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To the Graduate Council:

I am submitting herewith a thesis written by Tao Wu entitled "Production and Characterization of Fungal Chitin and Chitosan." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Svetlana Zivanovic, Major Professor

We have read this thesis and recommend its acceptance:

Frances Ann Draughon, Carl E. Sams

Accepted for the Council:
Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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recommend its acceptance:

Frances Ann Draughon

Carl E. Sams

Acceptance for the Council:

Anne Mayhew

Vice Chancellor and Dean of Graduate Studies

(Original signatures are on file with official student records)

Production and Characterization of Fungal Chitin and Chitosan

A Thesis Presented for the Master of Science Degree
The University of Tennessee, Knoxville

Tao Wu

August 2004

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Dedication

This thesis is dedicated to my dear wife, Xiaoyun Deng. Her encouragement and inspiration fuel the fulfillment of my goals.

Acknowledgments

I wish to thank everyone who made my masters education possible. I am very thankful to my major advisor Dr. Svetlana Zivanovic for inviting me to Department of Food Science at The University of Tennessee and introducing me such a rewarding project. I am also very thankful to my committee members Dr. Frances Ann Draughon and Dr. Carl E. Sams for a constant source of knowledge and support. I would like to thank Dr. William S. Conway in USDA for doing the bioassay for the fungal chitin and chitosan. I would like to thank Robert K. Moore III in Monterey Mushrooms, Inc. for providing the mushrooms as the experiment materials. I would like to thank Ming Huang, a former graduate student in Food Science and Technology department for her previous research in the mold growth. I would like to thank Philipus Pangloli, Willie Taylor, Kelly, Jason for giving me help on microbial work or providing experiment materials. I would also like to thank Shari, Shuang, Ibrahim and Gunnar for their help during the study and research.

Abstract

Chitin is a biopolymer. Theoretically, it is a homopolymer of acetyl-glucosamine. Chitosan is the deacetylated product of chitin. Chitin and chitosan have many applications in water purification, agriculture, food and pharmaceutical industries. Currently, industrial production for chitin and chitosan is from the shell wastes. The objective of this study was to evaluate the feasibility of chitin and chitosan production from fungal sources.

The accumulation of chitin and chitosan during *A. bisporus* storage and *Aspergillus niger* and *Mucor. rouxii*. Growth was evaluated by total glucosamine determination. Chitin and chitosan were extracted from biomass by alkali and acid treatment and yield and composition (glucosamine and glucan) of extracted material were analyzed. The Degree of acetylation (DA) and crystallinity were analyzed by HPLC and FT – IR, respectively. The antimicrobial activity against *Salmonella* Typhimurium DT104 2576 and the induction of apple defense response against *Botrytis cinerea* and *Penicillium expansum* were also evaluated.

Results showed *Agaricus bisporus*, *Aspergillus niger* and *Mucor rouxii* have excellent potentials to be used for chitin and chitosan production on industrial scale with maximum chitin/chitosan content of 19 %, 12 % and 20 % in dry mycelia, respectively. Fungal chitin from all 3 species had lower DA compared with shrimp chitin. Native fungal chitosan had DA comparable with commercial available chitosan. Fungal chitin and chitosan did not show significant antimicrobial activity against *Salmonella Typhimurium* DT 104 in TSB with pH 4.5 at 25 ºC. Chitin and chitosan extracted from 3 species showed strong ability to induce apple defense reaction against *Botrytis cinerea* and *Penicillium expansum.*

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Part 1

Introduction and literature review

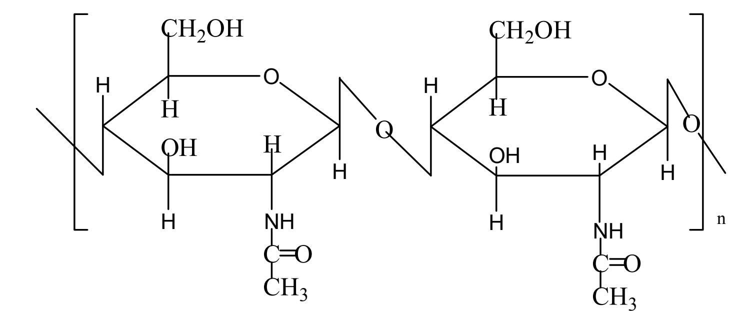
DEFINITIONS AND OCCURRENCE OF CHITIN AND CHITOSAN

DEFINITIONS AND CHEMICAL STRUCTURES

Biopolymer is a term commonly used for polymers which are synthesized by living organisms (1). Biopolymers originate from natural sources and are biologically renewable, biodegradable and biocompatible. Chitin and chitosan are the biopolymers that have received much research interests due to their numerous potential applications in agriculture, food industry, biomedicine, paper making and textile industry. Chitin is a polysaccharide, made of N-acetyl-D-glucosamine units connected by β (1→4) linkage (Figure 1.A). When the acetyl-D-glucosamine units in chitin lose acetyl groups, the molecule is called chitosan (Figure 1.B). However, commonly available chitin and chitosan are not strict homopolymers but they exist as co-polymers (Figure 1.C). This results in the interchangeable usage of chitin and chitosan definitions in the literature. Generally, a biopolymer with dominant N-acetyl-D-glucosamine fraction is recognized as chitin and one with dominant glucosamine fraction as chitosan. Furthermore, chitin is insoluble in most common solvents, while chitosan is soluble in diluted acid solutions.

OCCURRENCE AND BIOLOGICAL FUNCTIONS IN NATURE

Chitin is a characteristic compound found in fungi and some animals. In animals, chitin mainly exists in the shells of crustaceans and mollusks, in the backbone of squids and in the cuticle of insects. Long chitin molecules are associated with proteins by covalent bonds and together they form a complex structural network. On crustacean's shells, calcium carbonate deposits into the network contributing to strength of the shells and protection of the organisms (2). In fungi, chitin exists in the cell wall of spores and hyphae. It is associated with glucan molecules in form of microfibrils, which are embedded in an amorphous matrix and provide the framework in cell wall morphology (3). Chitosan is not native to animal sources, but a small number of fungi, such as *Mucor*, *Absidia* and *Rhizopus* species have chitosan as one of the structural components in the cell wall (3).



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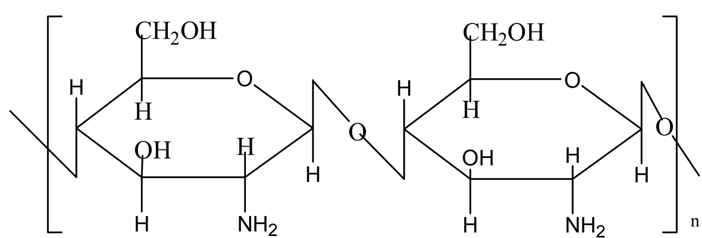

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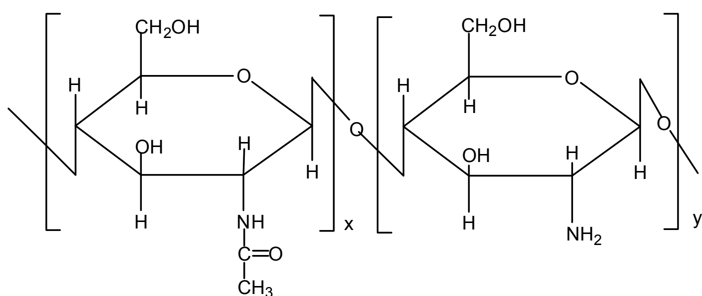

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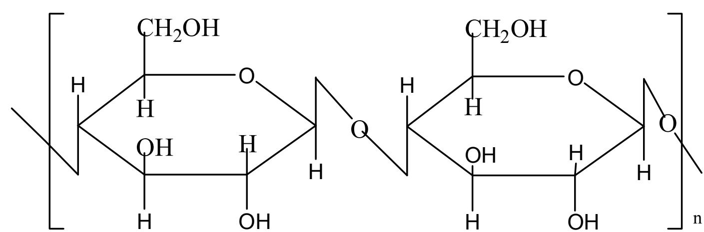

D

Figure 1. Structure of chitin, chitosan and cellulose. A – Chitin, B – Chitosan, C – Copolymer of chitin and chitosan, D – Cellulose.

The amount of chitin in animal and fungi is specific to species, age and environmental conditions where the organism exists. Chitin content in the dry shells of crabs, lobsters and shrimps ranges from 14 to 27 % (4), while in the fungal cell wall it varies from 2 to 42 %, the lowest value corresponding to yeasts, and the highest values to Euascomycetes (3).

CHITIN AND CHITOSAN PRODUCTION

INDUSTRIAL PRODUCTION OF CHITIN AND CHITOSAN

Chitin is the most abundant biopolymer on earth next to cellulose. It is estimated that its annual biosynthesis reaches 100 billion tons (5). The best available source of chitin is the seafood waste, primarily the crabs and shrimps shells. The annual worldwide production of crustacean shells has been estimated as 1.2×10^6 tons (6).

Crustacean chitin is naturally closely associated with proteins, minerals, lipids, and pigments. The industrial process consists of three basic steps: demineralization to remove calcium carbonate; deproteinization to remove protein; and decoloration to remove pigments (7). Demineralization is generally performed with HCl at a concentration of 0.275 – 2 M at a temperature of 0 - 100 °C for 1 - 48 h (7). Deproteinization is typically performed with 1 M NaOH at 65 - 100 °C for 1 - 72 h (8) and decoloration is carried out by ethanol, acetone, or hydrogen peroxide (7).

Based on the basic steps, many industrial procedures have been developed for the demineralization and deproteinization parameters. Thus Percot et al. (9) found that demineralization and deproteinization were completely achieved within 15 min at ambient temperature in 0.25 M HCl and within 24 h in 1M NaOH at a temperature around 70 °C without damage to the molecular weight or the degree of acetylation (DA), respectively.

Chitosan is generally produced from chitin by treatment with concentrated sodium or potassium hydroxide solution (40-50 %) at 80 - 150 °C (7). The production of chitosan from crustacean shells includes the following steps: (a) washing, drying and milling of

crustacean shells, (b) protein separation, (c) extensive washing, (d) demineralization, (e) extensive washing, (f) deacetylation, and (g) washing and drying (7).

PRODUCTION FROM FUNGAL SOURCES

Production of chitin and chitosan from fungal mycelium has recently received increased attention due to significant advantages. For example, while crustacean waste supplies are limited by seasons and sites of fishing industry, fungal mycelium can be obtained by convenient fermentation process that does not have geographic or seasonal limitations (10); fungal mycelia have lower level of inorganic materials compared to crustacean wastes, and thus no demineralization treatment is required during the processing (11); crustacean chitin and chitosan may vary in the physico-chemical properties, while fungal chitin and chitosan have relatively consistent properties because of the controlled fermentation conditions (12); fungal chitin and chitosan are apparently more effective in inducing the plant immune response and are potentially more suitable for agricultural applications (13).

Many fungal species, including *Absidia glauca*, *Absidia coerulea*, *Aspergillus niger*, *Mucor rouxii*, *Gongronella butleri*, *Phycomyces blakesleeanus*, *Absidia blakesleeana*, *Rhizopus oryzae*, *Trichoderma reesei* and *Lentinus edodes* have been investigated for the production of chitin and chitosan (10 – 12, 14 - 19). Among all investigated species, the most commonly researched one is *M. rouxii* (10, 14, 16) and quantities of chitin and chitosan in its mycelia can reach 35 % of cell wall dry weight (16).

Fungi are usually harvested at their late exponential growth phase to obtain the maximum yield for chitin and chitosan. Although fungi can be grown on solid media, cultivation for chitin and chitosan isolation is usually carried out in the yeast peptone glucose broth (YPG), potato dextrose broth (PDB) or molasses salt medium (MSM) (14). Chatterjee et al. (14) compared the performance of different media and did not find significant difference in the yield and physico-chemical properties of chitosan and chitin obtained.

Extraction process from fungal sources is similar to industrially utilized except that no demineralization treatment is required due to low mineral content in fungal mycelia (16). Generally, the extraction procedure consists of three steps: (1) alkaline treatment to remove protein and alkali soluble polysaccharides; (2) acid reflux to separate chitin and chitosan; and (3) precipitation of chitosan under alkaline conditions.

Removal of proteins by alkaline treatment is commonly performed with 1N NaOH at 95 °C from 1 to 6 h or at 121 °C from 0.25 h to 1 h (8). Separation of chitosan by acid treatment is usually carried out by 2 to 10 % acetic or hydrochloric acid at 95 °C for 3 to 14 h. For example, Synowiechi et al. (16) used 2 % NaOH at 90 °C during 2 h for alkali treatment and 10 % acetic acid at 60 °C during 6 h for acid reflux during extraction of chitin and chitosan from *M. rouxii*. Hu et al. (18) adopted autoclaving at 121 °C in both alkaline and acid treatments of *Absidia glauca mycelia*. However, the temperature and time of acid treatment had to be reduced to 25 °C and 1 h to avoid the depolymerization of chitosan during extraction from zygomycetes strains (19).

Most of the studies in this field concentrate on the fermentation processes to produce fungal mycelia for chitin and chitosan extraction (10 – 12, 14 - 19). Relatively few studies have focused on the fungal waste from industrial fermentations or mushroom industry (17). However, considering the amount of waste that accumulates during processing, citric acid industry and mushroom industry, specifically from *Agaricus bisporus* growing practices, can provide plenty of raw materials for fungal chitin and chitosan production.

Citric acid is the most widely used organic acid in food, beverage and pharmaceutical industries. The industrial production is based on *A. niger* submerged fermentation. The current world requirements for citric acid are estimated to be 400,000 tons per year (20). Taking into account that 20 % dry mycelium waste is produced under industrial fermentation conditions, approximately 80,000 tons of *A. niger* mycelium waste accumulates every year (21). Managing this waste presents an extra expense for the producers and alternative solutions for mycelium disposal have been evaluated. One of the potential outputs for the spent mycelia is in feed supplements. However, this type of feed seems to be difficult to compete with the other low price feeds.

White common mushroom, *Agaricus bisporus,* is the most consumed mushroom in the U.S. In last several years the production has been relatively constant and sales totaled 382 million kilograms in the 2002/03 season (22). The waste accumulated during mushroom production and harvest consists mainly of stalks and mushrooms of irregular dimensions and shape. Depending on the size of the mushroom farm, the amount of waste ranges between 5 and 20% of the production volume. This waste material results in approximately 50,000 metric tons per year that currently has no application (personal communication).

PHYSICO-CHEMICAL PROPERTIES AND ANALYSIS

Properly processed, highly purified chitin and chitosan are white and odorless. Their chemical structures are similar to those of cellulose (Fig1.A – Fig 1.D). The only difference is that the 2-hydroxy group of the cellulose has been replaced with an acetamide or amino group in chitin or chitosan, respectively. Therefore, the physico-chemical properties and research methodology for all three biopolymers are presumably similar. For example, chitin and chitosan are insoluble in the common organic and inorganic solvents, but soluble in salt organic mixtures of LiCl-N,N-DMAc, which is a common solvent for cellulose.

CHITIN AND CHITOSAN QUANTIFICATION AND PURITY DETERMINATION

Since chitin and chitosan are polysaccharides consisting of acetyl-glucosamine and glucosamine units, hydrolysis results in the cleavage of the glycoside bonds and deacetylation of N-acetyl-D-glucosamine monomers. The amount of end products, glucosamine units, can be used to estimate amount of chitin or chitosan in the analyzed material. This approach has been applied in agriculture to measure crops and food contamination with molds or in fermentation industry to measure fungal biomass (23 - 26).

The hydrolysis can be performed by acid or appropriate enzyme treatments. Hydrolysis with acid cleaves the β-(1→4) glycosidic bonds, removes the acetyl-group and produces glucosamine. The efficiency of acid hydrolysis is affected by many factors, such as the type and concentration of acid, the time and temperature for hydrolysis, and the type of material analyzed. The concentrations of acid, hydrolysis time, and temperature have been investigated by many researchers. For *Lentynus edodes* fruitbodies grown on sawdust, hydrolysis with 6 N HCl for 2 h at 110 °C resulted in 100 % recovery of glucosamine and longer hydrolysis resulted in glucosamine breakdown (24). However, Nilsson and Bjurman (1998) found that the best recovery for the same material was achieved when hydrolysis was carried out at 90 °C for 48 h with 6 N HCl (25). Plassard et al. (1982) applied 6N HCl at 80 °C for 16 hr for hydrolysis of mycelia of basiodiomycetes (26) and achieved a complete hydrolysis for the material analyzed.

Chitin can be also efficiently hydrolyzed to oligomers by chitinases (E.C. 3.2.1.14). The oligomers are usually further hydrolyzed to monomeric N-acetylglucosamine units by chitobiase (N-acetylglucosaminidase: E.C. 3.2.1.20). However, the enzymatic hydrolysis may be hindered by the presence of organic or inorganic constituents such as calcium carbonate or proteins (27). Consequently, enzymatic hydrolysis must be preceded by chemical treatments to eliminate interferences (27).

The quantification of end product of hydrolysis, glucosamine, can be performed by **c**olorimetric methods and chromatographic methods. One of the first colorimetric methods to determine glucosamine was developed by Elson and Morgan (28). In short, the samples were heated with acetylacetone in boiling water for 15 min; after cooling, Enrlich's reagent (*p*-dimethylaminobenzadehyde) was added and 30 minutes later, the color was compared to that of standards. Tsuji et al. (29) modified this method by using 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) and ferric chloride, as reagent and indicator, respectively. Although glucosamine can be determined by high performance liquid chromatography (30) or gas chromatography (31), colorimetric method has been proved to be simple, reproducible and sensitive for the chitin analysis in food and agricultural products.

The actual content of chitin can be calculated from the glucosamine content determined. Assuming that all units of the biopolymer were acetylated, chitin content (M) can be calculated as: M = $m \times 203/179$, where '*m*' presents the mass of glucosamine determined by analysis, '203' is molecular weight of acetylglucosamine anhydride (g/mol), and '179' is molecular weight of glucosamine (g/mol).

In fungal cell walls, chitin and chitosan molecules are associated with β-glucan chains through covalent and extensive hydrogen bonds (3). Thus, the residual glucan content in extracted fungal chitin and/or chitosan may reach significant levels and it is an important parameter of purity of the extracted product. β-glucan is the polymer of (1→3)-β-D-glucose and its content is most often estimated by colorimetric methods, such as anthrone method (32).

DEGREE OF ACETYLATION (DA)

Degree of acetylation is defined as the fraction (expressed in %) of acetylated glucopyranose units of the polymer chain. DA is a key property that influences the physical and chemical characteristics of chitin and chitosan including solubility, chemical reactivity and biological activity. For example, only the modified chitin samples with the degree of acetylation of about 50 % were soluble in water and those with either lower or higher DA were insoluble (33). Chitosan molecules with lower level of acetylation (DA < 15 %) are strongly positively charged in aqueous solution with pH bellow 6 (34).

Various techniques have been proposed to determine the degree of acetylation for chitin and chitosan. The methods include infrared spectroscopy (IR) (35, 36), ^{13}C solid-state NMR (37), ultraviolet spectrometry (38), potentiometric titration (39), and high pressure liquid chromatography (HPLC) (40). Among these techniques, IR is the most utilized one because of its convenience in minimal sample preparation, but IR requires the precise calibration using a wide spectrum of chitin and chitosan standards with known DA. The ^{13}C solid-state NMR appears to be the most reliable technique and is often used as the reference method (37), but it is not available in many laboratories due to the high cost of the instrument. Ultraviolet spectrometry and potentiometric titration are

techniques that require dissolved samples and, thus, are not applicable for chitins and chitosans with DA > 50 %.

HPLC method has been successfully developed to determine the DA for chitin and chitosan samples (40). The analysis is conducted in two steps. Firstly, the sample is hydrolyzed in a mixture of sulfuric and oxalic acid at 155 °C for 1 h. During the hydrolysis, acetic acid is released from acetylated units and subsequently quantified by HPLC (second step). The DA is calculated from the amount of acetic acid released from the acid hydrolysis. A typical HPLC spectrum for DA analysis of chitin and chitosan obtained in our laboratory is depicted in Figure 2. The amount of acetyl groups liberated (m_x) is calculated according the equation (1):

$$m_x = K \times \frac{Hx}{His} \times m_{is} \qquad (1),$$

where 'K' is the response factor, 'Hx' and 'His' are the heights of the acetic acid and internal standard peaks, respectively, and m_{is} is the amount of internal standard. The DA is calculated according to equation (2):

$$DA\ (\%) = \frac{161x}{43 - 42x} \times 100 \qquad (2),$$

where x was determined as $x = \frac{mx}{M'}$ and M' was mass of glucosamine in the material analyzed.

CRYSTALLINITY AND FTIR ANALYSIS

Depending on the source from which it was extracted, chitin has been found in three polymorphic forms: α-, β-, and γ-chitin. The polymorphic forms differ in the arrangement of the chains within the crystalline regions, which implies different networks of hydrogen-bonds. α-chitin refers to anti-parallel arrangement of adjacent chains, β-chitin to parallel arrangement, while γ-chitin represents the mixed arrangement of chitin molecules (41). The crystallinity of chitins and chitosans influences the diffusion properties and the accessibility of internal sorption sites (42). Availability of active groups, either amino or acetylamido groups, significantly impact biological activities (42).

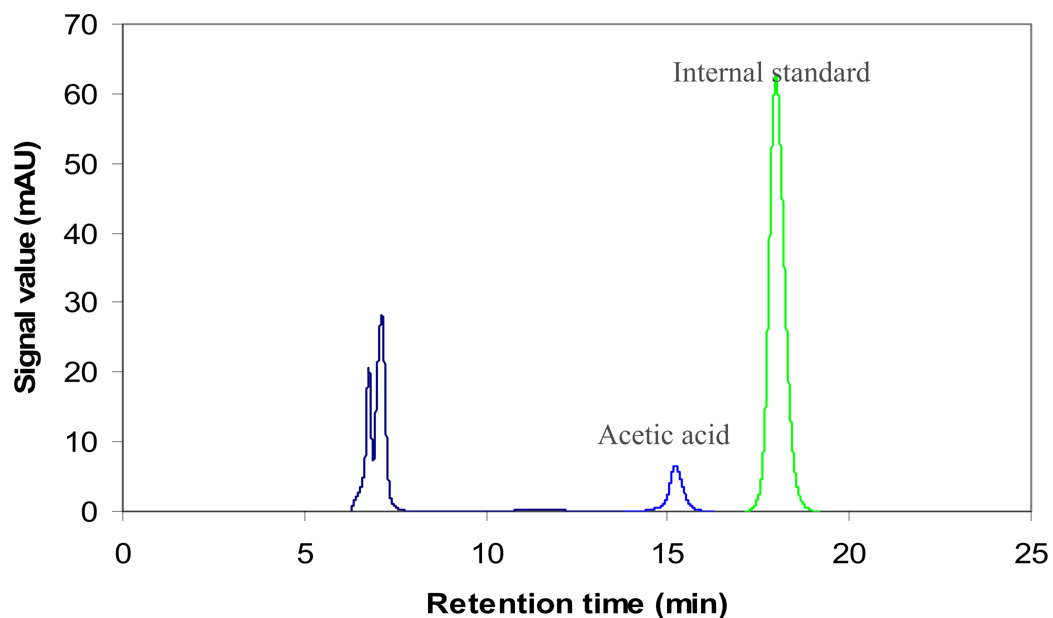

Figure 2. HPLC chromatograph obtained during DA analysis of chitin and chitosan. First peak with the retention time of 6.3 min represents oxalic acid. Blue peak with Retention time of 15.3 min represents acetic acid. Green peak with retention time of 18.1 min represents proprionic acid used as an internal standard.

The crystallinity of chitin can be determined by x-ray diffraction (43). However, good estimation of crystallinity development in chitin samples has been achieved with FT-IR (43). Infrared spectroscopy is a powerful tool used in organic and polymer chemistry. Band at 1379 cm^{-1} has been assigned to CH bending with some OH-bending contributions (43). The ratio of intensities of the bands at 1379 cm^{-1} and 2900 cm^{-1} has been suggested as the crystallinity index for chitin and chitosan (43) (Figure 3). The tendency of crystallinity change was consistent with the results of X – ray and NMR measurements (43).

APPLICATIONS OF CHITIN AND CHITOSAN IN FOOD PRESERVATION AND PLANT PROTECTION

ANTIMICROBIAL ACTIVITY AND APPLICATIONS IN FOOD PRESERVATION

It has been shown that chitosan posses strong antimicrobial activity against both gram-positive and gram-negative bacteria, including the foodborne pathogens, such as *Escherichia coli*, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Listeria monocytogenes* (44- 47). Two theories have been proposed for the antimicrobial mechanism of chitosan. Based on one, interaction between positively charged chitosan molecules and negatively charged microbial cell membranes results in the disruption of the cytoplasmic membrane and, ultimately, leakage of intracellular constituents (46). By the other theory, chitosan oligosaccharides easily permeate into the nucleus of eukaryotic cell and interfere with the transcription of RNA and the synthesis of proteins (47). However, chitosans with high molecular weight (above 100 kDa) generally express stronger antibacterial activity than chitosan oligomers (45).

ELICITOR EFFECT TO INDUCE PLANT DEFENSE RESPONSE AND APPLICATIONS IN PLAN PROTECTION

Botrytis cinerea and *Penicillium expansum* are the two major plant pathogens that cause considerable losses in wide varieties of harvested commodities, including apples,

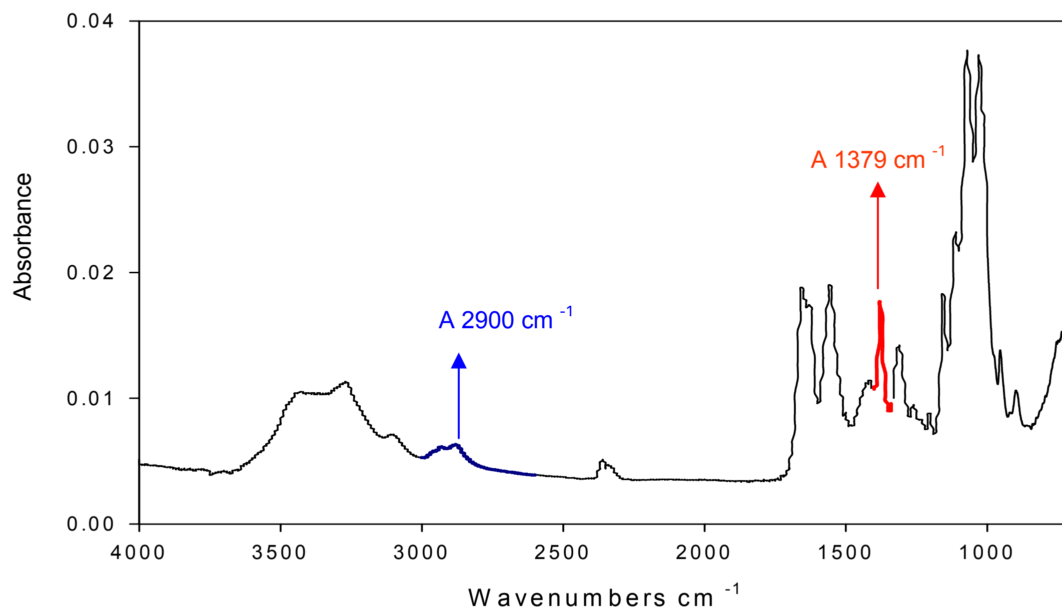

Figure 3. FTIR spectrum of chitin.

* Peaks used for crystallinity analysis are labeled as A1379 cm^{-1} and A2900 cm^{-1}.

grapes, and peaches (48 - 52). Application of chemical fungicides is the most common procedure used for postharvest disease control. However, the public concerns about the pesticide residues, specially on fresh fruits and vegetables, are increasing and demand for more natural approach is currently very strong. Therefore, the biological control of fungal diseases has been extensively studied (48- 52, 53). For example, three saprophytic yeasts, *Cryptococcus laurentii, Cryptococcus infirmominitus* and *Rhodotorula glutinis* were tested as the biocontrol agents against *P. expansum* on pears by Chand-Goyal et al. (54). The combination of *C. laurentii* and *C. infirmo-miniatus* whole cell suspensions was the most effective treatment and it reduced the *P. expansum* incidence on pears to 9 % compared to water treatment control. Teixido et al. (50) found that postharvest treatment with *Candida sake* resulted in effective control of *Penicillium expansum* on apples during cold storage. Vero et al. (51) also found that *Cryptococcus laurentii* and *Candida ciferrii* reduced the incidence *Penicillium expansum* up to 80 % at 25 °C. Furthermore, fumigation of apples for 7 days with volatiles produced by fungus *Muscodor albus* gave a complete control of *Botrytis cinerea* and *Penicillium expansum* in fruits (52). Bacterium, *Pantoea agglomerans* was also found to be very effective agent for control of *Botrytis cinerea* and *Penicillium expansum* in wounded pears even at concentration of 2×10^{7} CFU ml^{-1} (53).

Chitin and chitosan have shown the ability to act as potent agents to elicit higher plant defense reactions and inhibit the growth of several phytopathogenic fungi and bacteria (54). Chitosan was found to be effective in reducing the amount of polygalacturonases produced by *Botrytis cinerea* and caused severe cytological damage to the invading hyphae in bell pepper fruit (55). Chitosan induced the synthesis of phytoalexin in rice leaves, which is a potent suppressor of fungal growth (56). In the cucumber plants sprayed with chitosan or chitin before the inoculating of *Botrytis cinerea*, chitosanase and peroxidase activity increased and growth of *Botrytis cinerea* was successfully inhibited (57).

OBJECTIVES OF THIS STUDY

The overall objective of this research was to evaluate the possibility of production and application of chitin and chitosan from fungal sources. Specifically, the objectives were (1) to determine yield of chitin and chitosan in three fungal species (*Agaricus bisporus, Aspergillus niger,* and *Mucor rouxii*) and (2) to determine physico-chemical and biological properties of extracted material.

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Part 2

Chitin and chitosan – value added products from mushroom waste

This chapter is a lightly revised version of a paper by the same name submitted to the Journal of Agriculture and Food Chemistry in May, 2004 by Tao Wu, Svetlana Zivanovic, F. Ann Draughon, and Carl E. Sams.

My use of "we" in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) most of sampling, chitin and chitosan extraction, and physico-chemical properties determination, (2) most of the data gathering and interpretation, (3) most of the writing.

ABSTRACT

Accumulation of chitinous material in *Agaricus bisporus* stalks was determined during post-harvest storage at 4 °C and 25 °C. The chitinous material was extracted after alkali treatment and acid reflux of alkali insoluble material and analyzed for yield, purity, degree of acetylation (DA), and crystallinity. Total glucosamine content in mushroom stalks increased from 7.14 % dry weight (dw) at harvest (day 0) to 11.00 % dw and 19.02 % dw after 15 days storage at 4 ºC and 5 days at 25 ºC, respectively. The yield of crude chitin isolated from stalks stored at 25 ºC for 5 days was 27.00 % dw and consisted of 46.08 % glucosamine and 20.94 % neutral polysaccharides. The DA of fungal chitin was from 75.8 % to 87.6 %, which is similar to commercially available crustacean chitin. The yield of crude fungal chitin of 0.65 % to 1.15 % on a fresh basis indicates potential for the utilization of these mushroom by-products.

KEYWORDS: *Agaricus bisporus*; chitin; chitosan; glucosamine; waste utilization; biobased products; value-added products

INTRODUCTION

White common mushroom, *Agaricus bisporus,* is the most consumed mushroom in the U.S. Production of *Agaricus* mushrooms has been relatively constant and sales totaled 311 million kilograms in the 2002/03 season (1). Waste accumulated during mushroom production and harvest consists mainly of stalks and mushrooms of irregular dimensions and shape. Depending on the size of the mushroom farm, the amount of waste ranges between 5 and 20% of production volume. This results in approximately 50,000 metric tons of waste material per year with no suitable commercial use. Waste disposal creates environmental problems for producers due to the large volume and volatile degradation products. However, *Agaricus bisporus* is rich in chitinous biopolymers that can be utilized as a biopesticide (2, 3, 4), antimicrobial agent (5, 6), coating material (7), or for water purification (8).

Fungal chitinous biopolymers, or glucosaminoglycans, consist mainly of chitin and chitosan. Besides being found in fungal cell walls, chitin is the major structural biopolymer in crustacean shells, squid skeleton, and the cuticle of insects. Currently, commercial chitin and its deacetylated derivate, chitosan, are produced from shrimp and crab shells as by-products of the sea-food industry. However, the conventional industrial isolation of chitin from crustacean shells requires harsh solvents and high temperature treatments and has seasonal supply as well as geographical limitations. Fungal biotechnology offers advantages in the production of fungal chitin and chitosan over classical processing procedures because chitinous material can be produced in a controlled environment all year round, and the extraction process is simpler and requires less harsh solvents.

Fungal cell walls are composed of neutral polysaccharides, chitin, chitosan and glycoproteins with minor amounts of galactoamine polymers, polyuronides, melanin and lipids (9, 10). In *A. bisporu*s, the neutral polysaccharides make up approximately 50 % of the cell wall dry weight (11, 12, 13). Highly branched β-1,3/β-1,6 glucan and linear α-1,3 glucan are accumulated at the outer layer of the cell wall or as an extracellular amorphous gel that hold hyphae together (14). The major cell wall polysaccharide, linear β-1,3

glucan, provides 15 to 30 % of the total cell wall polysaccharides and has a strong tendency for hydrogen bonding and formation of crystalline microfibrils with chitin (15). Chitin, 2-acetamido-2-deoxy-β(1→4)-D-glucan, is the second most abundant polymer in the fungal cell wall. It is structurally very similar to cellulose but contains acetamino, instead of a hydroxyl group on the C2 atom glucose monomer. The chitin content in *Agaricus* cell walls has been reported to be: 13.3 to 17.3 % (11), 35 % (16), 20 to 38 % (12), and 43 % (17). This broad range of concentrations can be explained by the fact that chitin content significantly increases during the mushroom life-cycle (12, 18) and post-harvest storage (19). In cell walls of fungi, chitin usually occurs in a complex with β-1,3 glucan. Formation of wall microfibrils starts with hydrogen bonding between homolog linear molecules and continues with covalent linkages between glucan and chitin (20). While crystalline microfibriles of β-1,3 glucan and chitin are insoluble in common solvents, other wall polymers (α-1,3 and β-1,3/1,6 glucans, proteins and glycoproteins) are easily removable by alkali extraction.

Chitosan, 2-amino-2-deoxy-β(1→4)-D-glucan, is also one of the structural biopolymers in fungal cell walls. However, its content depends primarily on the taxonomy of the fungi. Thus, the chemotype of the cell wall in Zygomycetes consist of a chitosan-glucan complex, while in Homobasidiomycetes, Euascomycetes, and Deuteromycetes it contains chitin-glucan (10). Chitosan is commercially produced by deacetylation of chitin from crustacean shells, but the technology requires strong alkali, high temperatures, and a long processing time (21). Since late 1970s, when White et al. (22) proposed a laboratory-scale method for the isolation of chitosan from mycelia of *M. rouxii*, different protocols have been developed to utilize fungal biomass rather than crustacean shells for production of chitosan (23-27). Mycelia of various fungi including *Absidia coerulea, Absidia glauca, A. niger, Colletotricum lindemuthianum, Gangronella butleri, M. rouxii, Phycomyces blakesleeanus, Pleurotus sajo-caju, Rhyzopus oryzae, Lentinus edodes,* and *Trichoderma reesei* have been suggested as alternative chitosan sources to crustaceans (25, 26, 28, 29, 30, 31). However, due to variations in extraction procedures reported in the literature, it is impossible to reliably compare different fungal species as potential chitin/chitosan sources. Nevertheless, regardless of the fungal species

or the method used, extraction of fungal chitinous material always starts with a dilute alkali treatment to remove proteins, glycoproteins and branched polysaccharides. In a subsequent step, chitosan is isolated from alkali insoluble material by acid extraction, and chitin and β-glucan remain as alkali/acid insoluble residue.

Fungal chitin and chitosan potentially differ from those isolated from crustaceans in molecular weight, degree of acetylation, and distribution of charged groups. These differences can promote their functional properties and enhance bioactivity. However, research is needed to evaluate the most economical way of obtaining chitinous material from fungal sources. The objectives of this study were to determine the content and properties of chitinous material in *Agaricus bisporus* stalks and to design a process for extraction of these bioactive polymers from mushroom waste.

MATERIALS AND METHODS

Mushroom samples. White button mushrooms, *Agaricus bisporus,* were donated from Monterey Mushrooms, Inc., Loudon, TN. Whole fruit bodies were harvested in the "closed cap" stage with a cap diameter of 30 ± 5 mm and transported to the laboratory within one hour after harvest. Immediately upon arrival at the laboratory, stalks were separated from the caps, packed in paper bags (100 g stalks per bag, one bag representing one replication), and stored at 4 °C and 25 °C. Triplicate samples were taken for analysis after 0, 1, 2, 3, 4, and 5 days storage at 25 °C and after 0, 3, 6, 9, 12, and 15 days at 4 °C. At each sampling time, fresh weight losses and dry weight content were determined and the rest of the stalks were freeze-dried, ground to a powder with a Thomas Wiley mill (Thomas Co., Philadelphia, PA), sieved through a #40 mesh, and stored in a desiccator at room temperature for further analyses and extraction.

Commercial chitins and chitosans obtained from crustacean shells were provided by Primex, Co. (Iceland) and purchased from ICN Biomedicals, Inc. (Aurora, OH), Fluka (Luausanne, Switzerland), and Aldrich (Milwaukee, WI).

Glucosamine determination. The procedure for glucosamine determination was based on the 3-methyl-2-benzothiazolone hydrazone hydrochloride (MBTH) colorimetric

assay of Tsuji et al. (32) with minor modifications. Chitin and chitosan standards and samples at various stages of extraction were hydrolyzed with 6 M HCl at 110 ºC. Hydrolysis by strong acid causes depolymerization of the polysaccharides and deacetylation of acetylglucosamine units, resulting in free glucosamine residues (33). However, the time necessary for complete hydrolysis of each type of the chitinous material depends on the level of crystallinity between the macromolecules and was determined in preliminary experiments. Hydrolyzate was neutralized with sodium acetate (34), and glucosamine was deaminated with nitrous acid to yield the anhydromannose. After the reaction, the excess nitrous acid was neutralized with ammonium sulfamate, and MBTH and $FeCl_2$ were added to produce a blue color complex with anhydromannose. The absorbance was measured at 650 nm. A calibration curve was prepared from glucosamine chloride standards (10 to 150 μg/ml).

Extraction of chitinous material. Chitinous material was obtained after treatments with alkali and acid by a procedure adapted from Rane and Hoover (27) and Synowiecki and Al-Khateeb (35) (Figure 1). The freeze-dried stalk powder was stirred with 1 M NaOH (w:v/1:40) and refluxed at 95 ºC for 30 min to extract proteins, alkali-soluble polysaccharides and small molecules (e.g., monosaccharides, phenolics, amino acids, and salts). The slurry was centrifuged (12,000 g, 20 min, 22 °C) and alkali insoluble material (AIM) was washed two times with deionized water and 95 % ethanol. After the final centrifugation, the alkali insoluble residue was freeze-dried and ground to a fine powder. To obtain insoluble crude fungal chitin, AIM was refluxed in 2 % acetic acid (w:v/1:100) for 6 h at 95 ºC. The acid-treated slurry was centrifuged (12,000 g, 20 min, 22 °C) and washed with d.i. water and ethanol as described for AIM. Chitin, if present, would remained as insoluble residue (crude chitin), and chitosan, if present, would be extracted with aqueous acetic acid and precipitated from the supernatant after adjustment of pH to 10.

Characterization of extracted chitinous material. Glucosamine and glucan contents in AIM, insoluble residue, and supernatants were determined by the MBTH (32) and the anthrone method (36), respectively. The FTIR analysis was performed with a Nexus-670 FT-IR spectrometer using attenuated total reflection (ATR) sampling

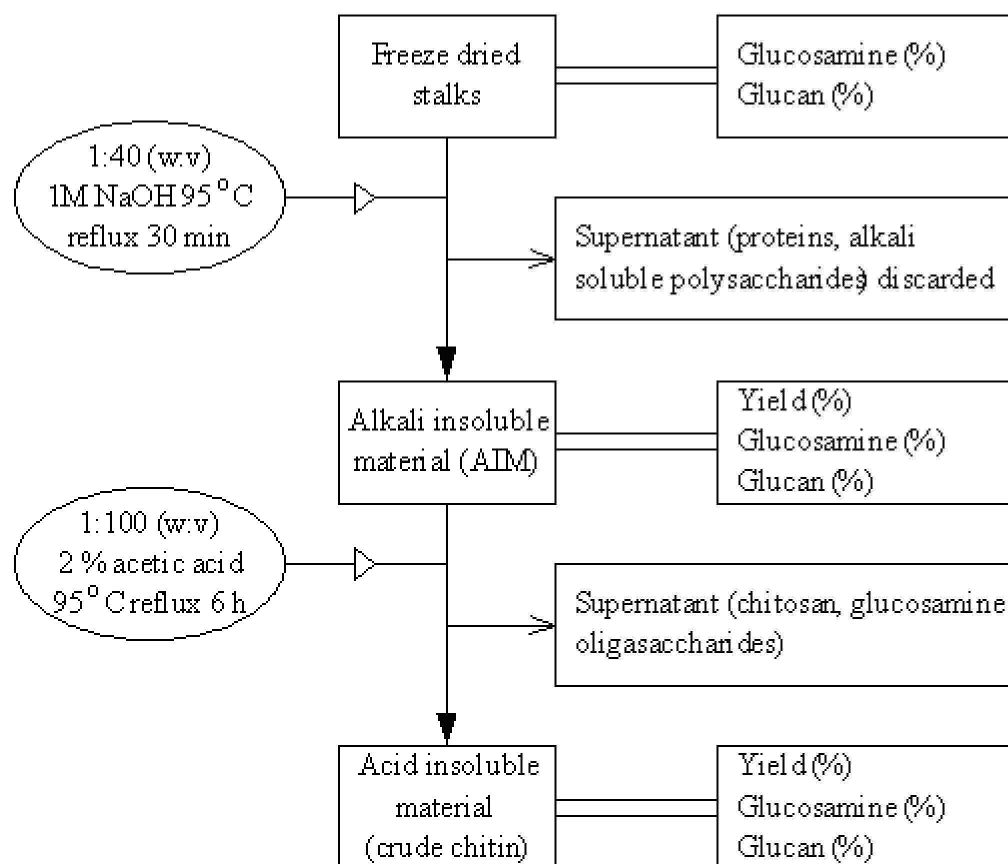


Figure 1. Extraction of chitinous material from *Agaricus bisporus* stalks.

accessory in the range 700 to 4000 cm^{-1} (ThermoNicolet, Mountain view, CA). Degree of acetyletion (DA) was determined after hydrolysis of chitin/chitosan samples following the method of Niola et al. (37). Hydrolysis was performed in 12 M H_2SO_4, with an oxalic and propionic acid standard mixture, at 155 ºC for 1 h by using vacuum hydrolysis tubes and heating blocks (Pierce Biotechnology, Inc., Rockford, IL). Acetic acid liberated during hydrolysis was analyzed on a HPX 87H column (BioRad, Hercules, CA) using a HPLC system with PDA detector (Dionex, Sunnyvale, CA). The mobile phase was 10 mM H_2SO_4, the flow rate 0.60 ml/min, the injection volume 10 µl, and absorbency was monitored at 210 nm.

RESULTS AND DISCUSSION

Fresh weight losses and dry weight content. Fresh weight of mushroom stalks declined during storage, more rapidly at 25 °C than at 4 °C (Figure 2, A). The average fresh weight loss was 35.65 % after 5 days at 25 °C and 26.50 % after 15 days at 4 °C. The weight reduction of fresh mushrooms due to extensive moisture loss has previously been reported (38). Moisture loss presents a significant problem in marketing of fresh mushrooms and is commonly reduced by modified atmosphere packaging. However, the reduction of fresh weight in mushroom waste has no adverse effects on waste handling, and is advantageous due to the reduction of waste to be processed. However, it appears that losses during storage were caused not only by water evaporation but also due to dry weight reduction (Figure 2, B).

Dry weight also declined more rapidly at the higher storage temperature. At harvest, fresh stalks contained 7.79 % dw. However, dry weight declined to 6.79 % after 15 days at 4 °C, and to 4.27 % after 5 days at 25 °C. Dry weight decrease can be explained by biochemical processes, such as respiration, that continue within the cells even after harvest (39). Although this fact can have negative implications on potential utilization of the mushroom waste for extraction of structural biopolymers, an increase in chitin content in cell walls may compensate for the decrease in total dry matter (19).

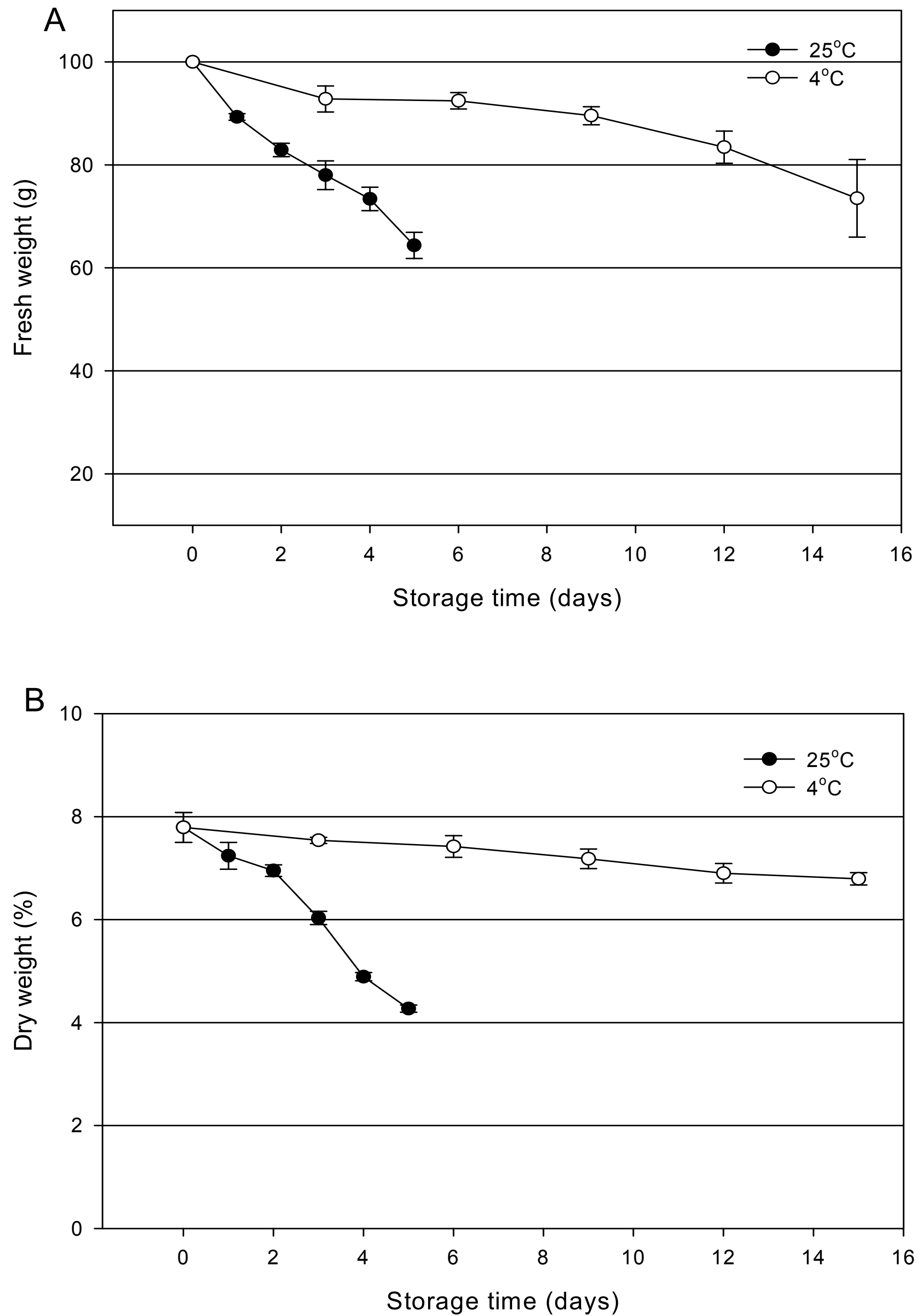


Figure 2. Fresh weight losses (A) and dry weight content (B) in mushroom stalks during storage at 4 °C and 25 °C. Error bars represent standard deviation of 3 replications.

Total glucosamine content. Chitin and chitosan are linear polysaccharides consisting of N-acetyl-D-glucosamine and D-glucosamine units present in different ratios in the polymers. Regardless of the method used for determination of chitin and chitosan concentration, a complete hydrolysis of the polymers is needed prior to the analysis. Acid hydrolysis results in cleavage of the glycoside bonds between (acetyl)glucosamine molecules and in deacetylation of N-acetyl-D-glucosamine monomers (33). The amount of end products, glucosamine residues, can be easily determined and used to estimate the amount of chitin and chitosan in the analyzed material. However, type of material tested, type and concentration of the acid, and temperature and time of hydrolysis influence the recovery of glucosamine. According to Cousin (40), the optimum hydrolysis of chitinous material from *Lentinus edodes* occured with 6 M HCl, for 2 hours at 110 ºC. However, 2 h were insufficient for complete hydrolysis of the materials used in this study. To determine the optimum hydrolysis time for complete depolymerization of the fungal material, we examined glucosamine recovery during 18 h of hydrolysis in 6 M HCl at 110 °C. Since the extended crystallinity can reduce the accessibility of the polymers by acid molecules, commercial chitin and chitosan standards were included in the study. The results showed that maximum recovery of glucosamine was achieved after 3-hour hydrolysis of chitosan and freeze dried stalks and after 12-hour hydrolysis of chitin and alkali insoluble material (Figure 3). With prolonged time of hydrolysis, the amount of detected glucosamine decreased, apparently due to degradation of liberated monomer under harsh conditions of hydrolysis. Thus, the hydrolysis time for further analysis was 3 h for freeze dried stalks and 12 h for AIM and fungal chitin. However, the absolute recovery of glucosamine from highly crystalline chitin was much lower than from chitosan. Analyzing commercial chitosan standard, we determined 93.02 ± 4.55 % glucosamine after 3 h hydrolysis, and a maximum of 68.66 ± 2.42 % from chitin standard although the hydrolysis lasted 12 h (Table 1).

The glucosamine content in mushroom stalks increased from 7.14 % dw at day 0 (at harvest) to 11.00 % and 19.02 % dw after 15 days at 4 ºC and 5 days storage at 25 ºC, respectively (Figure 4, B). Calculated on a fresh weight basis, the maximum glucosamine content of 0.88 % was reached in the stalks stored at 25 ºC after 4 days (Figure 4, A).

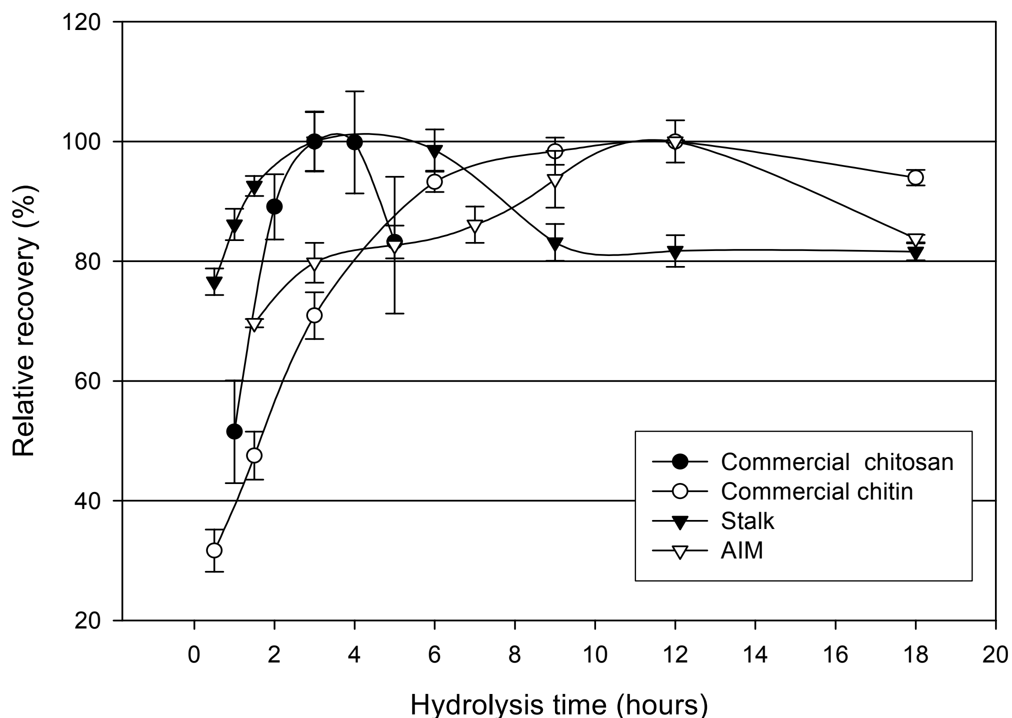
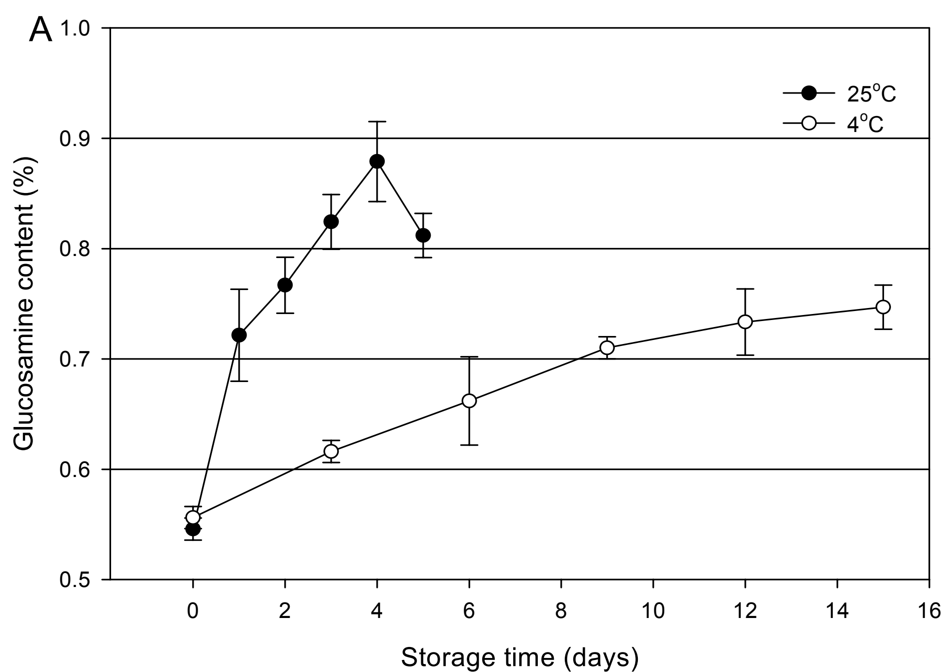


Figure 3. Effect of hydrolysis time on recovery of glucosamine from commercial chitin and chitosan, freeze-dried mushroom stalks obtained at harvest, and alkali insoluble material (AIM) from stalks stored 5 days at 25 °C. All samples were hydrolyzed in triplicates in 6 M HCl at 110 °C. Glucosamine recovery represents the ratio of detected glucosamine at a particular point and maximum detected glucosamine in the sample. Error bars represent standard deviation of 3 replications.

Table 1: Effects of hydrolysis time on glucosamine extracted from mushroom sources.

Time (h)	**Glucosamine** (%)			
	Chitosan[a]	**Chitin**[b]	**Stalks**[c]	**AIM**[d]
0.5	—[e]	21.74 ± 2.42	5.36 ± 0.16	—
1.0	47.93 ±7.98	—	6.03 ± 0.18	—
1.5	—	32.64 ± 2.75	6.48 ± 0.12	37.33 ± 0.37
2.0	82.87 ± 5.07	—	—	—
3.0	93.02 ± 4.55	48.69 ± 2.68	7.00 ± 0.35	42.74 ± 1.78
4.0	92.88 ± 7.93	—	—	—
5.0	83.22 ± 2.54	—	—	44.31 ± 6.12
6.0	—	64.03 ± 1.17	6.90 ± 0.24	—
9.0	—	67.54 ± 1.56	5.82 ± 0.22	50.21 ± 2.54
12.0	—	68.66 ± 2.42	5.72 ± 0.19	53.59 ± 0.12
18.0	—	64.51 ± 0.88	5.71 ± 0.10	44.90 ± 0.34

[a] Commercial chitosan; [b] Commercial chitin; [c] Freeze-dried stalks obtained at harvest; [d] Alkali insoluble material (AIM) extracted from stalks stored 5 days at 25ºC; [e] Not determined.



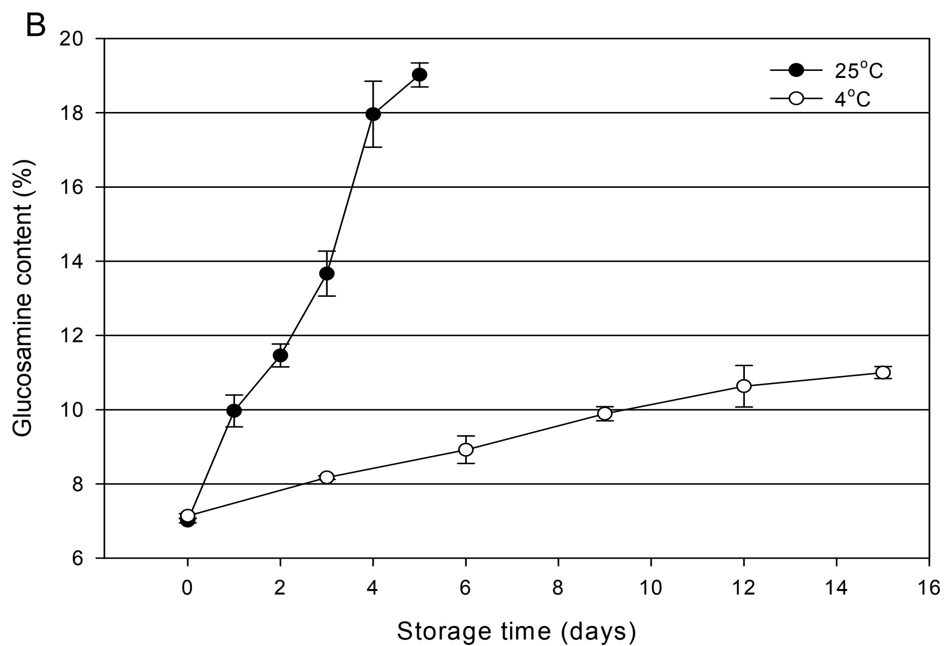


Figure 4. Total glucosamine content in mushroom stalks stored at 4 °C and 25 °C. The results are expressed on fresh weight (A) and dry weight (B) basis Error bars represent standard deviation of 3 replications.

More chitinous material was produced at 25 ºC than during storage at 4 °C (0.75 %). However, the content of chitinous material on fresh basis decreased after 4 days at 25 °C due to extensive dry weight loss and deterioration, either by endogenous enzymes and/or spoilage microorganisms.

The amount of chitin determined in stalks stored at 25 ºC for 5 days expressed on dry weight basis was 19.02 % (Figure 4, B) and was consistent with 21 % in *Agaricus bisporus*, 19.0 % in *Lactarius vellereus*, and 18.5 % in *Penicillium notatum* (10, 41).

However, quantity and solubility of wall polymers largely fluctuates with the development stage of mycelia (24). Experiments with *Absidia* spp., *Lentinus edodes*, *M. rouxii*, and *Rhizopus oryzae* have indicated that the highest accumulation of chitosan in hyphae walls was in the late exponential phase (29, 35, 42, 43). Thus, although *M. rouxii* is the most commonly explored fungi for chitosan extraction, the content of chitosan reported by different authors range from 8.8 to 44.5 % on a dry cell wall weight basis (8, 10, 22, 45). The total glucosamine level of 19.02 % on dry weight basis of *Agaricus bisporus* stalks is significant for commercial production considering the amount of waste generated during mushroom production.

Extraction and characterization of chitinous material. The freeze-dried samples obtained from stalks stored at 25 ºC for 5 days were used for the extraction and characterization of chitin and chitosan. The yield of alkali insoluble material was 32.03 % dw, and contained 43.84 % glucosamine and 17.92 % β-glucan (Table 2). Alkali extraction has usually been carried out in 1N NaOH by autoclaving a mycelia suspension at 121 °C for 15 min (26, 29, 30, 44). McGahren et al. (24) used milder conditions of alkali extraction but reported that protein impurities were still present. The AIM in our experiments contained no residual proteins, and extraction under atmospheric pressure is more practical for commercial applications. Boiling acetic acid further removed impurities, and the treatment resulted in 27.00 % crude chitin but did not yield any chitosan. Chitosan is commonly extracted from AIM by aqueous acids at temperatures from 25 °C to 121 °C during 1 to 14 h, with higher yields achieved with higher temperatures and longer treatments. (22, 25, 27, 31, 44). Nevertheless, the acid extraction of *A. bisporus* stalks did not yield any chitosan. This result was consistent with the results

Table 2: Yield and composition of material obtained during extraction of chitinous material from *Agaricus bisporus*.

Sample	Treatment	Yield[a]	Glucosamine[b]	Glucans[c]
Freeze dried stalks	Fresh[d]	(100)	7.14 ± 0.01	18.99 ± 1.16
	15 d 4 °C	(100)	11.00 ± 0.16	14.03 ± 2.88
	5 d 25 °C	(100)	19.02 ± 0.32	15.90 ± 2.92
Alkali insoluble material (AIM)	Fresh	12.65 ± 0.53	38.17 ± 2.12	34.99 ±1.18
	15 d 4 °C	17.47 ± 1.09	45.76 ± 1.89	22.07 ± 3.74
	5 d 25 °C	32.03 ± 0.42	43.84 ± 5.96	17.92 ± 2.24
Acid insoluble residue (crude chitin)	Fresh	8.30 ± 0.33	43.36 ± 1.61	46.27 ±2.21
	15 d 4 °C	13.90 ± 1.24	48.07 ± 1.86	26.79 ± 2.88
	5 d 25 °C	27.00 ± 0.39	46.07 ± 3.02	20.94 ± 1.75

[a] Yield expressed as % of dry weight of stalks; [b] Cell wall chitin was hydrolyzed and determined as glucosamine (% of analyzed material); [c] Cell wall neutral polysaccharides determined by anthrone reagent (% of analyzed material). Results are presented as mean values of three determination ± standard deviation; [d] Mushrooms were freeze dried and tested before any extraction treatments.

of Mol and Wessels (18) who found that chitinous material in *A. bisporus* cell wall is mainly in the acetylated form as chitin, contrary to *M. rouxii* where it is in the deacetylated, acid-soluble, chitosan form.

The acid insoluble residue, crude chitin, was apparently a chitin-glucan complex composed of 46.07 % glucosamine polymers and 20.94 % glucan (Table 2). The total amount of glucosamine in acid insoluble residue was lower than expected. The total glucosamine content in mushroom stalks after 4 days storage at 25 °C was 19.02 % (Figure 4, B) but only 12.44 % was obtained after alkali and acid treatments. The loss of (acetyl)glucosamine and its oligomers during extraction was excluded as the potential reason for the difference since no glucosamine was detected in either alkali or acid soluble material (data not shown). More likely, the difference may be attributed to crystallinity of chitin alone and/or within the chitin-glucan complex (18). Crystallinity would prevent accessibility of hydrochloric acid to crystallized chitin molecules and result in incomplete hydrolysis. If the maximum recovery of glucosamine from chitin standards is taken into account (68.66 %, Table 1), the detected glucosamine in crude fungal chitin would represent 67.10 % chitin. In other words, out of 19.02 % of total glucosamine originally present in *Agaricus bisporus* stalks, 18.11 % dw was extracted as crude chitin with a yield of 95.2 %.

The coexistence of glucan in crude fungal chitin has been reported (10, 20). The presence of β-glucan provides an additional benefit to the crude fungal chitin since the β-glucan has been shown to act as a potent elicitor that induces defense responses in several plants (4). Currently, only shrimp chitin has been registered as a nematocide (46) but the applications of fungal chitin-glucan complex as a biopesticide or plant growth regulator are promising, and this research is under way in our laboratories.

The **degree of acetylation** (DA) of crude chitin from stalks stored at 25 °C for 5 days was determined as liberated acetic acid after hydrolysis of insoluble residue in sulfuric and oxalic acid (36). The DA of crude chitin was 36.7 ± 1.7 %. However, when the β-glucan content was considered, the DA value of fungal chitin was calculated to be 79.7 ± 3.6 %, which is in the range of DA values of commercial crustacean chitin (70 to

90 %). Furthermore, the high DA value confirms that chitinous material of *Agaricus bisporus* is insoluble acetylated chitin rather than the deacetylated polymer, chitosan.

The **FTIR spectra** of chitin and chitosan standards, fungal AIM and chitin-glucan complex are shown in Figure 5. The spectra of AIM, chitin-glucan complex and crustacean chitin were similar, but quite different from that of chitosan. Several important characteristics in the spectra of AIM and chitin-glucan complex were observed. According to Focher et al. (47), the split of the amide I vibration band occurring at 1655 cm^{-1} indicates that the fungal chitin is in antiparallel, α-conformation. Furthermore, the weakening of the band occurring at 1655 cm^{-1} and absence of the band at 3100 cm^{-1} was associated with deacetylation and clearly differentiated chitosan standards from chitin and fungal chitinous material in our experiment. These results further confirmed that glucosaminoglycans of *Agaricus bisporus* were exclusively in a form of highly acetylated chitin molecules. The ratio of intensities of the bands at 1379 cm^{-1} and 2900 cm^{-1} has been suggested as the crystallinity index for chitin and chitosan (48).

As shown in Table 3, the crystallinity increased in the following order: commercial chitosan < fungal AIM < fungal chitin < commercial chitin. These results were expected because of the high DA of crude *Agaricus* chitin (79.7 %). The high degree of acetylation of chitin molecules (> 70 %) was associated with extended crystallinity while the low DA of chitosan molecules (< 30 %) resulted in a random distribution of the acetyl groups that did not allow significant development of the crystalline regions. The crystallinity index increased with purification of fungal material (fungal chitin > fungal AIM) mainly due to removal of interfering amorphous components. Similarly, Mol and Wessels (18) used X-ray diffraction to evaluate crystallinity of untreated, alkali and acid treated hyphae walls and found that X-ray patterns sharpened with purification and characteristic crystallinity peaks of α-chitin appeared after acid treatment of alkali-insoluble material.

Conclusion

We found that the chitin content in *Agaricus bisporus* stalks reached 19.02 % dw during post-harvest storage and could be efficiently extracted. Given the composition and

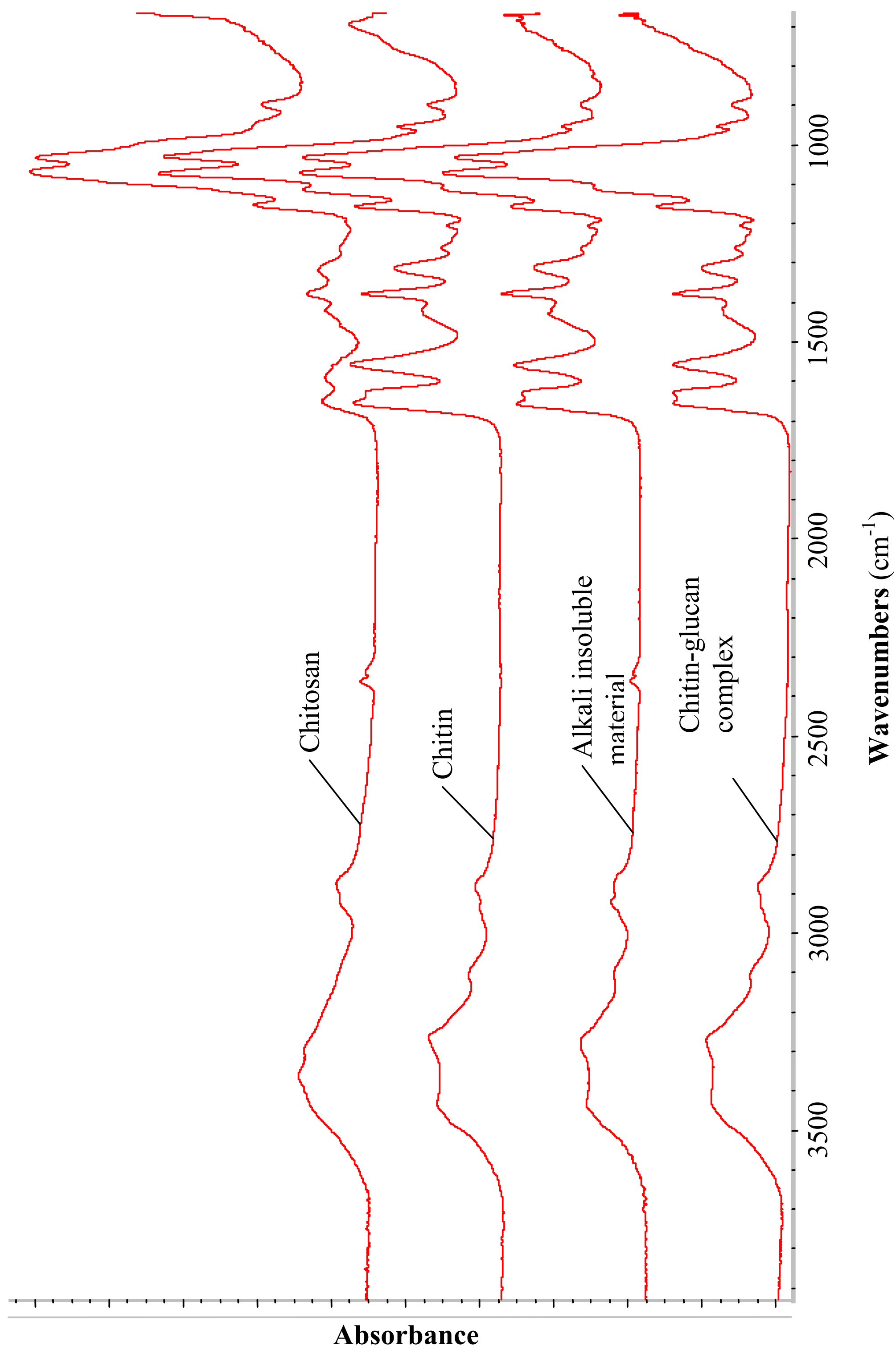


Figure 5. FTIR spectra of commercial chitin and chitosan, and alkali insoluble material (AIM) and acid insoluble residue (chitin-glucan complex) from *Agaricus bisporus*.

Table 3: Crystallinity of chitinous materials* determined by FTIR

Chitinous material	**Peak area**		
	$\mathbf{A_{1379\ cm^{-1}}}$	$\mathbf{A_{2920\ cm^{-1}}}$	$\mathbf{A_{1379\ cm^{-1}}/A_{2920\ cm^{-1}}}$
Chitin -1	0.15	0.12	1.25
Chitin -2	0.18	0.15	1.20
Chitin-glucan complex -1	0.22	0.29	0.76
Chitin-glucan complex -2	0.12	0.16	0.75
AIM -1	0.18	0.33	0.55
AIM -2	0.20	0.39	0.51
Chitosan -1	0.08	0.30	0.27
Chitosan -2	0.05	0.25	0.20
Chitosan -3	0.05	0.19	0.26
Chitosan -4	0.08	0.40	0.20
Chitosan -5	0.07	0.31	0.23

* Chitinous material included crustacean chitin (ICN and Primex), crustacean chitosan (Aldrich, Fluka, ICN, Primex), alkali insoluble material (AIM) and crude chitin (chitin-glucan complex) from *A. bisporus* stalks stored 5 days at 25 °C.

amount of mushroom waste annually accumulated by growers, the proposed procedure could yield 1000 metric tones of crude fungal chitin per year. The crude chitin from *A. bisporus* is composed of chitin-glucan complex that has potential to be used as a biopesticide, plant growth regulator, and feed additive. This research was the first step in our attempts to utilize the waste material from mushroom production as a value-added product. Future research will focus on production of fungal chitin from the waste and evaluation of its bioactive effects.

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Part 3

Physico-chemical properties and bioactivity of fungal chitin and chitosan

This chapter is a lightly revised version of a paper by the same name that is going to submit to the Journal of Agriculture and Food Chemistry in June, 2004 by Tao Wu, Svetlana Zivanovic, Carl E. Sams, F. Ann Draughon, William S. Conway:

My use of “we” in this chapter refers to my co-authors and myself. My primary contributions to this paper include (1) most of sampling, chitin and chitosan extraction, and physico-chemical properties determination, (2) most of antimicrobial activity determination (3) most of the data gathering and interpretation, (4) most of the writing.

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ABSTRACT

The chitinous material was extracted from mycelia of *Aspergillus niger* and *Mucor rouxii* grown in yeast peptone dextrose (YPD) broth for 15 and 21 days, respectively. The extracted material was characterized for purity, degree of acetylation (DA) and crystallinity, and tested for antibacterial and eliciting properties. The maximum glucosamine level determined in *A. niger* mycelium was 11.10 % dw and in *M. rouxii* it reached 20.13 % dw. Based on the stepwise extraction of freeze-dried mycelia, it appeared that *M. rouxii* mycelia contained both chitin and chitosan while *A. niger* had no chitosan in the hyphae wall. The yield of crude chitin from *A. niger* and *M. rouxii* was 24.01 % and 13.25 %, respectively, and yield of crude chitosan from *M. rouxii* was 12.49 %. Significant amounts (7.42 to 39.81 %) of glucan were associated with crude chitin and chitosan in both species and could not be eliminated during the extraction. The DA was 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 intensive than in corresponding materials from shrimp shells. The extracted chitin and chitosan reduced *Salmonella* Typhimurium DT104 2576 counts for 0.5 to 1.5 logs during 4-day incubation in tryptic soy broth at 25 °C. Furthermore, all tested chitinous materials from fungal sources significantly enhanced disease resistance in harvested apples against *Botritis cinerea* and *Penicillium expansum*.

KEYWORDS: ***Aspergillus niger*; *Mucor rouxii*; chitin; chitosan; degree of acetylation; crystallinity; antibacterial activity; disease resistance.**

INTRODUCTION

Chitin is the second most abundant polysaccharide on earth (Muzzerelli, 1977). Chitin and its deactylated product, chitosan, have received much interest for their application in agriculture, biomedicine, biotechnology and food industry due to their biocompatibility, biodegradability and bioactivity (1, 2, 3, 4, 5). Chitin is a characteristic constituent of crustacean shells, insect cuticles and fungal cell walls (6) and chitosan, although not native to animal species, presents as a major structural biopolymer in cell wall of some fungi, such as those from genera *Mucor*, *Absidia* and *Rhizopus* (6, 7).

Crustacean shells are the major commercial sources of chitin, which represents 14 - 27 % of the dry weight of shrimp and crab processing waste (8). Currently, chitin is industrially extracted from crab and shrimp shells obtained as a by-product of seafood industry, and chitosan is produced by deacetylation of chitin with concentrated NaOH (40 - 50 %) at 80 - 150 ºC (9). The production of chitin and chitosan from fungal mycelium has gained extensive attention in recent years due to potential advantages over currently applied process. For example, crustacean waste supplies are limited by seasons and sites of fishing industry, while fungal mycelium can be obtained by simple fermentation regardless on geographical location or season (10). Furthermore, fungal mycelia have lower level of inorganic materials compared to crustacean shells and no demineralization treatment would be required during the processing (11). Considering significant amounts of fungal-based waste materials accumulated in mushroom production and fermentation industries, and the expenses involved in managing the waste, production of highly functional value-added products may provide profitable solution to the industry. *Agaricus. bisporus* is one of the most favorite mushrooms worldwide and especially in the United 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). Just in the U.S. this results in approximately 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 *A. niger*. The annual world requirements for citric acid are estimated at 400,000 tons (12), what results in approximately 80,000 tons of *A. niger* mycelium waste per year (13).

Among various applications of chitin and chitosan in the food, agricultural and pharmaceutical industry, the uses of these biopolymers as antimicrobials in food preservation and as biopestides in agriculture 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). However, No and Jeon found that gram-positive bacteria were more susceptible to chitosan compared to gram-negative species (14 - 15). Two theories have been proposed for the antimicrobial mechanism of chitosan. By one, interaction between positively charged chitosan molecules and negatively charged microbial surface results in disruption of the cell membranes and leakage of proteinaceous and other intracellular constituents (16). The other hypothesis is based on the possible permeation of chitosan oligomers into microbial cell and its interference with the transcription of RNA and protein synthesis (17).

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 was found to effectively reduce polygalacturonases produced by *Botrytis cinerea* and caused severe cytological damages to the 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 the inoculation with *B. cinerea*, the activity of chitosanase and peroxidase increased and growth of *B. cinerea* was inhibited (21).

The objectives of the research presented here were to determine the yield and physico-chemical properties of chitin and chitosan from *A. niger* and *M. rouxii,* and to examine bioactivity of fungal chitin and chitosan against foodborne pathogen *Salmonella* Typhimurium, and plant pathogens *Botrytis cinerea* and *Penicillium expansum*.

MATERIALS AND METHODS

Commercial **chitins** and **chitosans** originated from crustacean shells were provided by Primex, Co. (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, USA). *Agaricus bisporus* chitin was prepared by the alkali and acid reflux method in our lab (22).

Growth of *Aspergillus niger* and *Mucor 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 ºC for 3 days, transferred to PDA, and incubated at 28 ºC for 5 days. Three plugs of each culture (plugged with 16 mm sterile test tube) were transferred to 1000 ml flasks containing 200 ml YPD (0.2 % yeast extract, 1.0 % peptone and 2.0 % glucose) medium. The inoculated flasks were incubated at 28 ºC 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 grounded to powder with a Thomas Wiley mill (Thomas Co., Philadelphia, PA), sieved through a #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 by a procedure adapted from Rane and Hoover (23) and Synowiecki and Al-Khateeb (24). The extraction consisted of two steps. The primary reflux with 1 M NaOH (w:v/1:40) at 95 ºC for 30 min removed proteins and resulted in alkali insoluble material (AIM) which was centrifuged (12,000 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 ºC for 6 h to separate acid soluble chitosan and insoluble chitin. Chitosan was precipitated from the supernatant after adjustment of pH to 10 with 1 N NaOH, filtered, washed with deionized water to reach neutral pH, and freeze-dried.

Characterization of extracted chitinous material. Yield of AIM, crude chitin and chitosan was determined gravimetrically. Glucosamine and glucan content in AIM,

insoluble residue, and supernatants were determined by the 3- methyl-2-benzothiazolone hydrazine (MBTH) (25) and the anthrone test (26), respectively. The FTIR analyses were performed with Nexus 670 FT-IR spectrometer using attenuated total reflection (ATR) sampling accessory in the range 700 to 4000 cm^{-1} (ThermoNicolet, Mountain view, CA). Degree of acetyletion (DA) was determined after hydrolysis of chitin/chitosan samples following the method of Niola et al. (27). Hydrolysis was performed in 12 M H_2SO_4, with an oxalic and propionic acid standard mixture, at 155 ºC for 1 h under vacuum (Pierce Biotechnology, Inc., Rockford, IL). Acetic acid liberated during hydrolysis was analyzed on HPX 87H column (BioRad, Hercules, CA) using HPLC system with PDA detector (Dionex, Sunnyvale, CA). Mobile phase was 5 mM H_2SO_4, flow rate 0.60 ml/min, injection volume 10 µl, and absorbance was monitored at 210 nm.

Assays for antibacterial activity. *Salmonella* Typhimurium DT104 2576 was obtained from culture collection of the Department of Food Science and Technology at the University of Tennessee. The bacterium was maintained on TSA slants at 4 ºC and activated at 32 ºC 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 Corporation, La Jolla, CA) to remove potential impurities. Each solution or suspension was added into Tryptic Soy Broth (TSB, Sparks, MD) to give a final chitin or chitosan concentration of 0.1 % (w/v). The pH of broth was adjusted to 4.5 with sterile 1 M HCl before autoclaving at 121 ºC for 15 min. Aliquots of 0.1 ml bacteria culture (10^9 CFU/ml) were inoculated into 10 ml broth supplemented with chitin or chitosan and incubated at 25 ºC for 48 h. The enumeration was carried out by stepwise dilutions with 0.1 % peptone water followed by plating on Typtic Soy Agar (TSA, Sparks, MD) and incubated for 24 h at 35 ºC.

Biocontrol bioassay. One month fresh 'Gala' apples free of rot or wounds were randomly assigned to different treatments. Plant pathogens *Botrytis cinerea* and *Penicillium expansum* were from the collection of the Produce Quality and Safety Laboratory, USDA, ARS, Beltsville, MD and were originally isolated from decayed

apples. The pathogens were routinely grown on potato-dextrose-agar (PDA) and virulence was maintained by periodic transfers through apple. The conidial suspensions used to inoculate the fruit were prepared from two-week-old cultures grown at 20 ºC 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 chitosan treatments were prepared as 2 % solutions (w/v) 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 hrs. After 3 days, the fruits were removed from storage, an identical wound was made 2 cm directly below the first wound and the 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 diameter of lesions was recorded after 4 and 7 days store at 25 °C. Ten apples were used per replication and each treatment was done in triplicates. Analysis of variance and contrasts between means were performed by Statistical Analysis System, Release 8.2 (SAS/STAT, 1996, SAS institute, Cary, NC, USA) PROC GLM and PROC GENMOD.

RESULTS AND DISCUSSION

Growth of *A. niger* and *M. rouxii* in YPD media at 28 ºC is presented in the Fig. 1. Biomass of *A. niger* increased rapidly in first 9 days and than slightly decreased, while *M. rouxii* growth was at a much slower rate. The maximum biomass dry weight of *A. niger* was 6.5 g/L, but for *M. rouxii* was only 1.7 g/L even after 21 days of incubation.

It has been suggested that the maximum yields of chitin and chitosan were obtained from fungi harvested at late exponential phase (28). Under conditions applied in our experiment, it appeared that the late exponential phase for *A. niger* was reached after 9 days while *M. rouxii* biomass did not significantly change during the 21 day-incubation. However, Tan et al. (28) reported that *M. rouxii* incubated at 25 °C with continues

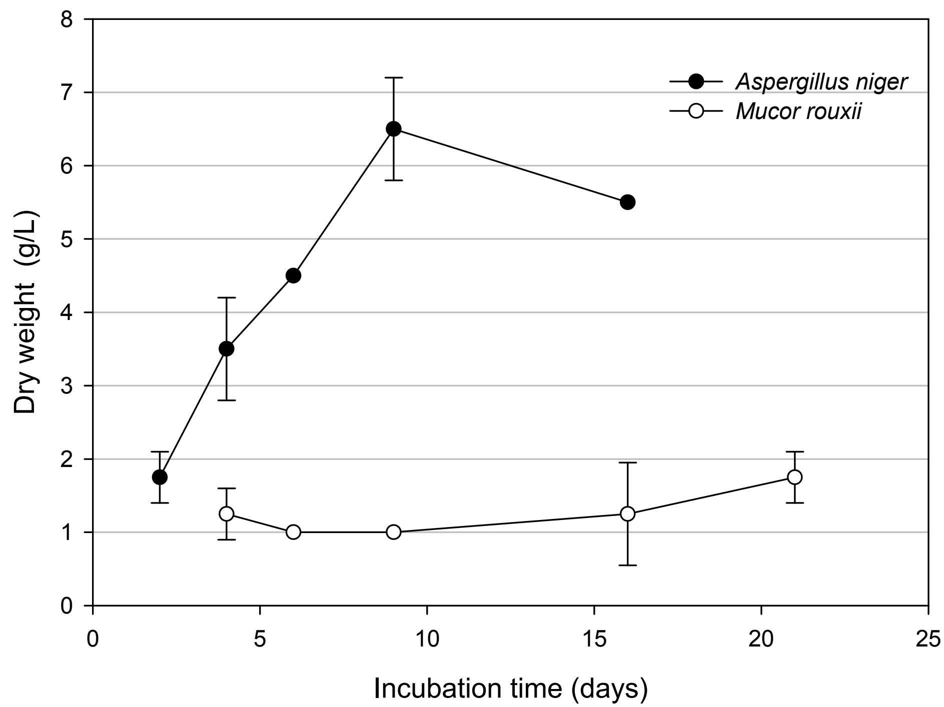
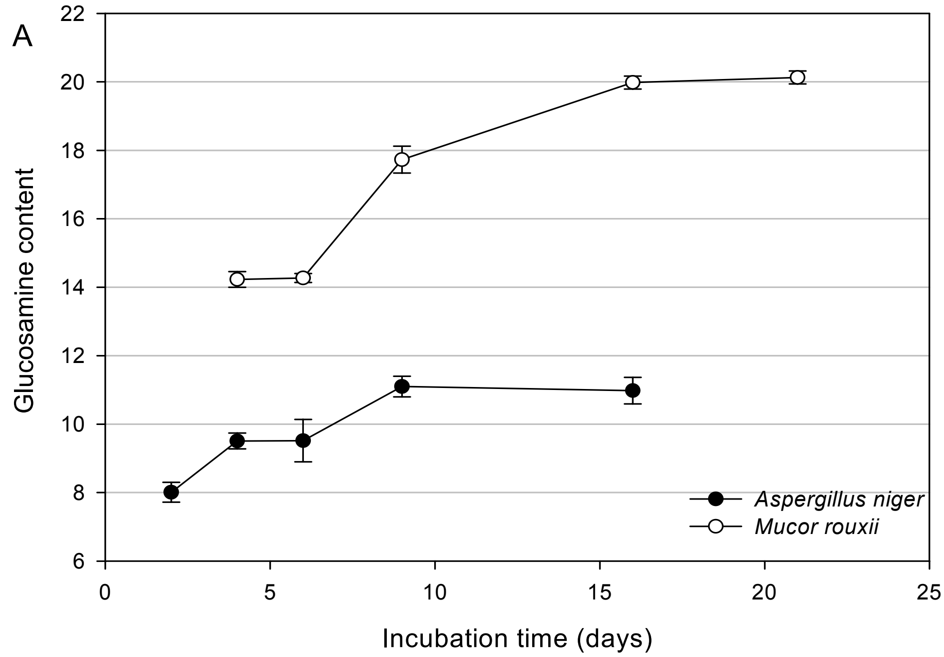


Figure 1. Growth of *A. niger* and *M. rouxii* in YPD media at 28 ºC. The results are expressed on dry weight basis. Error bars represent standard deviation of 3 replications.

shaking reached the late exponential growth phase after only 3 days producing more than 5 g/L of dry biomass. The possible reasons for the growth variation may be because of 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 glucosamine determination (29). The glucosamine content in *A. niger* dry mycelium increased from 8.01 % dw at day 2 to 11.1 % dw after 9 days, while in *M. rouxii* biomass it increased from 14.23 % dw at day 4 to 20.13 % dw after 21 days (Fig 2 - A). Although, *M. rouxii* had higher levels of total glucosamine in cell walls, *A. niger* appeared to have better potential to be used for extraction of chitinous material (Fig 2 - B).

Extraction and characterization of chitinous material. The freeze-dried mycelia of *A. niger,* collected after 9 day-incubation at 28 ºC, and of *M. rouxii,* collected after 21 days, were used for the extraction and characterization of chitin and chitosan. The yield of alkali insoluble material (AIM) was 36.35 % and 29.67 % for *A. niger* and *M. rouxii*, respectively (Table 1). Crude chitin, obtained as alkali and acid insoluble residue, from *A. niger* and *M. rouxii* was 24.01 % and 13.25 %, respectively. Considering the 'true' chitin content of 31.19 and 58.83 % in the crude extracts from *A. niger* and *M. rouxii*, respectively, it appears that practically the same level of chitin exist in the mycelia of both fungi (7.5 and 7.8 %, respectively). However, although 12.49 % chitosan was obtained from *M. rouxii* dry mycelia none was extracted from *A. niger*. The purity of *Mucor* chitosan was over 70 % and only 7.42 of β-glucan was co-extracted. This glucan content was lower compared to 39.81 and 9.48 % in the crude chitin fractions from *A. niger* and *M. rouxii*, respectively (Table 1). Extraction of fungal chitinous material in the form of chitin-glucan and chitosan-glucan complex has been detected before (30 - 32). For example, Machova et al (1999) determined total nitrogen content in chitin-glucan complex extracted from *A. niger* and reported that only about 30 % of the material was chitin (31). Optimizing conditions for extraction and deacetylation of chitinous material from *A. niger,* Muzzarelli et al (1980) found that chitosan-glucan yield varied between 25 to 71 % with 4 to 50 % chitosan content, depending on the applied procedure (30). This



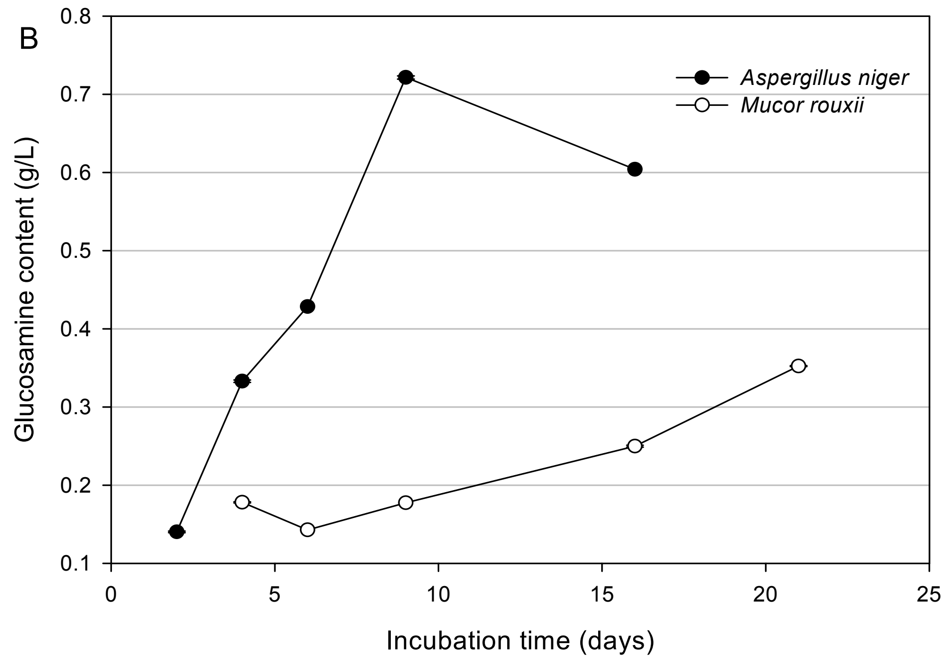


Figure 2. Total glucosamine content of *A. niger* and *M. rouxii* incubated at 28 ºC. (A) and (B) are expressed on mycelium dry weight and media volume basis, respectively. Error bars represent standard deviation of 3 replications.

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)	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)	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 % of dry weight of mycelium.

[b] Chitin and chitosan were hydrolyzed and determined as glucosamine (% of analyzed material).

[c] Neutral polysaccharides determined by anthrone reagent (% of analyzed material).

led to conclusion that chitosan may be obtained from *A. niger* industrial strain on a level of 3.5 to 14.8 % of biomass dry weight.

The glucosamine content in crude chitin and chitosan from *A. niger* and *M. rouxii* showed that 58.1 and 67.6 % of originally present glucosamine in mycelia, respectively, were extracted in a form of chitinous material. The difference may be attributed to the losses of soluble (acetyl)glucosamine oligomers during extraction and to crystallinity of chitin alone and/or within the chitin-glucan complex (33). The crystallinity would prevent complete hydrolysis necessary for glucosamine determination and result in underestimated content in the extracted material. Nevertheless, the yields obtained in our experiment are in the agreement with those of Arcidiacono and Kaplan (34), Muzzarelli et al., (30), 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 19.5 ± 0.97 %, respectively. DA results were indirectly proved by their solubility in 1 % acetic acid. *A. niger* chitin was insoluble, while *M. rouxii* chitin was partially soluble and *M. rouxii* chitosan was completely soluble. Similar values for *M.rouxii* chitosan were previously reported (10, 24, 34, 35). Thus, DA of chitosan isolated from *M. rouxii* were in the range from 8.5 to 39.7 %, depending on the processing conditions (34), while in the studies of Rone and Hoover (23) this range was much more narrow, from 10.5 to 11.1 %.

FTIR spectra and crystallinity. Infrared spectra of chitin and chitosan from *M. rouxii* and *A. niger* were similar to the IR spectra of chitosan and chitin produced from shrimps. The spectra had bands at 2900 cm^{-1} and 3000 cm^{-1} for NH bond stretching, 1650 cm^{-1} for C=O stretching, and 1557 cm^{-1} for NH vibration mode. The ratio of intensities of the bands at 1379 cm^{-1} and 2900 cm^{-1} has been used to estimate the crystallinity for chitin and chitosan (36). 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 expected because the high degree of acetylation of chitin molecules (> 70 %) has been associated with extended crystallinity (37) while 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 removal of interfering amorphous components. Similarly, Mol and Wessels used X-ray diffraction to evaluate crystallinity of untreated, alkali and acid treated hyphae walls and found that X-ray patterns sharpened with purification and characteristic crystallinity peaks of α-chitin appeared after acid treatment of alkali-insoluble material (38).

Antimicrobial activity of isolated chitin and chitosan against *Salmonella* Typhimurium DT104 2576 is shown in Table 2. Chitin and chitosan from *M. rouxii* and chitin from *A. niger* reduced bacterial counts in TSB by 0.96, 1.9, and 0.88 logs, respectively. Chitin previously extracted from stalks of button mushrooms, *Agaricus bisporus* (22), had similar effect as chitin from *A. niger*, while chitosan from *M. rouxii* was more effective than commercially available shrimp chitosan (1.72 logs). Although the same shrimp chitosan had much stronger antibacterial activity when used in oil-in-water emulsions (39), these results showed that simple extraction can provide fungal chitosan with same or higher efficiency as currently commercially available chitosan.

Induction of plant defense response. All fungal chitins and chitosan, as well as two commercial chitosans, significantly reduced lesions caused by both pathogens, compared to water controls (Fig. 3). For example, 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, while in chitin or chitosan pretreated samples were about 5 mm 4 days after inoculation and did not further increase. All differences between water control and chitin or chitosan treatments were statistically significant with $p < 0.001$. Although among all of chitin and chitosan treatments, water soluble chitosan had the best performance, differences between chitin and chitosan treatments were not significantly different at 0.05 level.

It has been previously reported that the disease resistance response in plants can be activated by inorganic compounds, such as phosphonate (40) and potassium phosphonate (41)), or organic compounds such as 2, 6-Dichloroisonicotinic acid (42), Acibenzolar (43, 44). Furthermore, shrimp chitosan induced defense responses in cucumbers, rice, strawberries and bell peppers (19 - 21). Our results showed that crude

Table 2: Antimicrobial activity of fungal chitin and chitosan against *Salmonella Typhimurium* DT 104 2576.

	Salmonella count Log CFU/ml	
Treatment	48 h	96 h
Water control	8.33 ± 0.03	6.86 ± 0.06
Acetic acid control	7.16 ± 0.09	6.55 ± 0.03
A. bisporus chitin	6.91 ± 0.05	6.11 ± 0.11
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
shrimp chitosan	6.50 ± 0.04	5.26 ± 0.07

* Agaricus bisporus chitin obtained from mushroom stalks (22)

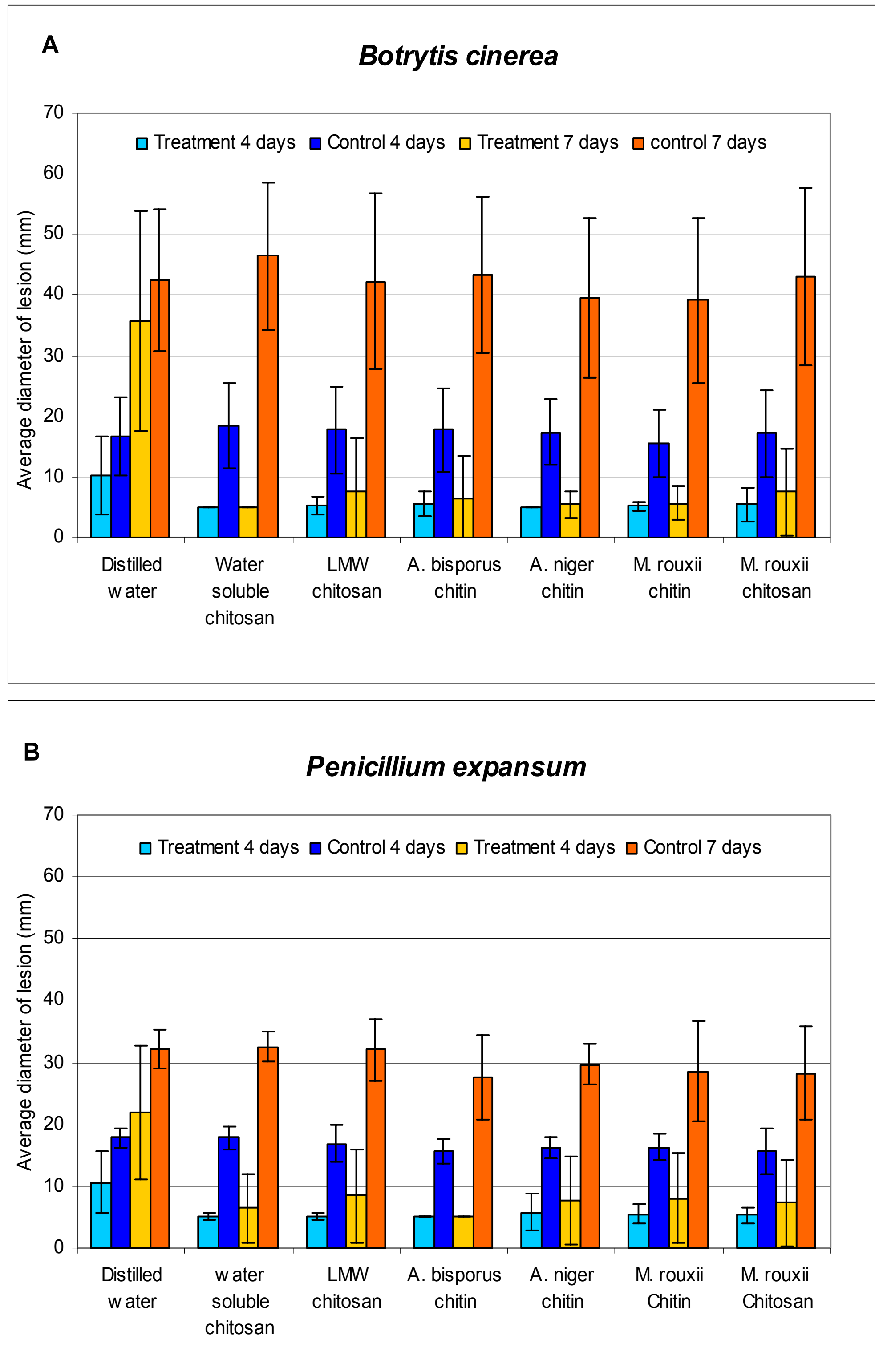


Figure 3. Effects of 2 % chitosan and chitin treatments on *B.cinerea* (A) and *P. expansum* (B) lesions on apple. Error bars represent standard deviation of 30 replications.

fungal chitin and chitosan has the same effect as commercially purified crustacean chitosan. This practically means that approximately 50,000 tones of mushroom waste, which annually accumulates just in the U. S., can be simply processed to yield about 500 tones (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 pharmaceutical industry can be easily utilized for production of these highly value-added products.

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Appendix

Appendix. List of symbols and Abbreviations

NaOH	Sodium hydroxide
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
FTIR	Fourier Transform Infrared
YPG	Yeast peptone glucose
PDB	Potato Dextrose
MSM	Molasses salt medium
DMAc/LiCl	*N,N*-dimethylacetamide/lithium chloride
MBTH	3 – methyl – 2 – Benzothiazolone hydrazone hydrochloric
NMR	Nuclear magnetic resonance
DA	Degree of acetylation
AIM	Alkali insoluble material
PDA	Photo Diode Array
ATR	Attentuated total reflection
TSA	Tryptic soy agar
TSB	Tryptic soy broth

Vita

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