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Physicochemical Properties and Bioactivity of Fungal Chitin and Chitosan

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Chitinous material was extracted from mycelia of *Aspergillus niger* and *Mucor rouxii* grown in yeast peptone dextrose broth for 15 and 21 days, respectively. The extracted material was characterized for purity, degree of acetylation, and crystallinity and tested for antibacterial and eliciting properties. The maximum glucosamine level determined in the mycelium of *A. niger* was 11.10% dw and in the mycelium of *M. rouxii* was 20.13% dw. On the basis of the stepwise extraction of freeze-dried mycelia, it appeared that *M. rouxii* mycelia contained both chitin and chitosan, whereas *A. niger* contained only chitin. The yields of crude chitin from *A. niger* and *M. rouxii* were 24.01 and 13.25%, respectively, and the yield of chitosan from *M. rouxii* was 12.49%. Significant amounts (7.42−39.81%) of glucan were associated with chitinous compounds from both species and could not be eliminated by the extraction method used. The degrees of acetylation were determined to be 76.53 and 50.07% for chitin from *A. niger* and *M. rouxii*, respectively, and 19.5% for *M. rouxii* chitosan. The crystallinity of fungal chitin and chitosan was estimated to be less intense than in corresponding materials from shrimp shells. The extracted chitin and chitosan in a concentration of 0.1% reduced *Salmonella* Typhimurium DT104 2576 counts by 0.5−1.5 logs during a 4 day incubation in tryptic soy broth at 25 °C. Furthermore, all tested chitinous materials from fungal sources significantly reduced lesions caused by *Botrytis cinerea* and *Penicillium expansum* in harvested apples.

KEYWORDS: ***Aspergillus niger*; *Mucor rouxii*; chitin; chitosan; antibacterial activity; disease resistance**

INTRODUCTION

Polysaccharide chitin and its deactylated product, chitosan, have received much interest for potential application in agriculture, biomedicine, biotechnology, and the food industry due to their biocompatibility, biodegradability, and bioactivity (*1*−*5*). Chitin is a primary constituent of crustacean shells, insect cuticles, and fungal cell walls (*6*). Chitosan, on the other hand, although not a component in animal species, is a major structural biopolymer in the cell walls of fungi, such as *Mucor*, *Absidia*, and *Rhizopus* genera (*6*, *7*).

Chitin is industrially extracted from crab and shrimp shells obtained as a byproduct in the seafood industry. The shells consist of chitin (14−27% dw), proteins, calcium, and small amounts of pigments (*8*). Under current processing conditions, calcium is removed by diluted hydrochloric acid, proteins are removed with sodium hydroxide, and the residue is usually bleached with potassium permanganate or hydrogen peroxide. Chitosan is produced by deacetylation of chitin with hot concentrated sodium hydroxide (40−50% NaOH at 80−150 °C) (*9*). The production of chitin and chitosan from fungal sources has gained increased attention in recent years due to potential advantages over the current source. For example, crustacean waste supplies are limited by seasons and fishing industry locations, whereas fungal mycelium can be obtained by simple fermentation regardless of geographical location or season (*10*). Furthermore, fungal mycelia have lower levels of inorganic materials compared to crustacean shells, and no demineralization treatment is required during processing (*11*). Considering the significant amounts of fungal-based waste materials accumulated in the mushroom production and fermentation industries, and the expenses involved in managing the waste, production of highly functional value-added products may provide a profitable solution to the industry. *Agaricus bisporus* is one of the most popular mushrooms worldwide and especially in the United

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States. Depending on the size of the mushroom farm, the amount of waste, which mainly consists of stalks and mushrooms with irregular dimensions and shapes, ranges between 5 and 20% of production volume (personal communication). In the United States alone, this results in ~50 000 metric tons of waste material per year with no suitable commercial use. On the other hand, citric acid is the most widely used organic acid in the food, beverage, and pharmaceutical industries as an acidifying or flavor-enhancing agent and is commercially produced by submerged fermentation with *Aspergillus niger*. The annual world requirements for citric acid are estimated at 400 000 tons (*12*), which results in ~80 000 tons of *A. niger* mycelium waste per year (*13*).

Among various potential applications of chitin and chitosan in the food, agricultural, and pharmaceutical industries, the uses of these biopolymers as food antimicrobials and biopesticides are especially attractive. Chitosan showed strong antimicrobial activity on both Gram-negative and Gram-positive bacteria, including *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Listeria monocytogenes* (*14*−*16*), but Gram-positive bacteria appeared to be more susceptible to chitosan compared to Gram-negative species (*14*, *15*). It has been suggested that the interaction between positively charged chitosan molecules and negatively charged microbial surfaces results in disruption of the cell membranes, leakage of intracellular constituents, and ultimately microbial cell death (*16*). Additionally, according to a second hypothesis, chitosan oligomers can penetrate into prokaryotic cells and interfere with the transcription of RNA and protein synthesis (*17*).

Crustacean chitin and chitosan have been shown to act as potent agents that elicit defense reactions in higher plants and, consequently, inhibit the growth of phytopathogenic fungi and bacteria (*18*). Chitosan effectively reduced polygalacturonases produced by *Botrytis cinerea* and caused severe cytological damage to invading hyphae in bell pepper fruit (*19*). It also induced the synthesis of phytoalexins in rice leaves and suppressed the growth of inoculated fungi (*20*). Similarly, when cucumber plants were sprayed with chitosan or chitin before inoculation with *B. cinerea*, the activity of chitosanase and peroxidase increased and the growth of *B. cinerea* was inhibited (*21*).

The objectives of the research presented here were to determine the yield and physicochemical properties of chitin and chitosan from *Aspergillus niger* and *Mucor rouxii* and to examine the bioactivity of fungal chitin and chitosan against the foodborne pathogen *S. typhimurium* and plant pathogens *B. cinerea* and *Penicillium expansum*.

MATERIALS AND METHODS

Commercial chitins and chitosans originating from crustacean shells were provided by Primex, Co. (Siglufjör''ur, Iceland) and purchased from ICN Biomedicals, Inc. (Aurora, OH), Fluka (Luausanne, Switzerland), and Aldrich (Milwaukee, WI). Other common chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). *A. bisporus* chitin was prepared previously according to the alkali and acid reflux method (*22*).

Growth of *A. niger* and *M. rouxii*. The *A. niger* and *M. rouxii* strains were obtained from the culture collection of the Department of Food Science and Technology at The University of Tennessee. The cultures were revived on potato dextrose broth (PDB) at 28 °C for 3 days, transferred to potato dextrose agar (PDA), and incubated at 28 °C for 5 days. Three plugs of each culture (plugged with a 16 mm sterile test tube) were transferred to 1000 mL flasks containing 200 mL of YPD (0.2% yeast extract, 1.0% peptone, and 2.0% glucose) medium. The inoculated flasks were incubated at 28 °C without shaking. Mycelium was harvested by vacuum filtration through Whatman no. 4 filter paper and washed with distilled water until a clear filtrate was obtained. The mycelia were lyophilized and ground to a powder with a Thomas Wiley mill (Thomas Co., Philadelphia, PA), sieved through a no. 40 mesh, and stored in a desiccator at room temperature for further analyses and extraction.

Extraction of Chitin and Chitosan. Chitinous materials from *A. niger* and *M. rouxii* mycelia were obtained according to a procedure described elsewhere (*22*−*24*). In short, the extraction consisted of two steps. The primary reflux with 1 M NaOH (w/v 1:40) at 95 °C for 30 min removed proteins and resulted in alkali insoluble material (AIM), which was centrifuged (12000*g*, 20 min, 22 °C) and extensively washed with deionized water. Freeze-dried AIM was further refluxed with 2% acetic acid (w/v 1:100) at 95 °C for 6 h to separate acid soluble chitosan and insoluble chitin. Chitosan was precipitated from the supernatant after adjustment of the pH to 10 with 1 N NaOH, filtered, washed with deionized water to reach a neutral pH, and freeze-dried.

Characterization of Extracted Chitinous Material. The yield of AIM, crude chitin, and chitosan was determined gravimetrically. Glucosamine and glucan contents in mycelia, AIM, and insoluble residue were determined by using the 3-methyl-2-benzothiazolone hydrazine (*25*) and the anthrone tests (*26*), respectively. The Fourier transform infrared spectroscopy (FT-IR) analyses were performed with a Nexus-670 FT-IR spectrometer using an attenuated total reflection (ATR) sampling accessory with a germanium crystal (ThermoNicolet, Mountain View, CA). The analyses were conducted between 700 and 4000 cm^{-1} with 64 scans and a resolution of 4 cm^{-1}. Calculations were performed using OMNIC 6.1 software (ThermoNicolet Co.) based on the ratio of peak area at 1379 and 2900 cm^{-1} in spectra obtained in the absorption mode (*27*). Degree of acetyletion (DA) was determined after hydrolysis of chitin/chitosan samples following the method of Niola et al. (*28*). Hydrolysis was performed in 12 M H_2SO_4, with an oxalic and propionic acid standard mixture at 155 °C for 1 h under vacuum (Pierce Biotechnology, Inc., Rockford, IL). Acetic acid liberated during hydrolysis was analyzed on an HPX 87H column (Bio-Rad, Hercules, CA) using a high-performance liquid chromatography (HPLC) system with a photodiode array (PDA) detector (Dionex, Sunnyvale, CA). The mobile phase was 5 mM H_2SO_4 at a flow rate 0.60 mL/min with an injection volume 10 μL, and absorbance was monitored at 210 nm.

Assays for Antibacterial Activity. *Salmonella* Typhimurium DT104 2576 was obtained from the culture collection of the Department of Food Science and Technology at The University of Tennessee. The bacterium was maintained on tryptic soy agar (TSA; Sparks, MD) slants at 4 °C and activated at 32 °C for 24 h before experiments. Chitosan solutions and chitin suspensions were prepared in 1% (v/v) acetic acid at a concentration of 1% (w/v). The chitosan solutions and chitin suspensions were stirred overnight at room temperature, and the chitosan solutions were filtered using miracloth (Calbiochem Corp., La Jolla, CA) to remove potential impurities. Each solution or suspension was added to tryptic soy broth (TSB; Sparks, MD) to give a final chitin or chitosan concentration of 0.1% (w/v). The pH of the broth was adjusted to 4.5 with sterile 1 M HCl before autoclaving at 121 °C for 15 min. Aliquots of 0.1 mL of the bacteria culture (10^9 CFU/mL) were added to 10 mL of broth supplemented with chitin or chitosan and incubated at 25 °C for 48 h. Enumeration of the bacterial population was carried out by stepwise dilutions with 0.1% peptone water, followed by plating on TSA and incubation at 35 °C for 24 h.

Biocontrol Bioassay. Cv. Gala apples that had been harvested 1 month previously and were free of rot or wounds were randomly assigned to different treatments. The test plant pathogens, *B. cinerea* and *P. expansum*, were from the collection of the Produce Quality and Safety Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD, and were originally isolated from decayed apples. The pathogens were routinely grown on PDA, and virulence was maintained by periodic transfers through apple. The conidial suspensions used to inoculate the fruit were prepared from 2-week-old cultures grown at 20 °C under constant fluorescent light and adjusted to a concentration of 1×10^4 conidia/mL using a hemacytometer. The fruits were surface disinfested with 70% ethanol and wounded 1 cm above the equator of the fruit with a 3-mm-diameter cork borer to a depth of 3 mm. The treatments were prepared as 2% (w/v) chitin or

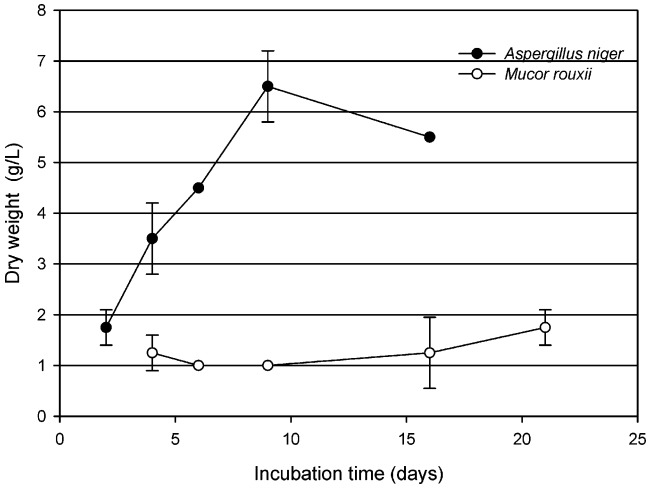

Figure 1. Growth of *A. niger* and *M. rouxii* in YPD media at 28 °C. The results are expressed on a dry weight basis per liter of medium. Error bars represent standard deviation ($n = 3$).

chitosan suspensions in distilled water, and 25 μL of a given treatment was pipetted into the wound. Apples were allowed to air-dry, packed in boxes with polyethylene liners, and stored at 25 °C for 72 h. After 3 days, the fruits were removed from storage, an identical wound was made 2 cm directly below the first wound, and both wounds were inoculated with 25 μL of a conidial suspension of either *B. cinerea* or *P. expansum* prepared as described above. Apples were again placed in tray-pack boxes with polyethylene liners, and lesion diameter was recorded after 4 and 7 days of storage at 25 °C. Ten apples were used per replication, and each treatment was done in triplicate.

Analysis of variance and contrasts between means were performed by Statistical Analysis System, release 8.2 (SAS/STAT, 1996, SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Growth of *A. niger* and *M. rouxii* in YPD media at 28 °C is presented in the **Figure 1**. The biomass of *A. niger* increased rapidly in the first 9 days and then slightly decreased, while *M. rouxii* growth was at a much slower rate. The maximum biomass dry weight of *A. niger* was 6.50 g/L, but was only 1.70 gL for *M. rouxii* even after 21 days of incubation.

It has been suggested that the maximum yields of chitin and chitosan were obtained from fungi harvested in the late exponential phase (*29*). Under the conditions of our experiment, it appeared that the late exponential phase for *A. niger* was reached after 9 days, whereas the *M. rouxii* biomass did not significantly change during the 21 day incubation period. However, Tan et al. (*29*) reported that *M. rouxii* incubated at 25 °C with continuous shaking reached the late exponential growth phase after only 3 days, producing >5 g/L of dry biomass. The possible reasons for the growth variation may be due to the different strains and incubation conditions used in the experiments.

Estimation of Chitin Content in *A. niger* and *M. rouxii*. The amount of chitin and chitosan in the analyzed material can be estimated by total (acetyl)glucosamine determination (*30*). The total (acetyl)glucosamine content in *A. niger* dry mycelium increased from 8.01% dw at day 2 to 11.10% dw at day 9, whereas in *M. rouxii* biomass it increased from 14.23% dw at day 4 to 20.13% dw at day 21 (**Figure 2A**). Although *M. rouxii* had higher levels of total glucosamine in the cell walls over the entire testing period, *A. niger* appeared to have a greater potential to be used for extraction of chitinous material due to a more abundant biomass production (**Figure 2B**).

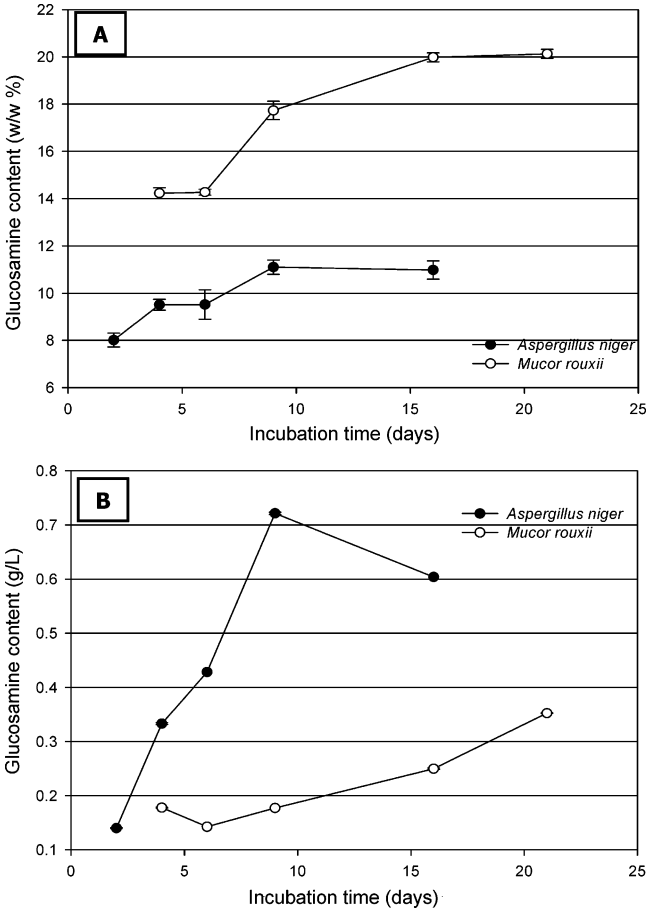

Figure 2. Total glucosamine content of *A. niger* and *M. rouxii* incubated at 28 °C expressed on mycelium dry weight (w/w %) (**A**) and media volume (g/L) (**B**) basis. Error bars represent standard deviation ($n = 3$).

Table 1. Yield and Composition of Material Obtained during Extraction of Chitinous Material from *A. niger* and *M. rouxii.*

	sample	yield[a]	glucosamine[b]	glucan[c]
A. niger	mycelium	(100.00)	12.87 ± 0.87	42.92 ± 1.16
	AIM	36.25 ± 1.03	22.05 ± 1.51	35.97 ± 1.19
	crude chitin	24.01 ± 0.47	31.19 ± 0.43	39.81 ± 1.42
M. rouxii	mycelium	(100.00)	24.69 ± 1.07	9.52 ± 0.11
	AIM	29.67 ± 1.00	50.13 ± 3.04	7.76 ± 0.09
	crude chitin	13.25 ± 1.01	58.83 ± 2.24	9.48 ± 1.03
	crude chitosan	12.49 ± 1.04	71.22 ± 1.45	7.42 ± 0.85

[a] Yield expressed as percent of dry weight of mycelium. [b] Chitin and chitosan were hydrolyzed and determined as glucosamine (percnet of analyzed material). [c] Neutral polysaccharides determined by the anthrone reagent (percent of analyzed material).

Extraction and Characterization of Chitinous Material. Chitinous material was extracted from freeze-dried mycelia of *A. niger* and *M. rouxii* collected after 9 and 21 day incubation periods at 28 °C, respectively. The yields of alkali insoluble material (AIM) from *Aspergillus* and *Mucor* were 36.35 and 29.67%, respectively, and yields of crude chitin, obtained as alkali and acid insoluble residue, from *A. niger* and *M. rouxii* were 24.01 and 13.25%, respectively (**Table 1**). Considering the "true" chitin contents of 31.19% in the crude extracts from *A. niger* and 58.83% from *M. rouxii*, it appeared that the same levels of chitin were extracted from the mycelia of both fungi (7.50 and 7.80%, respectively). However, 12.49% chitosan was obtained from *M. rouxii* dry mycelia and none from *A. niger*. The purity of *Mucor* chitosan was >71% (**Table 1**). Only 7.42%

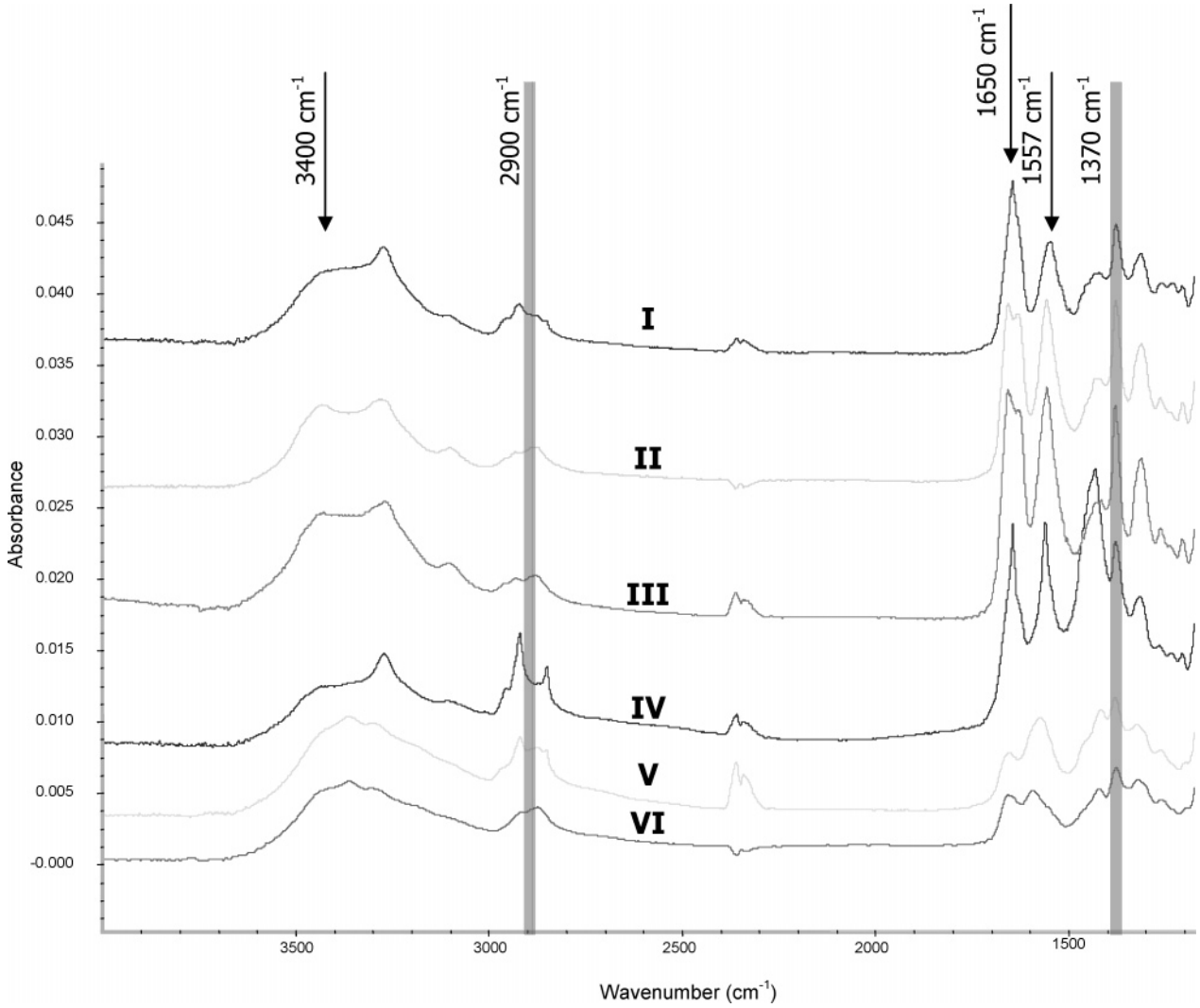

Figure 3. FT-IR spectra of fungal and commercial (crustacean) chitin and chitosan: (I) *A. niger* chitin; (II) *A. bisporus* chitin; (III) shrimp chitin; (IV) *M. rouxii* chitin; (V) *M. rouxii* chitosan; (VI) shrimp chitosan.

glucan was coextracted with chitosan, which was a lower amount compared to that in the crude chitin fractions (**Table 1**). Extraction of fungal chitinous material in the form of chitin−glucan and chitosan−glucan complexes has been previously detected (*31*−*33*). For example, Machova et al. (*31*) determined total nitrogen content in chitin−glucan complex extracted from *A. niger* and reported that only ∼30% of the material was chitin. Optimizing conditions for extraction and deacetylation of chitinous material from *A. niger*, Muzzarelli et al. (*31*) found that chitosan−glucan yield varied between 25 and 71% with 4−50% chitosan content, depending on the applied procedure.

The glucosamine content in crude chitin and chitosan from *A. niger* and *M. rouxii* showed that 58.10 and 67.60%, respectively, of the glucosamine originally present in mycelia were extracted in the form of chitinous material. The difference between total mycelial glucosamine and extracted chitinous material may be attributed to the loss of soluble (acetyl)-glucosamine oligomers during extraction and to the crystallinity of chitin alone and/or within the chitin−glucan complex (*34*). The extensive crystallinity of chitin could prevent its complete hydrolysis, necessary for glucosamine determination, and result in underestimated chitin content in the extracted material. Nevertheless, the yields obtained in our experiment are in agreement with those of Arcidiacono and Kaplan (*35*), Muzzarelli et al. (*31*), and Teng et al. (*4*).

The degree of acetylation (DA) values of *A. niger* chitin, *M. rouxii* chitin, and *M. rouxii* chitosan were determined to be 76.53 ± 2.40, 50.07 ± 0.48, and 19.50 ± 0.97%, respectively. DA results were indirectly proven by the solubility of the material in 1% acetic acid. *A. niger* chitin was insoluble, whereas *M. rouxii* chitin was partially soluble, and *M. rouxii* chitosan was completely soluble in the aqueous acid solution. Similar values for *M. rouxii* chitosan were previously reported (*10*, *24*, *35*, *36*). Thus, Arcidiacono and Kaplan (*35*) found that the DA of chitosan isolated from *M. rouxii* ranged from 8.50 to 39.70%, depending on the processing conditions, whereas in the studies of Rone and Hoover (*23*) this range was much narrower, from 10.5 to 11.10%.

FT-IR Spectra and Crystallinity. The infrared spectra of chitin and chitosan from *M. rouxii* and *A. niger* were similar to the IR spectra of chitosan and chitin produced from shrimp (**Figure 3**). The spectra had characteristic bands at 3400−3480 cm^{-1} that responded to OH-3 and CH_2OH intra- and intermolecular hydrogen bonds, 1650 cm^{-1} for amide I, and 1557 cm^{-1} for amide II vibrational mode. The ratio of intensities of the bands at 1379 and 2900 cm^{-1} has been used to estimate the crystallinity for chitin and chitosan (*27*). The crystallinity increased in the following order: *M. rouxii* chitosan (0.10) < commercial chitosan (0.20) < *A. niger* chitin (0.37) < *M. rouxii* chitin (0.38) < commercial chitin (1.25). These results were

Table 2. Antimicrobial Activity of Chitin and Chitosan from Fungal and Crustacean Sources against *S. typhimurium* DT 104 2576[a]

treatment	*Salmonella* count (Log CFU/mL) after 48 h	*Salmonella* count (Log CFU/mL) after 96 h
water control	8.33 ± 0.03	6.86 ± 0.06
acetic acid control	7.16 ± 0.09	6.55 ± 0.03
A. niger chitin	6.90 ± 0.08	6.10 ± 0.10
M. rouxii chitin	6.79 ± 0.35	6.02 ± 0.01
M. rouxii chitosan	6.72 ± 0.01	5.08 ± 0.17
A. bisporus chitin[b]	6.91 ± 0.05	6.11 ± 0.11
shrimp chitosan	6.50 ± 0.04	5.26 ± 0.07

[a] Initial count at time 0 h for all treatments was 6.98 Log CFU/mL. [b] *A. bisporus* chitin obtained from mushroom stalks (*22*).

expected because the high DA of chitin molecules (> 70%) has been associated with extended crystallinity (*37*), whereas the low DA of chitosan molecules (<30%) resulted in a random distribution of the acetyl groups that did not allow significant development of broad crystalline regions. The crystallinity index also increased with purification of fungal material (fungal chitin > fungal AIM) mainly due to the removal of interfering amorphous components. Mol and Wessels (*38*) used X-ray diffraction to evaluate the crystallinity of untreated and alkali- and acid-treated hyphae walls and found that X-ray patterns sharpened with purification and that characteristic crystallinity peaks of α-chitin appeared after acid treatment of alkali-insoluble material.

Antimicrobial activity of isolated chitin and chitosan against Gram-negative, foodborne pathogen *Salmonella* Typhimurium DT104 2576 is shown in **Table 2**. Chitin and chitosan from *M. rouxii* and chitin from *A. niger* reduced bacterial counts after 96 h in TSB by 0.96, 1.90, and 0.88 logs when compared to initial count, respectively. Chitin previously extracted from stalks of button mushrooms, *A. bisporus* (*22*), had effects similar to that of chitin from *A. niger*, whereas chitosan from *M. rouxii* was more effective than commercially available shrimp chitosan (1.72 logs). Although the same shrimp chitosan had stronger antibacterial activity when used in oil-in-water emulsions (*39*), these results showed that simple extraction from fungal biomass can provide fungal chitosan with the same or higher efficiency as currently commercially available chitosan.

Induction of Plant Defense Response. All fungal chitins and chitosan, as well as commercial chitosan, significantly reduced lesions on apples caused by plant pathogens, *B. cinerea* and *P. expansum* (**Figure 4**). For example, the diameter of lesions caused by *B. cinerea* in control apples pretreated with only water increased from 10 mm on day 4 to 36 mm on day 7, whereas lesions in chitin- or chitosan-pretreated samples were ~5 mm 4 days after inoculation with the pathogen and did not increase further during the experiment. The differences in lesion diameter between the water control and chitin or chitosan treatments were statistically significant with $p < 0.001$, but were not significant among chitin and chitosan treatments ($p > 0.05$). The reduction of lesion diameter appeared to be a

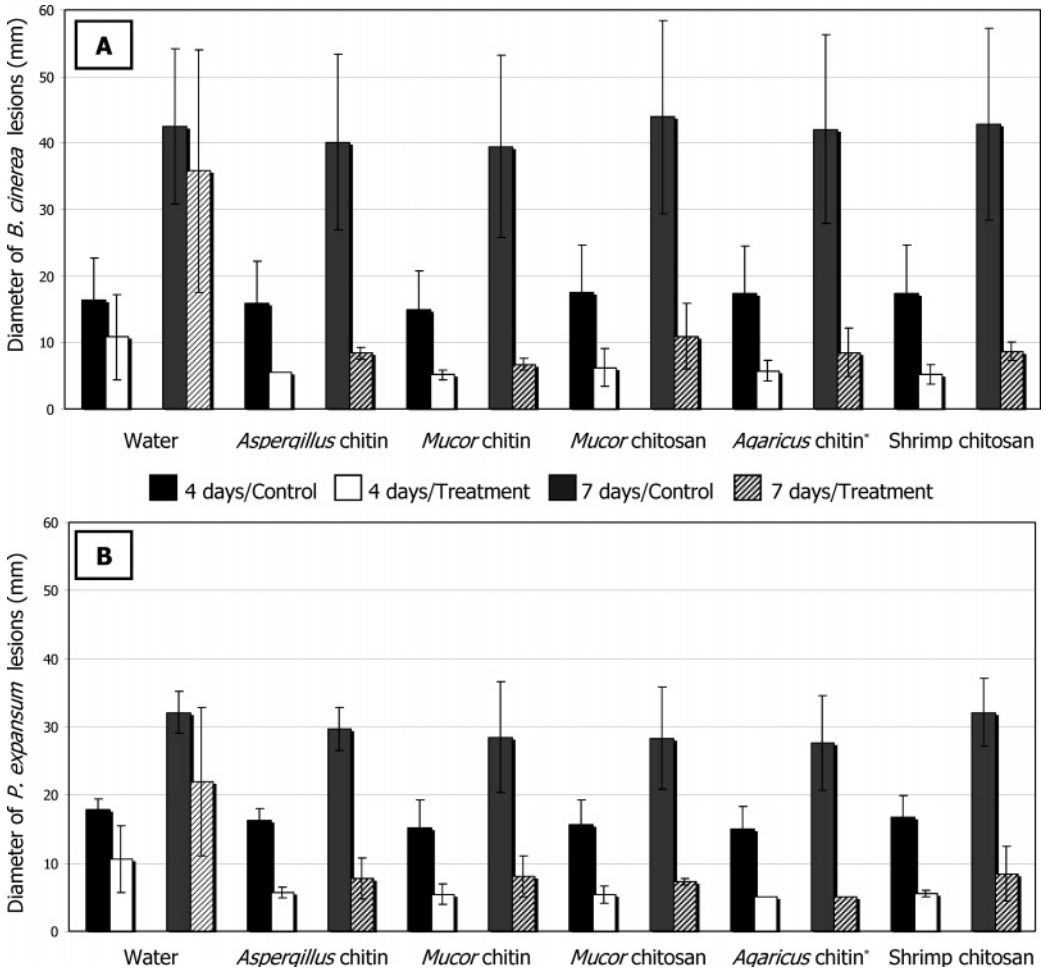

Figure 4. Effects of 2% chitosan and chitin treatments on severity of decay of apples by *B. cinerea* (**A**) and *P. expansum* (**B**): control, inoculated wounds; treatments, wounds treated with chitin, chitosan, or water and inoculated with the pathogen 3 days after treatment; *, *A. bisporus* chitin obtained from mushroom stalks (*22*). Error bars represent standard deviation ($n = 30$).

direct effect of chitosan addition, although available literature indicates the possibility for both systematic (*20*) and direct effects (*19*).

It has been previously reported that disease resistance response in plants can be activated by inorganic compounds, such as phosphonate (*40*) and potassium phosphonate (*41*), or by organic compounds such as 2,6-dichloroisonicotinic acid (*42*) and acibenzolar (*43*, *44*). Furthermore, shrimp chitosan induced defense responses in cucumbers, rice, strawberries, and bell peppers (*19*−*21*). Our results showed that crude fungal chitin and chitosan had the same effect as commercially purified crustacean chitosan. From a practical aspect, this means that ∼50 000 tons of mushroom waste, which accumulates annually in the United States, can be processed to yield ∼500 tons (*22*) of highly effective fungal chitin that can be used as a biopesticide. In the same way, fungal waste from citric acid production and/or from the pharmaceutical industry can be easily utilized for the production of these high-value-added products.

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