               | 2.5      | 5   | 0.896                |
| 11          | 50               | 3        | 3.6 | 0.753                |
| 12          | 50               | 3.5      | 4.6 | 1.624                |
| 13          | 50               | 4        | 5.4 | 1.231                |
| 14          | 50               | 4.5      | 4   | 1.465                |
| 15          | 55               | 2.5      | 4.6 | 1.359                |
| 16          | 55               | 3        | 5.4 | 1.035                |
| 17          | 55               | 3.5      | 4   | 1.235                |
| 18          | 55               | 4        | 5   | 0.957                |
| 19          | 55               | 4.5      | 3.6 | 0.869                |
| 20          | 60               | 2.5      | 4   | 0.634                |
| 21          | 60               | 3        | 5   | 0.521                |
| 22          | 60               | 3.5      | 3.6 | 0.397                |
| 23          | 60               | 4        | 4.6 | 0.824                |
| 24          | 60               | 4.5      | 5.4 | 0.349                |

Mean value 1 = 1.234, 1.024, 1.358
Mean value 1 = 1.124, 1.024, 0.954
Mean value 1 = 1.295, 1.427, 1.123
Mean value 1 = 1.257, 1.012, 1.567
Mean value 1 = 0.954, 0.859, 1.459

Range = 0.248, 0.169, 0.201
As shown in Table 1, the range of pH, temperature and time becomes smaller in turn, so the major and minor relationship of the influence of each factor on the conversion rate is temperature > pH > time. The temperature has the greatest influence on the experimental results, followed by the pH. The influence of time of enzyme hydrolysis is the smallest. According to the order of influence degree from large to small and the mean value of each factor, the optimal values of each factor are selected one by one. Eventually, the optimal values of each factor are temperature of 50 °C, pH of 4.8 and time of 3.5 h.

3.2. Verification test

The reaction conditions were controlled at the temperature of 50 °C, the pH of 4.8 and the time of 3.5 h and the amount of enzyme added was 50 U·mg⁻¹. The concentration of reducing sugar obtained at this time was 1.59 mg·mL⁻¹, which was the optimum value in the orthogonal experiments. Therefore, the most suitable conditions for the preparation of low molecular weight chitosan oligosaccharide by cellulase hydrolysis are as follows: the reaction temperature of 50 °C, the pH of 4.8 and the hydrolysis time of 3.5 h and the amount of enzyme added is 50 U·mg⁻¹.

3.3. Analysis and Results of Molecular Weight Determination

The determination and calculation of molecular weight before degradation is shown in Table 2, and the determination and calculation of molecular weight after 3.5 h of degradation is shown in Table 3.

### Table 2. Determination and Calculation of Molecular Weight before Degradation.

| Project | Solvent NaAc-HAc | Chitosan solution |
|---------|------------------|------------------|
| Apparent viscosity/mPa·s | 1.12 | 59.6 |
| The first time flowing through the capillary/s | 58 | 59.7 |
| The second time flowing through the capillary/s | 57.9 | 59.6 |
| The third time flowing through the capillary/s | 57.9 | 59.6 |
| Average value/s | 57.9 | 59.6 |
| Concentration/g·cm⁻³ | 0 | 0.010 |
| Relative viscosity η_r | 1.029 | 0.029 |
| Increased specific viscosity η_sp | 0.029 | |

Using a one-point empirical formula \([\eta]=\left[\eta_{sp}+3\ln\eta_r\right]/4C\), \([\eta]=31.47\;\text{cm}^3\cdot\text{g}^{-1}\). According to Mark-Houwink. The formula \([\eta]=KM^a\), wherein \(K = 1.\;424 \times 10^{-4}\;\text{cm}^3\cdot\text{g}^{-1}\), \(a = 0.96\), whereby the molecular weight \(M\) can be calculated to be 33 523.14.

### Table 3. Determination and Calculation of Molecular Weight after Degradation for 3.5h.

| Project | Solvent NaAc-HAc | Low molecular weight chitosan solution |
|---------|------------------|--------------------------------------|
| Apparent viscosity/mPa·s | 2.85 | 59.85 |
| The first time flowing through the capillary/s | 52.13 | 58.12 |
| The second time flowing through the capillary/s | 52.03 | 58.12 |
| The third time flowing through the capillary/s | 52.15 | 59.38 |
| Average value/s | 52.1 | 60.31 |
| Concentration/g·cm⁻³ | 0 | 0.0028 |
| Relative viscosity η_r | 1.115 | |
| Increased specific viscosity η_sp | 0.091 | |
Using a one-point empirical formula \([\eta] = [\eta_{sp} + 3\ln\eta_r]/4C\), \([\eta] = 31.47 \text{ cm}^3\cdot\text{g}^{-1}\). According to Mark-Houwink Formula, the molecular weight can be calculated to be 4514.68. The solubility was measured to be 1.2.

4. Conclusion
The major and minor relationship between the factors affecting the conversion of chitosan oligosaccharide prepared by cellulase hydrolysis is temperature > pH > time. The optimum conditions are as follows: at the reaction temperature of 50 °C, the pH of 4.8 and the hydrolysis time of 3.5 h.

Under these reaction conditions, the content of reducing sugar is the highest, which is 1.59 mg·mL\(^{-1}\), and the economic benefit is the best.

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