

Molecular Weight Distribution of Chitosan Isolated from *Mucor rouxii* under Different Culture and Processing Conditions

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The isolation of chitosan from a fungal source offers the potential of a product with controlled physicochemical properties not obtainable by the commercial chemical conversion of crustacean chitin. A variety of culture and processing protocols using *Mucor rouxii* were studied for their effects on biomass yield and chitosan molecular weight. Weight-averaged molecular weight determined by gel permeation chromatography ranged from 2.0×10^5 to approximately 1.4×10^6 daltons. The chitosan yield ranged from 5% to 10% of total biomass dry weight and from 30% to 40% of the cell wall. Of the culture parameters studied, length of incubation and medium composition effected biomass production and molecular weight. Modification of the processing protocol, including the type and strength of acid, and cell wall disruption in acid prior to refluxing were used to optimize the efficiency of chitosan extraction.

The degree of deacetylation of fungal and commercial chitosans was compared using infrared spectrometry, titration, and first derivative of UV absorbance spectrometry. The chitosan obtained directly from the fungal cell wall had a higher degree of deacetylation than commercial chitosan from the chemical conversion process.

Key words: chitin • chitosan • *Mucor rouxii* • polysaccharides

INTRODUCTION

Chitosan, a cationic polymer consisting of (1,4)-linked 2-amino-deoxy- β -D-glucan, is rarely found in nature. The primary occurrence is as a cell wall component of some fungi, particularly the zygomycetes, in addition to chitin. Chitosan has been identified in the genera *Mucor*,³ *Phycomyces*,⁷ and *Saccharomyces*,⁵ among others.

Commercially, chitosan is derived by the chemical deacetylation of chitin from waste crustacean exoskeletons with strong alkali. This harsh conversion process, as well as variability in source material, leads to inconsistent physicochemical characteristics.¹ Physicochemical properties of chitosan isolated directly from a fungus may be manipulated by control of fermentation and processing parameters. Additional control through genetic manipulation of the fungal system may be possible in the future. The list of applications for chitosan is extensive, including adhesives, food processing, paper and textile additives, wound-healing accelerants, and wastewater treatment.⁸ For these and other potential applications, control of physicochemical characteristics (e.g.,

weight-averaged molecular weight and degree of acetylation) is often critical due to effects on mechanical and chemical properties when chitosan is processed into films, powders, and fibers.

Chitosan produced from crustacean chitin ranges from near 0% to 50% degree of acetylation⁶; chitosan isolated from the *Mucor rouxii* cell wall has been reported to be 5%–50% acetylated.¹² The chitosan content of the *M. rouxii* cell wall is 33% for mycelia and 28% for yeastlike forms.³

Previous work by White et al. on chitosan isolation from *M. rouxii* focused on optimization of polymer extraction without regard to molecular weight.¹² The objective of this study was to determine the yield, molecular weight (MW) distribution, and degree of acetylation of chitosan isolated from the *M. rouxii* mycelial cell wall under different growth and extraction conditions.

MATERIALS AND METHODS

Strain

Mucor rouxii (ATCC 24905) was maintained at 4°C on agar slants containing (g L⁻¹) yeast extract, 3.0; peptone, 10.0; and glucose, 20.0; pH 5.0 (YPG medium). For long-term storage, a spore stock was prepared by washing 72 h YPG plates with sterile water containing 0.05 g L⁻¹ Tween 80. The spores were centrifuged at 3000g for 10 min followed by washing twice with sterile water and once with mineral salts solution consisting of (g L⁻¹) KH₂PO₄, 0.7; K₂HPO₄, 0.7; MgSO₄ · 7H₂O, 0.7; (NH₄)NO₃, 1.0; NaCl, 0.005; FeSO₄ · 7H₂O, 0.002; ZnSO₄ · 7H₂O, 0.002; and MnSO₄ · H₂O, 0.002. The spores were suspended and titered in mineral salts and lyophilized in the presence of an osmotic stabilizer, 120 g L⁻¹ sucrose or 100 g L⁻¹ skim milk, and stored at -70°C.

Culture Conditions

Two complex liquid media, YPG and BG, and one defined liquid medium, TVB, were examined. The BG medium, pH 5.0, consisted of (g L⁻¹) nutrient broth, 8.0; yeast extract, 0.1; glucose, 5.0; KCl, 0.1; MgSO₄ · 7H₂O,

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0.25; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.002; and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.00029. The YPG medium was as described excluding agar. The pH of complex media was monitored twice daily and adjusted if necessary by the addition of 1N NaOH. The TVB medium contained (g L^{-1}) glucose, 20.0; $(\text{NH}_4)_2\text{SO}_4$, 1.4; KH_2PO_4 , 2.0; CaCl_2 , 0.3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3; molybdc acid 85%, 0.01; and 1.0 mL trace metal solution containing (g L^{-1}) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5.0; ZnCl_2 , 1.66; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2.0; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1.96; and 10.0 mL 12M HCl. A 0.05M citrate buffer was used to pH the medium to pH 4.4 when constant pH was required. Glucose was prepared separately and added aseptically after autoclaving.

Batch cultures involved 2800-mL Fernbach vessels containing 750 mL medium. Inocula were prepared by homogenizing two 16-mm agar plugs from 72-h BG plates (for BG broth) or YPG plates (for YPG and TVB broth) in 50 mL sterile water. The homogenates were added to the Fernbach flasks and incubated at 25°C, 125 rpm. The 10-L batch cultures were grown in 14-L Magnaferm fermentor vessels (New Brunswick Scientific, New Brunswick, NJ) at 25°C, 1200 rpm agitation, and 5 L min^{-1} sterile air. Prior to autoclaving, 1 mL dimethylpolysiloxane antifoam (Union Carbide Corp., Sistrville, WV) was added. A 10% (v/v) inoculum for the 10-L cultures was prepared by inoculating a 24-h, 1-L batch culture with a YPG plug homogenate, prepared as described above. Dissolved oxygen was measured using a YSI model 51A dissolved oxygen meter (Yellow Springs Instrument Co., Yellow Springs, OH).

Hypchal Wall Isolation and Chitosan Extraction

Fungal biomass was collected by vacuum filtration using Whatman no. 40 filter paper and twice washed with distilled water. An aliquot was dried overnight at 100°C in preweighed aluminum pans to obtain biomass dry weight. Biomass yield was expressed as a w/w percentage based on initial glucose concentration. The progress of chitosan extraction was monitored by light microscopy of cell wall material treated with Lugol's stain, which contains (g L^{-1}) I_2 , 40; and KI, 60. The cell wall material appears pink to violet, debris from the cytoplasm yellow, and intact mycelia reddish brown.¹²

Extraction of chitosan from the cell wall was a modification of the procedure developed by White et al.¹² (Fig. 1). The changes included elimination of lyophilization of NaOH-treated cell wall material, homogenization of cell wall material by Waring blender in acetic acid prior to refluxing, and increasing the cell-wall-to-acid ratio to 1:100. Modifications were made to the procedure providing they did not adversely affect MW or yield of chitosan.

Analytical Methods

Chitosan weight-averaged MW and dispersity (weight-averaged/number-averaged MW) were determined on a Waters 150-C ALC/GPC gel permeation chromatography

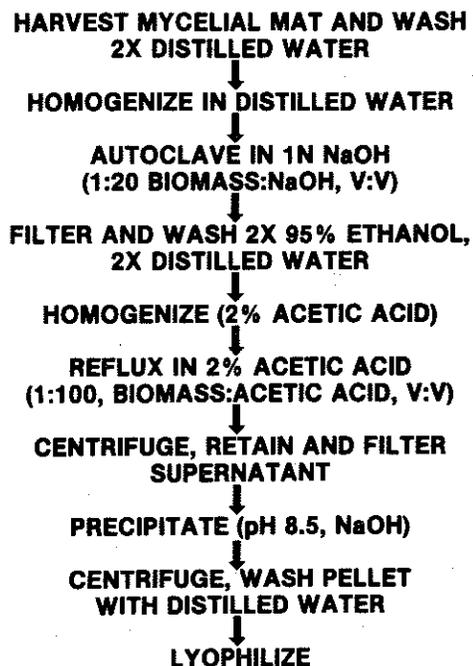


Figure 1. Extraction procedure for isolation of chitosan from *M. rouxii*.

graph (Waters Chromatography Div., Millipore Corp., Milford, MA). All MW data are reported as weight-averaged MW. The calibration curve consisted of a series of polysaccharide (linear α -D-glucan) standards ranging in MW between 1.2×10^4 and 8.53×10^5 daltons (Polymer Laboratories Ltd., Church Stretton, United Kingdom). Weight-averaged MW was determined using a Bio-Gel (Bio-Rad Laboratories, Richmond, CA) TSK-60 column followed in tandem by two TSK-50 columns, resolving from 4.0×10^3 to 8.0×10^6 daltons. Standards and samples, 1.0 g L^{-1} , were solubilized in 8.2 g L^{-1} sodium acetate, 0.5 g L^{-1} sodium azide, and 0.35N acetic acid.

Three methods were evaluated for determining degree of deacetylation of chitosan: infrared (IR) spectrometry,¹¹ first derivative of UV absorbance spectrum,⁹ and titration. To prepare samples for titration, 10.0 g L^{-1} chitosan was solubilized in 0.1N HCl standardized with 10.6 g L^{-1} Na_2CO_3 , with gentle shaking overnight. Viscosity was reduced by the addition of 10 mL water purified by reverse osmosis. Titration against 0.1N NaOH was performed on an automatic titration system (ABU80 Autoburette, TTT80 titrator, and PHM standard pH meter, Radiometer America, Cleveland, OH). Glucosamine and N-acetyl glucosamine (Sigma Chemical, St. Louis, MO) were used as 100% and 0% deacetylated controls, respectively.

RESULTS

Complex Medium

Initially, the two complex media YPG and BG were compared in 750-mL batch cultures over 168 h (Fig. 2). The MW of chitosan isolated from BG medium was a

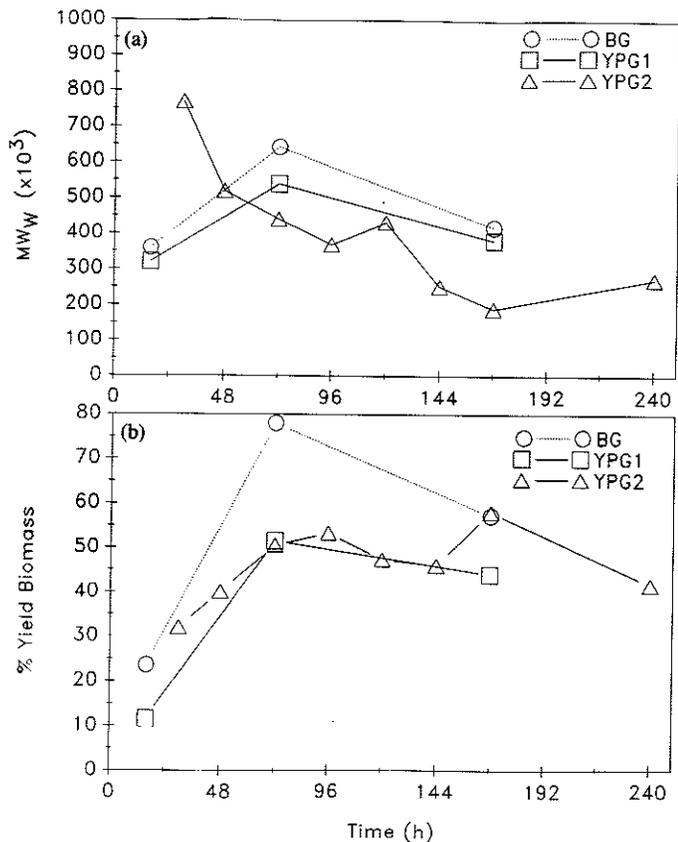


Figure 2. Effect of incubation in 750-mL complex batch cultures on (a) weight average MW and (b) biomass yield.

maximum of 6.43×10^5 , and from YPG1 it was 5.39×10^5 , both occurring at 72 h. The maximum yield of biomass also occurred at 72 h, 78% for BG and 51.5% for YPG medium. A second study (YPG2) involved processing 750-mL YPG cultures every 24 h for 168 h, with an additional sample at 240 h. This study more accurately determined the effect of incubation time on MW and biomass yield by sampling more often than YPG1. During the second study the maximum MW was 7.69×10^5 at 24 h, which gradually declined to 2.0 to 3.0×10^5 after 144 h. Biomass yields increased for 72 h before stabilizing in the range of 45%–55%.

In 10-L batch cultures MW increased rapidly, reaching a maximum of approximately 1.0×10^6 – 1.1×10^6 before declining after 72 h (Fig. 3). Although maximum biomass yields were similar, the 10-L cultures reached 50.2% yield by 30 h, compared to 50.5% at 72 h in the 750-mL batch cultures.

Defined Medium

The defined medium TVB was studied over a 168-h period in 750-mL batch cultures (Fig. 4). In general, chitosan MW in defined medium was significantly higher than in complex media. A maximum MW of 1.44×10^6 occurred at 120 h, later than peak values in the YPG medium. The biomass yield in defined medium increased for 72 h and stabilized between 31% and 36%. Although the incubation time to reach maximum biomass levels was similar to YPG medium, the values were reduced 20%–30%. A maximum MW of 1.47×10^6 was attained in 10-L batch systems by 48 h, compared to 1.44×10^6 in 120 h in the 750-mL cultures. Maximum biomass levels were similar in the two systems. However, the 10-L batch reached this level in 48 h, compared to 72 h for 750-mL cultures.

pH

In controlled pH experiments, YPG and BG batch cultures were adjusted to the initial pH of 5 twice daily for a period of 72 h. Cultures without pH adjustment served as controls. In BG, pH control decreased biomass yield from 80.0% to 66.6% and MW from 6.43×10^5 to 5.33×10^5 . In YPG, pH adjustment reduced biomass yield from 48.5% to 40.0%, while the MW remained unchanged at approximately 6.5×10^5 .

A second experiment to determine optimal pH was conducted in YPG medium that was adjusted to a constant pH (3.0, 4.0, 5.0, or 6.0) twice daily (Fig. 5). Medium without pH adjustment served as the control. Chitosan MW from pH-adjusted cultures was not significantly different than the control, which had a final

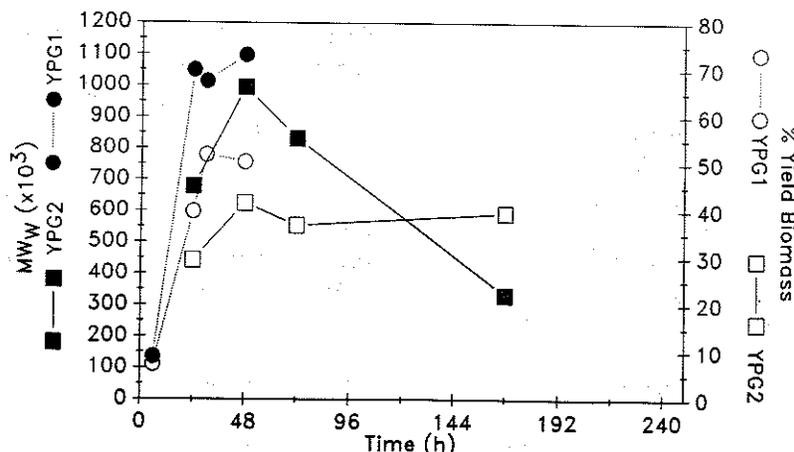


Figure 3. Effect of incubation in 10-L YPG batch cultures on weight average MW and biomass yield.

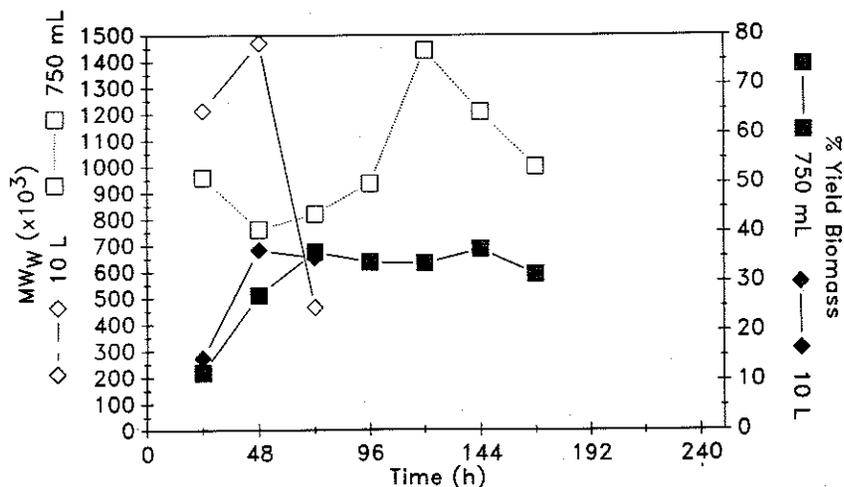


Figure 4. Effect of incubation in TVB batch cultures on weight average MW and biomass yield.

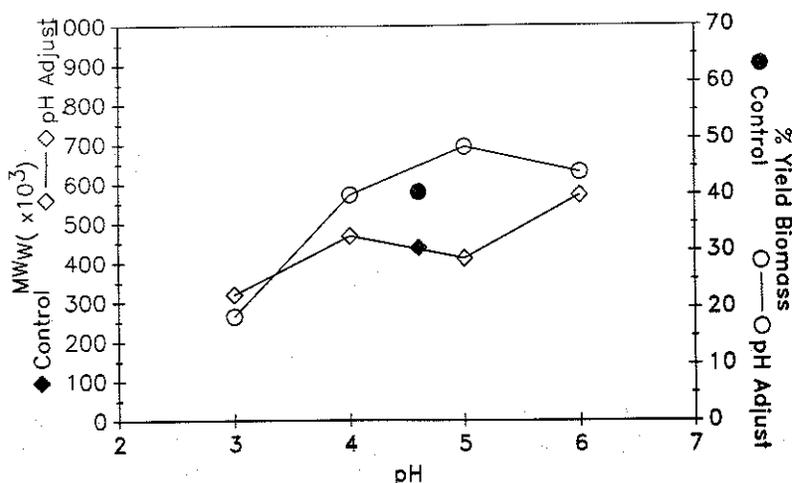


Figure 5. Effect of pH control in YPG batch cultures on weight average MW and biomass yield. control culture was not pH adjusted (initial pH 5.0).

pH of 4.6. Optimal pH for biomass production was 5.0, while maximum MW occurred at pH 6.0. Adjustment to pH 3.0 hindered growth and reduced MW. Cultures with an initial pH of 3.0, 4.0, 5.0, and 6.0 without pH adjustment had a final pH after 72 h of 3.09, 4.92, 6.89, and 6.65, respectively.

Medium Composition

To examine the effect of the components of YPG medium, each component was either eliminated or doubled in concentration in 72-h, 750-mL shake cultures. The control generated 51.5% biomass yield and a MW of 3.27×10^5 . Removal of peptone or yeast extract reduced biomass to 30.6% and 39.7% respectively. A twofold increase of peptone to 20.0 g L^{-1} resulted in a 50.5% biomass yield, and doubling the yeast extract to 6.0 g L^{-1} resulted in a 47.5% yield. Neither were significantly different from the control, possibly due to glucose limitation. The MW was not affected by doubling the glucose to 40.0 g L^{-1} (3.65×10^5) or by the absence

of peptone (3.64×10^5); however, doubling peptone or yeast extract increased MW to 4.62×10^5 and 4.61×10^5 , respectively.

Hyphal Wall Isolation and Chitosan Extraction

Several modifications were made to the extraction procedure developed by White et al.¹² Storage studies indicate that harvested mycelia may be refrigerated at 4°C up to 18 days without adversely affecting MW. Reduction of the 1:40 (w/v) ratio (wet biomass:1N NaOH) to 1:20 as well as elimination of the lyophilization of NaOH-treated cell wall material were found to reduce processing time without affecting chitosan extraction or MW. A 1:10 (w/v) ratio (dry cell wall:acid) of 1N HCl, 10% acetic, or 10% formic acid to extract chitosan was used by White et al.¹² At 1:10, HCl charred the sample, whereas the acetic acid was completely absorbed. Further examination showed that a 1:100 ratio was required for extraction, and acetic acid was chosen because HCl discolored the chitosan.

Lyophilized cell wall material was extracted with different acetic acid concentrations. This study took place prior to the elimination of lyophilization of the NaOH-treated cell wall material from the protocol. The amount of chitosan extracted with 1*N*, 0.7*N*, and 0.35*N* acetic acid were (w/w) 41.3%, 33.8%, and 47.5% of the cell, respectively. To determine length of extraction, cell wall material was extracted three times for 30 min, with fresh acid used for each step. No additional chitosan was obtained after a total of 1 h. The amount of chitosan isolated was increased by homogenizing cell wall material in a Waring blender for 3 min in 0.35*N* acetic acid at (w/v) 1:100 cell wall:acid prior to refluxing. The control cell wall material, not homogenized, yielded only (w/w) 32.7% chitosan (7.2% of total biomass), compared to 43% chitosan (9.5% of biomass) for the homogenized sample.

Deacetylation Analysis

Infrared spectrometry proved to be an unreliable method for determining degree of deacetylation due to difficulty in calibration in the high acetyl content range as well as unacceptable variability between samples from the same source. The titration method resulted in large standard deviations within given samples, possibly due to solution viscosity, foaming, and sudden precipitate formation (Table I). The degree of deacetylation of fungal chitosans determined by first-derivative UV spectrometry ranged from 87% to 92%, while commercial chitosan from crustacea waste ranged from 79% to 85%.

DISCUSSION

A number of growth and extraction parameters influenced biomass production and weight-averaged MW,

Table I. Degree of deacetylation of commercial and fungal chitosans.

Sample	Percentage of deacetylation \pm SD	
	Titration	First derivative UV spectrometry
Commercial		
Sigma (114F-0141)	84.5 \pm 25.3	79.6 \pm 0.5
Sigma (47F-0226)	ND ^a	84.7 \pm 1.8
Protan (Seacure)	ND	80.5 \pm 1.8
Fungal (<i>M. rouxii</i>)		
(63)TVB 0.5 L h ⁻¹	85.0 \pm 18.2	91.0 \pm 0.3
(33)YPG pH 5	91.4 \pm 2.6	88.6 \pm 1.1
(37)YPG/biotin	75.8 \pm 10.3	ND
(75a)TVB-c 0.5 L h ⁻¹	60.3 \pm 4.9	ND
(85)TVB-c 48 h 1:20	72.6 \pm 1.8	ND
(85)TVB-c 48 h 1:40	70.8 \pm 0.7	ND
(31)YPG pH 3	84.0 \pm 11.3	ND
(47)YPG 144 h	84.5 \pm 1.3	ND
(49)YPG 72 h	ND	87.1 \pm 1.4
(83)TVB-c 72 h 1:20	ND	91.5 \pm 0.7
(83)TVB-c 72 h 1:40	ND	88.2 \pm 2.9

^a ND, No data.

including length of incubation, medium composition, type and strength of acid, and homogenization of cell wall material before refluxing.

For growth studies in complex media, MW increased rapidly for 72 h and then declined with further incubation. The decline of MW with time suggests that chitosan is turned over, degraded, or modified in the cell wall. Chitosanases have been reported in the strain of *M. rouxii* being investigated,¹⁰ with autolysis beginning at 168 h. It is not clear if chitosanases are responsible, since the MW decline begins prior to 168 h. The formation of arthrospores may account for the change in MW with increased incubation time. Arthrospores form as environmental stresses occur and are difficult to break apart as observed by Lugol's stain. In addition, during the early stages of arthrospore formation, the hyphal cell wall continues to surround the arthrospore.² As formation proceeds, the hyphal wall and possibly the chitosan are degraded, accounting for the MW decline.

Mucor rouxii in the 10-L batch cultures grew more rapidly than the 750-mL batch cultures. Impellers in the 10-L system may transfer nutrients and dissolved oxygen more efficiently than by shaking in the small batch cultures. In addition, the volume of the small batch cultures may have been too large for the culture vessel to allow optimal mixing and transfer of nutrients. The dissolved oxygen level in the 750-mL and 10-L cultures was at saturation, approximately 8.0 ppm. Efficient dissolved oxygen transfer would also counteract the effects of carbon dioxide, which promotes the formation of the yeastlike instead of the hyphal form of the fungus.³ Adjusting the pH to a constant level over the range of 4.0–5.0 in complex media did not appear to influence MW or yield of chitosan. At pH 3.0, reduced growth rate most likely accounted for the reduction of MW, while at pH 6.0 MW increased slightly.

Extraction steps are important in terms of optimizing yield and maintaining MW. White et al.¹² used 1*N* HCl based on polymer yield without consideration of degree of acetylation or MW. In this study, HCl was evaluated but not used since the cell wall material became discolored and appeared to be degraded during the extraction. Acetic acid at different concentrations effected polymer yield; 0.35*N* acetic acid extracted more chitosan than 0.70*N* or 1*N*. Cell wall homogenization in acid prior to refluxing increased extraction efficiency, possibly by making more chitosan accessible to extraction.

Several steps in a published extraction protocol¹² were modified or eliminated to improve the efficiency without reducing MW or yield of chitosan. Storage of the mycelium at 4°C, reduction of the cell wall:NaOH ratio to 1:20, and elimination of cell wall lyophilization decreased processing time and reagent requirements.

Degree of deacetylation data generated by first-derivative UV spectrometry for a given sample were more consistent than the other methods evaluated; however, some aspects cited in the method⁹ could not be duplicated. Peak absorbance of *N*-acetyl glucosamine

standards was not always at 199 nm; peak wavelength steadily increased with increased *N*-acetyl glucosamine concentration; and measurements at 202 nm (zero-order crossing point) were not independent of acetic acid concentration. Increased acetic acid concentration caused a reduction of peak height and an increase of peak wavelength in samples and standards. Therefore, these data are used as comparative but not absolute values.

There is room for additional improvement of the extraction procedures described, especially to reduce the amount of insoluble material present in some samples. In addition, comparison of MW values determined over extended periods of time will require an improved gel permeation chromatography column packing which eliminates interaction with chitosan. Due to its cationic nature, some chitosan irreversibly binds to the column packing, eventually reducing column efficiency. Increasing the ionic strength of sodium acetate in the mobile phase from 0.1M to 0.3M in an attempt to reduce this interaction results in nonreproducible MW data and could not be used.

CONCLUSIONS

Culture conditions and processing steps critical in the isolation and purification of chitosan from the fungal cell wall of *M. rouxii* were examined. These culture and processing factors were correlated to the yield and MW of chitosan. Length of incubation and medium composition were important factors that influenced biomass yield and chitosan MW. Processing protocol modifications, including strength and type of acid, and cell wall disruption in acid prior to refluxing were used to optimize extraction efficiency. Chitosan ranging in MW from 2.0×10^5 to 1.4×10^6 was produced under different culture conditions. Degree of deacetylation ranged from 60% to 92%, and polydispersity ranged between 5.0 and 8.0. The relatively high polydispers-

ity reflects the heterogeneous distribution of polymer chain lengths, possibly due to the cell wall extraction procedure.

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