EFFECT OF NAOH TREATMENT CONDITIONS ON THE NATURE OF FUNGAL CHITOSAN-GLUCAN COMPLEX AND CHITOSAN PRODUCTION

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Abstract

Cell wall of *Gongronella butleri* USDB 0201 mainly composes with chitin, chitosan, glucan and chitin/chitosan-β-glucan complex. Free chitosan could be isolated by treatment with 1 M NaOH, followed by extraction with 0.35 M acetic acid at 95°C for 5 h. Most of the chitin/chitosan in the fungal cell wall forms a complex with β-glucan in a very rigid myceliar matrix and can not be extracted under the conditions mentioned. In order to isolate more chitosan, it was demonstrated that the complex had to be (partially) detached from the myceliar matrix, subsequently the chitosan can be liberated from the chitosan-β-glucan complex. The myceliar matrix was treated with different NaOH and acidic acid conditions. It was observed that this matrix can be broken down by 11M NaOH at 45°C for 13 h followed by 0.35 M acetic acid at 95°C for 5 h. This is attributed to the breakdown of intramolecular hydrogen bonds or rearrangement of the polymers in the matrix. In order to isolate chitosan, the chitosan-β-glucan complex was treated with various enzymes. Termamyl, Type LS enzyme is able to split the chitosan-β-glucan complex and to liberate the chitosan. However enzyme action failed to isolate chitosan from myceliar material treated with only 1 M NaOH. Therefore the NaOH treatment condition is essential for the extraction of chitosan from fungal cell wall.

Keywords: Chitosan-glucan complex, Fungal chitosan, NaOH treatment, Termamyl Type LS enzyme

Introduction

Chitin is a natural polysaccharide, which consists of a copolymer of N-acetyl-D-glucosamine and D-glucosamine residues, linked by β-1,4 glucosidic bonds. The deacetylated form of chitin is chitosan. Chitosan with a molecular weight of 5-50 kDa was effective in cholesterol absorption [1], semipermeable membrane [2], antifungal and plant growth promotion [3] and enhance protocorm like body formation in orchid tissue culture [4]. The isolation of chitosan from fungal source could be permitted to obtain a low molecular weight chitosan (10-50 kDa with high polydispersity, 3-

5)[5]. Therefore fungal source may be an alternative chitosan source for agriculture and medical applications.

Several methods have been developed for the extraction of chitosan from fungal mycelia [6, 7, 8, 9, 10]. However yield of chitosan produced from the fungal mycelia is very low. A bottleneck for isolation of high yield pure chitosan from fungi cell wall is the binding of fungal chitosan to cell wall 1,3-β glucan [10]. The binding to 1,3-β glucan interferes the chitosan extraction. In 1994, Muzzarelli et al., developed a single alkaline extraction method to obtain a chitosan-glucan complex[9]. High yield of chitosan could be isolated from the chitosan-glucan complex, which obtained from the mycelia treated with 11 M NaOH, with the help of Termamyl enzyme [10]. Therefore NaOH treatment step is one of the critical points for the extraction of chitosan from fungal source.

In this research, sweet potato pieces were used as solid support and carbon source for the growth of fungus *Gongronella butleri* USDB 0201. Fungal mycelia were harvested at the end of fermentation and treated with different concentration of NaOH to obtain the alkaline insoluble materials. The nature of alkaline insoluble materials was studied under various acid treatment conditions. Finally, chitosan was extracted from the alkaline insoluble materials obtained from different NaOH treatments and compared the yield of chitosan.

Materials and methods

Gongronella butleri USDB 0201, which is belong to the class of Zygomycetes, was obtained from the Department of Biological Sciences, National University of Singapore. The strain was maintained on 3.9 percent potato dextrose agar (PDA) slants at 4^{o}C. The spore suspension for inoculation was prepared from a 10 day culture of *G. bulteri* grown on PDA plates at 30^{o}C.

Effect of acid treatment conditions on extraction of chitosan

Chitosan was extracted using a method proposed by Tan *et al.*, (1996), modified from Shimahara *et al*., (1988). Dried mycelia powder (1 g) was treated with 40 ml of 1 M sodium hydroxide and sodium borohydride, 0.05 g to prevent oxidation. The mixture was autoclaved at 121^{o}C for 15 min and centrifuged at 16000 g for 5 min to sediment the alkali insoluble material (AIM). The AIM was washed twice with distilled water, washed once with 95 % ethanol and dried. Chitosan was extracted from AIM with 200 ml of 0.35 M acetic acid per g dried AIM at various temperatures for 5 h (Table 1). The mixture was centrifuged at 16000 g for 5 min and the supernatant was collected. The supernatant was adjusted to pH 8 -9 with 2 M NaOH to precipitate the chitosan. The suspension was centrifuged at 16000 g for 5 min. The chitosan pellet was collected and washed twice with distilled water, once with 95 % ethanol and freeze-dried.

Effect of NaOH concentration, temperature and time on decomposition of chitosan-glucan complex

Dry mycelia powder (1 g) was treated with 40 ml of sodium hydroxide in various concentrations and sodium borohydride, 0.05 g at various treatment temperatures and times (Table 2). Alkaline insoluble materials were collected from each treatment and washed with water until neutral pH. The dried AIM, 1 g was treated with 200 ml of 0.35 M acetic acid at 95^{o}C for 5 h. The nature of chitosan-glucan matrix was studied.

Extraction of chitosan from chitosan-glucan complex

The fungus was grown on sweet potato (*Manihot esculenta*) pieces supplemented with mineral and urea. Mycelia was harvested at the end of fermentation and dried at 45^{0}C [5]. Dried mycelia were treated with 10 ml of 11 M NaOH per g mycelia at 45^{o}C for 13 h. Dried AIM (1 g) was treated with

200 ml of 0.35 M acetic acid at 95^{o}C for 5 h. The AIM suspension was adjusted to pH 4.5 with 1 M NaOH and treated with 4 % Termamyl, Type LS Novo (v/v of AIM suspension) and incubated in a shaking water-bath at 65^{o}C, 200 rpm for 3 h. The resultant AIM suspension was centrifuged at 1600 g for 15 min to obtain a clear chitosan solution. Chitosan was precipitated by adjusting the pH of the supernatant to 8-9 with 1M NaOH solution [10].

Characterization of chitosan

The degree of deacetylation was determined by first derivative ultraviolet spectrophotometry [13] with the modification of Tan *et al*. (1998)[14]. Relative molecular weight was determined by gel permeation chromatography (GPC) in a Waters HPLC equipped with Ultrahydrogel 2000, 1000 and 500 columns and a Waters 410 Differential Refractometer Detector. The solvent used was 0.2M acetic acid/0.1M sodium acetate buffer, pH 4.05 with the flow rate of 0.6ml/min. Dextrans of various molecular weights ranging from 9.9 x 10^3 to 2 x 10^6 Da were used as standards. IR-spectrum was recorded using an FTIR instrument (model 2000, Perkin Elmer, Norwalk, CT) scanning between 400 and 4000 cm^{-1}.

Results and discussions

Fungus, *Gongronella butleri* USDB 0201 was grown under solid substrate fermentation conditions. Fungal mycelia were harvested at the end of fermentation and dried. In the fungal mycelia, chitin/chitosan consists in 3 forms: free chitosan, free precursor chitin and chitosan/chitin covalently bound to glucan. The individual chains of chitin are aggregated into microfibrils by hydrogen bonds. These chitin microfibrils are cross-linked to the β-glucan and form a major component of the cell walls of most fungi [15]. Moreover the glucan chains together with hydrogen bonding among chitin chains could result in a rigid cross-linked network in the cell wall [16]. In Zygomycetes, chitosan is synthesized by deacetylation of chitin with the action of chitin deacetylase enzyme [17, 18]. The free chitosan could be extracted easily from the fungal mycelia. However decomposition of chitosan-glucan complex matrix and breaking the covalent bond between the chitosan and glucan steps are necessary for the extraction of chitosan from chitosan-glucan complex in fungal cell wall. Therefore the nature of chitosan-glucan complex obtained from the various treatment conditions was studied.

Effect of acid treatment conditions on extraction of chitosan

The fungal mycelia were treated with 1M NaOH at autoclave conditions 121^{o}C for 15 min. The resultant alkaline insoluble materials were very rigid. The alkaline insoluble materials were treated with 0.35 M acetic acid at various treatment temperatures for 5 h. The treatment at 95^{o}C for 5h gave a high chitosan yield but alkaline insoluble materials could not break down under this condition (Table 1). Extraction at 95^{o}C results in 3.14 g CTS per 100 g mycelia more than double of the amount extracted at 50^{o}C (1.44 g CTS/100 g mycelia). However some turbid particles also extracted out together with chitosan into acetic acid solution at 95^{0}C. These particles may be chitosan-glucan complex. The yield of chitosan decreased to 1.71 g CTS/100g of mycelia after 14 h incubation at 95^{o}C. Hu *et al*., 1999 reported that the high temperature treatment can be more efficient to isolate chitosan. However chain degradation of chitosan was observed when the acid extraction step was carried out in 2% acetic aicd or 1M hydrochloric acid at autoclave condition for 15 min [7]. Therefore the conditions, temperature 95^{o}C for 5 h were selected to study the decomposition of chitosan-glucan complex.

Table 1: Effect of acetic acid extraction temperature on the yield of fungal chitosan

Temperature (^oC)	CTS (g/100 g of mycelia)
50	1.44
60	2.00
80	2.46
90	2.79
95	3.14

Note: Fungal mycelia were grown on the sweet potato pieces impregnated with 250 ml of soymilk per kg of sweet potato at 100^{o}C for 15 min. After that the sweet potato pieces were sterilized at 121^{o}C for 20 min together with the mineral solution (Nwe *et al*., 2002) adjusted to pH 2 with 5 % H_2SO_4. The sterilized sweet potato pieces were inoculated with 1.05 x 10^8 spores/kg SS and incubated under supply of humidified air 0.2 l/min at 26 ± 2^{o}C for 7 days.

Effect of NaOH treatment conditions on decomposition of chitosan-glucan complex

Mycelia powders were treated with various concentrations of NaOH; 1.77 M (M1), 5.08 M (M2), 6.75 M (M3), 8.41 M (M4) 11.73 M (M5) for various time intervals at various temperatures. Alkaline insoluble materials (AIM) were collected and treated with 0.35 M acetic acid solution at 95^{o}C for 5 h. The residual acid-alkaline insoluble materials obtained from M1, M2 and M3 treatments were very rigid (Figure 1a). However turbidity was observed in the acid extraction solutions obtained from M4 and M5 treatments (Table 2). All AIM obtained from M5 treatment was totally broken down in the 0.35 M acetic acid within 5h at 95^{o}C (Figure 1b). Other conditions were not supported to break down the AIM in the acetic acid solution completely. Therefore the best alkaline treatment conditions for fungal chitosan production are 11.73 M NaOH at 46.2^{o}C for 13.5 h.

Table 2: Effect of NaOH treatment conditions on the decomposition of alkaline insoluble material (AIM)

Treatment	Temperature (^oC)	NaOH concentration (M)	Time (h)	Decomposition of AIM in acetic acid solution
1	79	1.77	13.50	-
2	46	1.77	13.50	-
3	79	5.08	4.93	-
4	46	5.08	4.93	-
5	95	6.75	13.50	-
6	63	6.75	13.50	-
7	30	6.75	13.50	-
8	79	8.41	22.07	+
9	46	8.41	22.07	+
10	79	11.73	13.50	++++
11	46	11.73	13.50	++++

Remark: Fungal mycelia were grown on the sweet potato pieces impregnated with 1 liter of mineral solution supplemented with 10 g peptone, pH 3.75 per kg of sweet potato at 100^{o}C for 15 min. Afterthat the sweet potato pieces were sterilized at 121^{o}C for 20 min. The sterilized sweet potato pieces were inoculated with 1.05 x 10^8 spores/kg SS and incubated under supply of humidified air 0.6 l/min 30^{o}C for 4 days.

Muzzarelli *et al.,* (1980) concluded that the residue obtained after treatment of *Aspergillus niger mycelia* with 40% NaOH consists of a chitosan-glucan complex, insoluble in acetic acid [19]. Therefore there are needed to remove glucan from the turbid solution to get pure chitosan (Figure 1b). Termamyl enzyme was used to remove the glucan from the chitosan-glucan complex

suspension [10]. However Termamyl enzyme could not accessible to cleave the bond between the chitosan and glucan in the chitosan-glucan complex obtained from the 1 M NaOH treatment due to it rigid structure (Figure 1c).

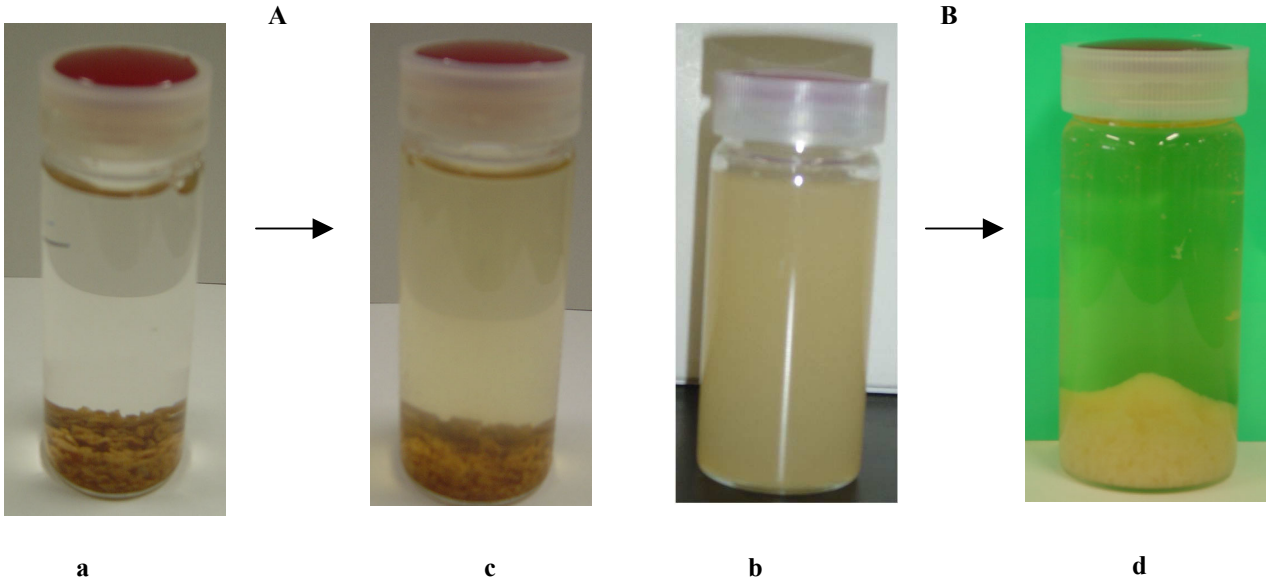

Figure 1: The nature of chitosan-glucan complex obtained from the fungal mycelia treated with 1M (A) and 11M NaOH (B) at 45^{o}C for 13 hr and treated with 0.35 M acetic acid at 95^{0}C for 5 h (a, b) followed by Termamyl treatment (c and d)

Extraction of chitosan from chitosan-glucan complex

The amount of dried mycelia harvested at the end of fermentation was 42 g/kg solid substrate. Chitosan was extracted from the dried mycelia. The yield of chitosan 1.74 g/100 g of mycelia was obtained from the mycelia treated with 1 M NaOH at 45^{o}C for 13 h and extracted with 0.35 M acetic acid at 95^{0}C for 5 h. The yield of chitosan was increased to 8.09 g/100 g of mycelia when the mycelia were treated with 11 M NaOH at 45^{o}C for 13 h and treated with 0.35 M acetic acid at 95^{0}C for 5 h followed by Termamyl treatment. In the treatment with 1 M NaOH, chitin is not deacetylated and not extracted. Chitosan as much present in the fungal cell wall was extracted. The yield of chitosan obtained from the fungal mycelia was increased about 4 times after treatment with 11 M NaOH. In this condition, chitin present in the mycelia cell wall is converted into chitosan and extracted during acid treatment and chitosan/chitin covalently link to glucan also extracted out with the help of Termamyl enzyme.

However the amount of chitosan decreased to 7.59 g/100 g of mycelia when the mycelia were treated with 11 M NaOH at 95^{o}C for 5 h. This confirms that chitosan is degraded during the treatment with hot concentrated NaOH to the mycelia.

The degree of deacetylation of fungal chitosan was about 87 % and number average molecular weight was about 55 kDa. The IR spectrum of the fungal chitosan is shown in Figure 2.

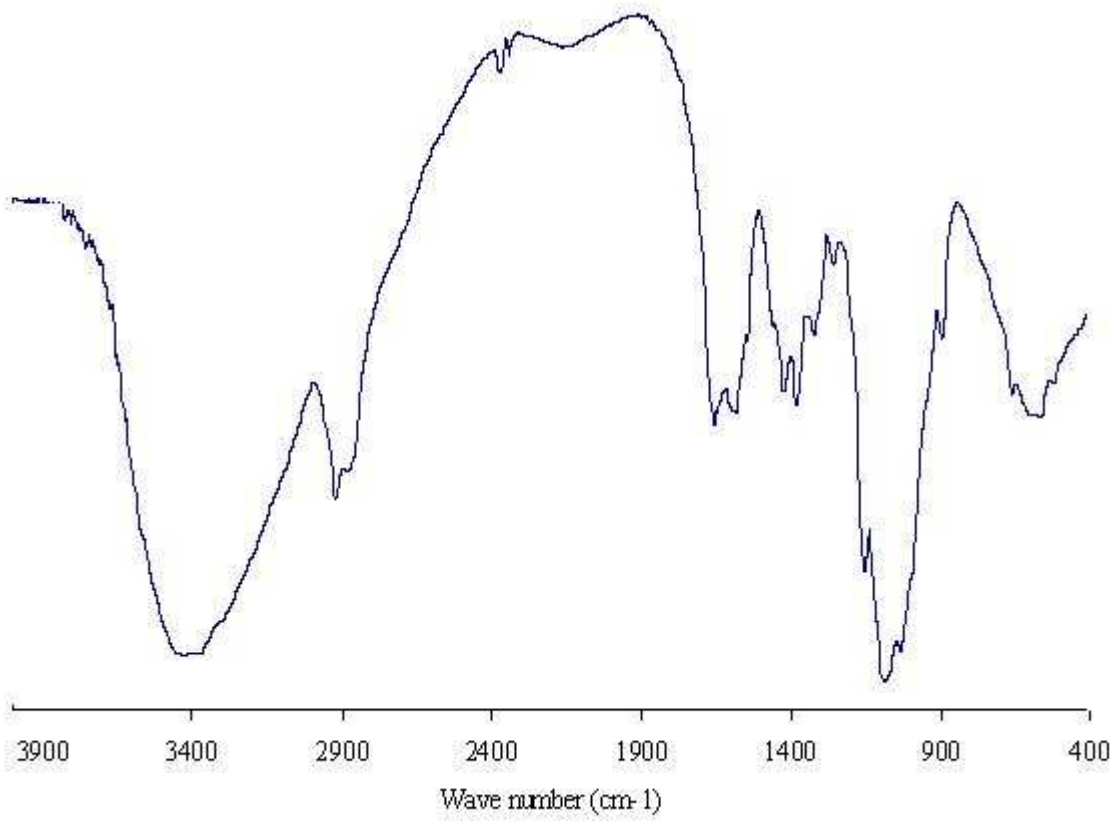

Figure 2: Infrared spectrum of fungal chitosan

Therefore the chitosan extraction procedure is an important technique for high yield production of fungal chitosan. Termamyl, Type LS enzyme is very useful. Better quality of fungal chitosan could be produced by using this enzymatic chitosan extraction method and by using food grade enzyme with high activity. Fungal chitosan has a high degree of deacetylation, low viscosity, low molecular weight, very low ash content and it can easily dissolve in some dilute organic acid. With these properties, we expected to use this fungal chitosan in the agriculture and pharmaceutical industry.

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