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Process optimization of chitosan extraction and deacetylation in ants

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Abstract: In this experiment, ants were used to extract lipids and proteins, and then decolorized. Finally, the alkali immersion method was used to remove acetyl groups and extract chitosan. The effect of alkali immersion method on the deacetylation of chitosan was determined by different temperature, treatment time and material-liquid ratio, so as to obtain the best conditions of deacetylation of chitosan. The experimental results show that the optimal condition for deacetylation of chitosan is 85℃, time 4 h, material-liquid ratio of 1:25. Chitosan has physiological functions such as bacteriostasis, fat reduction, anticancer, and improving immunity. It contains excellent cell affinity, degradability and biocompatibility, as well as various health functions such as enhancing immunity, improving digestive function, reducing cholesterol, regulating human acid-base metabolism balance, and eliminating harmful heavy metals.

1. Introduction

The raw material of chitin production is shrimp and crab shell, in which the content of chitin is 20%~30%. The method is mainly traditional chemical acid and base method, EDTA method and enzymatic method developed in recent years. In recent years, the focus of research and development has gradually shifted towards resource insects and fungi. Ants have strong adaptability, fast growth rate, strong reproductive ability, are not limited by regional environment, can achieve high density, low cost three-dimensional breeding, can save money and reduce costs. Therefore, on the basis of the analysis of ants, from the extraction method and process of chitosan extraction is studied, and then get the best extraction scheme.

2. Background and meaning

2.1 Research Background

Chitosan, also known as chitin, is the product of deacetylation of acetyl group in chitin. It is more soluble than chitin. It is a branched chain polymer compound [1] of N-acetyl-D-glucosamine monomer connected by β -1, 4-glycosidic bond. A large amount of chitin exists in the exoskeleton of the species of animals such as ants, the carapace of marine arthropods, the shells and bones of molluscs, the cell membranes of fungi and algae, and the cell walls of higher plants. Chitin is an important natural macromolecule, second only to cellulose in content. It is a recyclable renewable

resource, around the world, most of these natural polymers are in some coastal countries [2].

Chitin and chitosan have physiological effects such as bacteriostasis, fat reduction, anticancer, and improving immunity[3]. They contain excellent cell affinity, degradability and biocompatibility. They are used in food processing, textile, environmental protection, beauty and health care, medical material manufacturing and other fields. Chitosan has a variety of health functions such as enhancing immunity, improving digestive function, reducing cholesterol, regulating human acid and base metabolism balance [4]. After chitosan treatment, fruits and vegetables can form a film on the surface to inhibit atmospheric exchange, thus delaying fruit senescence, reducing decay, and increase the storage time of [5]. Chitosan composite film has obvious preservation effect on cooled pork, effectively extends the shelf life of cooled pork, and provides a theoretical basis for its application in fresh food preservation [6]. Chitosan composite coating film can effectively reduce the decay rate and weight loss rate of winter jujube, maintain good hardness, reduce the consumption of soluble solids, titratable acid, vitamin C and other nutrients, and maintain the high POD activity [7] in the fruit. Chitosan and plant compound for compound preservation, has a wide range of raw material sources, low cost, non-toxic and harmless to human body and other advantages [8]. The treatment of cassia bark compound clove extract combined with chitosan coating film has a good effect in inhibiting the titrating acid content, malondialdehyde content, and increasing the activity of polyphenols oxidase, and maintains the water content of summer black grape [9] to a certain extent. Chitosan and derivatives are not easily dissolved in aqueous and alkali solutions, but they can be dissolved in low concentration of inorganic acid. After dissolution, they will show a sticky state, and most of them can be slowly dissolved in acidic solution, forming chitosan molecules after dissolution. In addition, it has good biocompatibility, strong degradability, and good film forming ability, and has been widely used in the clinical medical field, and chitin and its derivatives have been used in the slow and sustained release of drugs [10]. Because of the advantages of wide source, low price and many response sites, in cancer treatment, due to the continuous deepening understanding of cancer, chitosan nanoparticles can use passive targeting, active targeting and stimulation-response to improve the effect of drugs on cancer cells [11]. Researchers modified chitosan and its derivatives to make chitosan more effective and safe for plant pest control, food preservation, crop fertilization and plant growth regulation, and make it play an increasingly important role in food preservation, feed improvement, organic fertilizer and the development and research of forest copper, toxic maid, glyphosate pesticide sustained-release drugs[12]. In recent years, with the understanding of chitosan and its wide application in industry.

As early as in the early 1980s, China put forward the recycling of shrimp shells. After years of practice and exploration, with the acid-base method as the core, it continued to optimize and improve it, and laid a technical foundation for industrialization. The raw material of the traditional chitin production method is shrimp and crab shell, in which the content of chitin is 20%~30%. The methods are mainly the traditional chemical acid and base method, EDTA method and [13] enzymatic method developed in recent years. In recent years, the focus of research and development has gradually shifted to resource insects and fungi, preparing chitin and chitosan by traditional acid and base method; extracting chitin from cicada shed, with a yield of 34.3%, much higher than shrimp shell, removing protein and calcium salt by bee and cooking chitosan by 50%NaOH alkali; preparing chitin by cicada, pipa and Chinese rice locust. The acid-base method is mostly used to extract chitin from insects, but different from shrimp and crab, the amount of inorganic salt, protein and fat is different from shrimp and crab shells. Due to the different extraction and processing methods and raw materials used, the chitin samples are different in all aspects. Ants have strong adaptability, fast growth rate, strong reproductive ability, and are not limited by regional environment, and can achieve high-density, low-cost three-dimensional breeding, which can save money and reduce feeding costs. Therefore, on the basis of the analysis of ants, from the extraction

method and process of chitosan extraction is studied, and then get the best extraction scheme.

2.2 Study significance

In the background of increasingly serious energy crisis and increasingly serious environmental pollution, the traditional process of chitin production needs to be improved. Many scientific research institutes have developed many new products by using new technologies such as resource treatment and enzymatic method, and have made great progress. Insects have become an important resource, and have shown broad prospects for utilization. The extraction of chitin and chitosan from insects has great potential for development. First, there are many kinds of insects on land, many of which can be used as raw materials. On Earth, there are about ten million species of insects that have been discovered, occupying about half of the living species. In addition, insects have the characteristics of rapid growth, season, region, resources, easy to artificial cultivation. In addition, extracting chitin from insects can obtain a lot of protein, vitamins, minerals and trace elements, which is a new method for many birds with one stone. Ants are a very abundant biological resource with more than 12000 species in 21 subfamilies known worldwide and more than 1000 species in 7 subfamilies known in China. As a tonic for both medicine and food, ants not only have high medicinal value, but also are precious food. As early as in the Heavenly Palace of the Zhou Dynasty, it was recorded that "meat sauce is for the son of heaven", in the Ming Dynasty.

The Compendium of Materia Medica, a work of Li Shizhen, also has detailed records of the ant population and affirms the value of ants in eating. Modern scientific research has also found that the body of ants is rich in protein and various nutrients, and has good effects on the human body, so ants are called the "nutrition treasure house" [14]. Ants have a high protein content, higher than 62.4 percent and 56.6 percent of fish and poultry, and about the same amount as cow liver. In addition, the ant body also contains a lot of vitamin B1, B2, C, D, E and other vitamins, they also have calcium, iron, phosphorus, manganese, selenium, zinc, more than 20 kinds of trace elements, a certain biological activity of triterpenoids compounds, formic acid, alkaloids, interleukin-2 dopamine derivatives are good for human body nutrients, their pharmacological activity is very obvious [15]. So far, a large number of experimental results show that the ants can improve the body's immunity, the acute and chronic bronchitis, diabetes, ankylosing spondylitis and other diseases have very good therapeutic effect, can also play an anti-aging, anti-inflammatory, yan, analgesic, sedative effect, and its preparations in clinical also achieved very good effect.

At present, in the reported insect chitin-chitosan, ant chitin-chitosan research development is still less. Therefore, the study of ant chitin-chitosan extraction is of great significance to further expand the source of chitin production, use ants to create residual value and add development species for ant products.

2.3 Main tasks

The main task of this paper is the changbai mountain black ants as research material, the changbai mountain black ant degreasing, decorin, decolorization, and then the decolorizing ants after acetyl chitosan, by selecting different time, temperature, liquid than the alkali soak to remove acetyl, take single factor orthogonal experiment, to explore the best conditions, get the best condition of the chitosan deacetylation.

3. Materials and Methods

3.1 Materials

3.1.1 Experimental materials

Black ants (scientific name black thorn ants) from Changbai Mountain area were selected as raw materials and purchased from Bozhou Kangmei Traditional Chinese medicine Market.

3.1.2 Main Reagents

Methyl orange, hydrochloric acid, sodium hydroxide and hydrogen peroxide are all pure analytical reagents and were purchased from Sinophol Chemical Reagent Co., LTD. Petroleum ether (30-60℃) was purchased from Tianjin Zhiyuan Chemical Reagent Co., LTD.

Neutropsin, trypsin, papain, pepsin, were purchased from McLean Biology Co., Ltd.

3.1.3 Main instruments

For example, common instruments and manufacturers are listed in Table 1.

Table 1: Name and manufacturer

Instruments and models	Manufacture factory
Digital display drum air drying box BGZ 140	Shanghai Boxun Instrument Co., LTD
Analytical balance, BSA224S-CW	Beijing Sidis Scientific Instrument Co., LTD
Digital constant temperature water bath HH-3A	Changzhou Guoyu Instrument
Low-temperature high-speed freezing centrifuge H1850R	Shanghai Lu Xiangyi Instrument Co., LTD
Multi-function induction cooker, C21-RT2160	Guangdong Midea Co., Ltd
Cryogenic cold storage grinder JX-CL	Shanghai Jingxin Industrial Development Co., LTD
SOCZ GY-QZ CDY	Shanghai Guiyong Electronics Co., Ltd
PH meter phs-25	Shanghai Lemagnetic Instrument Co., LTD
Ultrapure water unit, Milli-Q	American Millipore, Inc

3.2 Experimental method

Chitosan was extracted from ants in four steps, including defat, deprotein, decoloring and deacetylation.

3.2.1 Ant skim

The black ants from Changbai Mountain were washed repeatedly to filter out debris, debris and impurities, and then dried at 65℃. After drying, grind it into fine powder with a mortar or grinder, then put 1 g of fine powder into a filter paper bag, Put the filter paper bag with Changbai Mountain ant sample into a drum drying box with a temperature of 105℃ for a period until the quality remains unchanged; When weighing reaches a certain value, remove the filter paper bag with Changbai Mountain ant sample, and put it into the extraction bottle. Researchers can add a certain amount of petroleum ether to a glass receiving bottle that has been dried to a constant weight;In the Soxhlet extraction device, the extraction bottle is separated from the receiving bottle, reflux extraction is adopted, and the extraction cycle is appropriate.After the extraction is completed, condensate is carried out to recover the petroleum ether, and then the receiving bottle is

disassembled and then recycled so that it can be used for secondary use and save costs.Finally, the researchers removed the extracted paper bag, poured out the ant powder inside, and then baked it for a while in a drum drying oven.

3.2.2 Ant deprotein

The researchers took 5 g of defatted ant powder and added it to 500 mL beaker, added 250 mL of distilled water, 0.1 g of neutral protease, 0.1 g of trypsin and 0.1 g of papain, and adjusted the pH to 8.2. The beaker was placed in a 55℃ constant temperature water bath and heated for 2 h.After the water bath was heated, the researchers placed beakers on an induction cooker to deactivate enzymes and then cooled them to room temperature.After cooling, he added 0.1g of pepsin, adjusted the pH to 5.2, and then placed the beaker in a 55℃ constant temperature water bath and repeated the above steps. After cooling, 0.1 g of pepsin was added, pH was adjusted to 5.2, and the beaker was placed in a 55℃ constant temperature water bath, and the above steps were repeated. After cooling, the ant powder was poured into a centrifuge tube and placed in a centrifuged at 3000 r/min for 15 min and the post-centrifuged sediment was washed with distilled water until it became neutral. It was then dried with a blast drying box at 65℃ and removed.

3.2.3 Ant discoloration

The deproteinized dried ant powder was added to a 50 mL centrifuge tube, then 30% H2O2 solution with a solid to liquid ratio of 1:20 and then heated in constant temperature at 80℃ water for 2 h. It was then removed, cooled to room temperature, centrifuged at 4000 r/min for 15 min, and the precipitate after centrifugation was washed with distilled water until it became neutral. It was then dried with a blast drying box at 65℃, dried and removed.

3.2.4 Ant deacetylation

The decolorized dried ant powder was added to a 50 mL centrifuge tube, then 40% NaOH solution was added to a thermostatic water bath to detach the acetyl group. The centrifuged precipitate was washed with distilled water until it became neutral and then placed on a blast drying box, dried at 65℃, taken out, dried and weighed with an electronic balance.

3.2.5 Deacetylated univariate experiment

Three factors of material-liquid ratio, extraction temperature and extraction time were selected as experimental factors to investigate the influence of the three factors on the deacetylation of ants in Changbai Mountain. Univariate test is one factor change, and other conditions are fixed. By measuring the value of deacetylation, the influence of changing factors on the experiment is explored.

The ranges of single factor experiment are: treatment temperature (55℃, 65℃, 75℃, 75℃, 85℃, 95℃), treatment time (2 h, 3 h, 4 h, 5 h, 6 h), material to liquid ratio (1:15,1:20,1:25,1:30,1:35); and determine the optimal range of each factor.

(1) Effect of temperature on deacetylation: 40%NaOH, 2 g substrate, material-liquid ratio was fixed at 1:25, the treatment time was 4 h, 55℃, 65℃, 75℃, 75℃, 85℃, 85℃ and 95℃ for comparative experiments.

(2) The effect of time on deacetylation: 40%NaOH, substrate 2 g, the material-liquid ratio was fixed at 1:25, the treatment temperature was 85℃, and the treatment time was 2 h, 3 h, 4 h, 5 h, 5 h and 6 h respectively.

(3) Effect of liquid ratio on deacetylation: 40%NaOH, 2 g substrate was used, the extraction time

was fixed at 4 h, the treatment temperature was 85℃, and the material-liquid ratio was 1:15,1:20,1:25,1:30,1:35 and 1:35.

3.2.6 Deacetylated orthogonal experimental design

According to the results of one-factor experiment, the liquid ratio, extraction temperature and extraction time were selected as the experimental factors, and the L9 (34) test was used for optimizing the level of orthogonal test factors is shown in Table 2.

Table 2: Level table of orthogonal test design factors

level	A time h	B temperature ℃	C liquid ratio	D liquid ratio
1	3	75	1:20	---
2	4	85	1:25	---
3	5	95	1:30	---

4. Determination method and analysis of deacetylation degree

4.1 Deacetylation

4.1.1 Methyl-orange titration method

The researcher weighed the chitin sample obtained from the experiment about 0.1g, weighed each sample in parallel to 3 parts, added HCL solution (0.1mol /L)5 mL in a 50 mL conical bottle, and its dissolution temperature reached (20±5) ℃, stir with a glass rod to make the sample completely dissolved, and then added 3 drops of methyl orange indicator.Titrated with NaOH (0.1 mol/L), the end point is when the solution changes from red to yellow.He then put three sets of samples into a drum drying oven to dry to a certain weight, and then measured their water content.. The calculation method is performed as follows:(3-1)In formula 3-1, C1 is the concentration of standard hydrochloric acid solution (mol/L); C2 is the concentration of sodium hydroxide standard solution (mol/L); V1 is the volume (mL) of hydrochloric acid standard solution added; V2 is the volume (mL) of sodium hydroxide standard solution for titration; G is the sample mass (g); W is the moisture (%) of the sample; 0.016 is the amino amount (g) equivalent to 1 mL1 mol/L hydrochloric acid solution.(3-2)In formula 3-2,9.94% refers to the total amine content in the cruitin.

4.1.2 and the potential titration method

0.1 g of chitosan was precisely weighed and added with 5 mL of 0.1 mol/L standard dilute hydrochloric acid solution and stirred with a glass bar until completely melted. Was titrated with a 0.1 mol/L standard NaOH solution while recording the corresponding pH. With V (NaOH) as the abscissa and pH as the ordinate, make the corresponding figure, the line extrapolated to the intersection of the abscissa is the volume of NaOH solution used at the equal point, the calculation method is the same as above.

4.2 Univariate outcome analysis

4.2.1 Effect of heating time on deacetylation degree

2 g of filter residue after deadipose, deprotein and decolorization were treated with 50 mL of 40% NaOH in 85℃ water bath for 2 h, 3 h, 4 h, 5 h, 5 h and 6 h, and the deacetylation was determined by the 3.1.1 method. From Figure 1, when other conditions remain unchanged, deacetylation degree gradually increases with time growth, reaching a maximum of 21.2% at 4 h, and

deacetylation degree begins to decrease after 4 h, thus showing that the optimal time of deacetylation treatment at 2-6 h is 4 h.

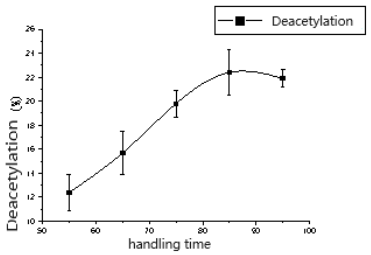

Figure 1: Deacetylation at different treatment times

4.2.2 Effect of temperature on deacetylation degree

The 2 g decalcification, protein, and decoloration were treated with 50 mL 40% NaOH at 55℃, 65℃, 75℃, 75℃, 85℃, 95℃ for 4 h, washed to pH of about 7, and deacetylation was determined by the 3.1.1 method.

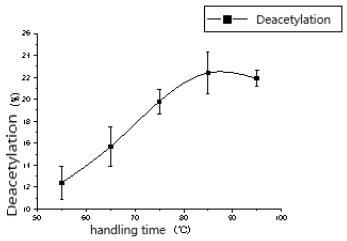

Figure 2: Deacetylation at different treatment temperatures

From Figure 2, under constant other conditions, the deacetylation degree gradually increased with increasing temperature, reaching a maximum value of 22.4% at 85℃, and the deacetylation degree began to decrease after 85℃. It follows that 85℃ is the optimal deacetylation treatment temperature.

4.2.3 Effect of liquid ratio on deacetylation

2 g of decalcified, deproteinized, and decolorized filter residue were treated with 40% NaOH for 4 h at 85℃ water bath at 1:15 g/mL, 1:20 g/mL, 1:25 g/mL, 1:30 g/mL, 1:35 g/mL ratio. The deacetylation was determined by 3.1.1 method.

As can be seen from Figure 3, under other conditions, the deacetylation gradually increases as the ratio of the material-liquid ratio increases, reaching the maximum value of 20.1% at 1:25, and the deacetylation degree begins to decrease after the material-liquid ratio is greater than 1:25. Thus, 1:25 is the optimal deacetyl liquid ratio.

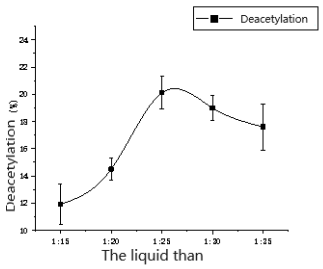

Figure 3: Deacetylation of different liquid ratios

4.3 Analysis of the orthogonal experimental results

Based on the single factor test, L9 (34) orthogonal test design was used to arrange the test to optimize the conditions for deacetyl extraction of chitosan by alkali immersion method. The test scheme and result analysis are shown in Table 3 below. According to the extreme difference analysis in Table 3, it can be concluded that the primary and secondary order of each factor on the deacetylation of chitosan is B> C> A, that is, the treatment temperature, followed by the material-liquid ratio, and finally the treatment time. According to the size of k value, the optimal extraction process condition of chitosan deacetylation is A2B2C2, that is, the experimental extraction condition is 22.8% under the treatment temperature of 85℃, the treatment time of 4 h and the material-liquid ratio of 1:25.

Table 3: Table of orthogonal experimental design of black ants in Changbai Mountain and their results

Number	A time h	B temperature ℃	C liquid ratio	Deacetylation%
1	1(3)	1(75)	1(1:20)	18.1
2	1	2(85)	3(1:30)	21.8
3	1	3(95)	2(1:25)	20.3
4	2(4)	1	3	19.5
5	2	2	2	22.8
6	2	3	1	20.7
7	3(5)	1	2	19.8
8	3	2	1	21.6
9	3	3	3	18.7
K_1	60.80	58.40	60.60	
K_2	62.90	65.50	63.10	
K_3	59.80	59.60	59.80	
k_1	20.27	19.47	20.20	
k_2	20.97	21.83	21.03	
K_3	19.93	19.87	19.93	
range R	1.03	2.37	1.10	
optimal portfolio	$A_2B_2C_2$			

5. Conclusion and discussion

In this experiment, chitosan was extracted from black ants as raw material, and the reaction conditions for acetyl extraction from black ants after deadipose, protein and discoloration were determined. The following conclusions were obtained: the treatment temperature was 85℃, the treatment time was 4 h, the liquid ratio was 1:25, and the deacetylation of chitosan reached 22.8%. The chitosan was less deacetylated and chitosan was darker than industrial chitosan. The first reason for the low deacetylation may be that the defatting and deproteinof black ants are not complete, which affects the deacetyl group. The lye used for the second deacetyl group is 40% NaOH, and the concentration of the lye will also have an effect on the deacetyl group. One reason for the darker color of chitosan may be the effect of the concentration of 30% hydrogen peroxide used for decolorization, depending on the concentrations. Another deacetylation reaction also produced a change in the color of the filter residue after decolorization. In addition, the ant chitosan titration method to measure the deacetylation instrument is easy to use, the operation is less difficult, easy to observe, the measured results are more accurate, but there are many factors affect the accuracy of the measurement results. It can be seen from equation 3-1 on the calculation of chitosan

deacetylation due to the accuracy of sample weight. Chitosan is a dry powder, which can easily absorb water during the weighing process, resulting in higher weighing results than the actual results, thus making the calculated deacetylation lower than the actual results. Therefore, the moisture should be removed as much as possible; before the weighing, put the sample into a 65℃ constant temperature drying box, dry for 10 h, the contact time between the sample and air should be shortened as much as possible. On titration, the lye should be added slowly and shaken adequately to avoid excessive dropping.

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