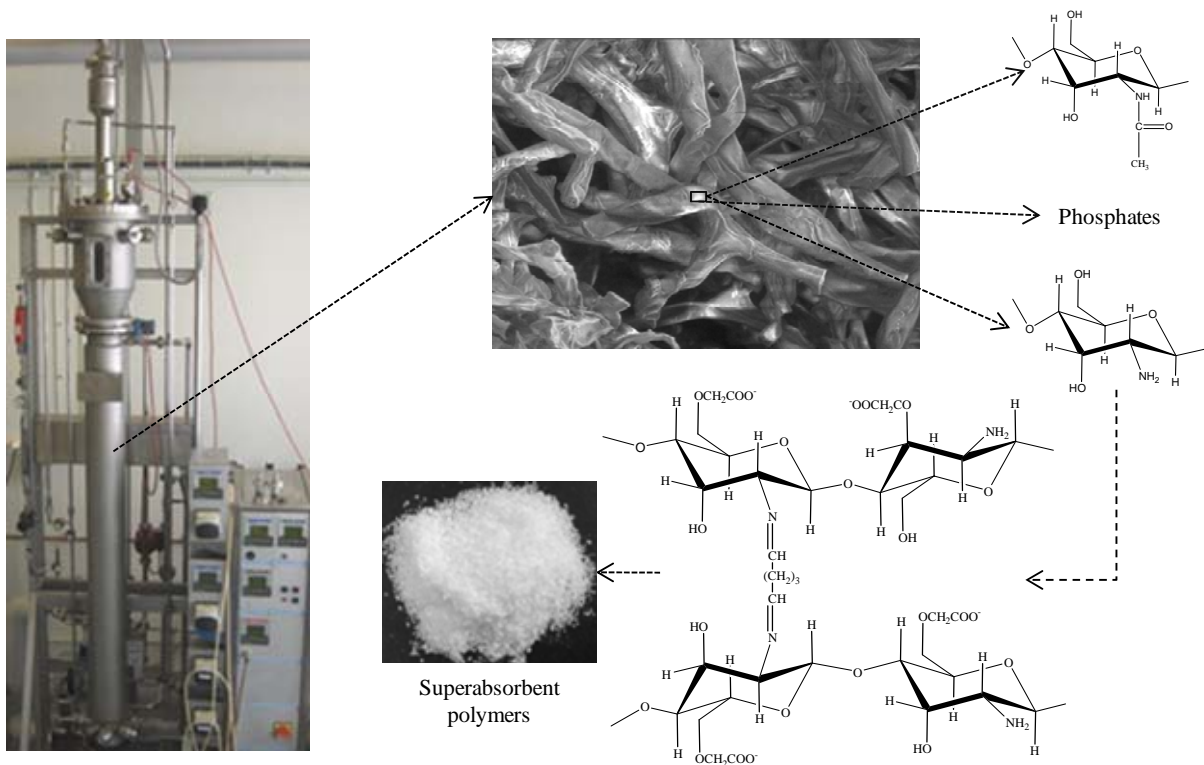


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Superabsorbent Polymers from the Cell Wall of Zygomycetes Fungi

AKRAM ZAMANI

Department of Chemical and Biological Engineering

CHALMERS UNIVERSITY OF TECHNOLOGY

Göteborg, Sweden 2010

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Department of Chemical and Biological Engineering
Chalmers University of Technology
412 96 Göteborg
Sweden
Telephone: +46 (0)31-7721000

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School of Engineering
University of Borås
501 90 Borås
Sweden
Telephone: +46 (0)33-4354000

Cover: Different steps in production of superabsorbent polymers from zygomycetes cell wall: Fungal cells production in an airlift bioreactor; Separation of cell wall ingredients including chitin, phosphates, and chitosan; Carboxymethylation and cross-linking of the cell wall chitosan to a hydrogel; Drying the hydrogel to superabsorbent polymers.

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Akram Zamani

Department of Chemical and Biological Engineering, Chalmers University of Technology, Göteborg, Sweden
School of Engineering, University of Borås, Borås, Sweden

Abstract

The present thesis presents new renewable, antimicrobial and biodegradable superabsorbent polymers (SAPs), produced from the cell wall of zygomycetes fungi. The cell wall was characterized and chitosan, being one of the most important ingredients, was extracted, purified, and converted to SAP for use in disposable personal care products designed for absorption of different body fluids.

The cell wall of zygomycetes fungi was characterized by subsequent hydrolysis with sulfuric and nitrous acids and analyses of the products. The main ingredients of the cell wall were found to be polyphosphates (4-20%) and copolymers of glucosamine and N-acetyl glucosamine, i.e. chitin and chitosan (45-85%). The proportion of each of these components was significantly affected by the fungal strain and also the cultivation conditions. Moreover, dual functions of dilute sulfuric acid in relation to chitosan, i.e. dissolution at high temperatures and precipitation at lowered temperatures, were discovered and thus used as a basis for development of a new method for extraction and purification of the fungal chitosan. Treatment of the cell wall with dilute sulfuric acid at room temperature resulted in considerable dissolution of the cell wall polyphosphates, while chitosan and chitin remained intact in the cell wall residue. Further treatment of this cell wall residue, with fresh acid at 120°C, resulted in dissolution of chitosan and its separation from the remaining chitin/chitosan of the cell wall skeleton which was not soluble in hot acid. Finally, the purified fungal chitosan (0.34 g/g cell wall) was recovered by precipitation at lowered temperatures and pH 8-10. The purity and the yield of fungal chitosan in the new method were significantly higher than that were obtained in the traditional acetic acid extraction method.

As a reference to pure chitosan, SAP from shellfish chitosan, was produced by conversion of this biopolymer into water soluble carboxymethyl chitosan (CMCS), gelation of CMCS with glutaraldehyde in aqueous solutions (1-2%), and drying the resultant gel. Effects of carboxymethylation, gelation and drying conditions on the water binding capacity (WBC) of the final products, were investigated. Finally, choosing the best condition, a biological superabsorbent was produced from zygomycetes chitosan. The CMCS-based SAPs were able to absorb up to 200 g water/g SAP. The WBC of the best SAP in urine and saline solutions was 40 and 32 g/g respectively, which is comparable to the WBC of commercially acceptable SAPs under identical conditions (34-57 and 30-37 g/g respectively).

Keywords: chitosan, zygomycetes fungi, cell wall, chitin, polyphosphates, dilute sulfuric acid, superabsorbent polymers, carboxymethyl chitosan, gelation, glutaraldehyde, drying, water binding capacity

List of publications

The thesis is mainly based on the results presented in the following articles:

- I. Zamani, A., Jeihanipour, A., Edebo, L., Niklasson, C., Taherzadeh, M. J. Determination of glucosamine and N-acetyl glucosamine in fungal cell walls. *Journal of Agricultural and Food Chemistry* 2008; 56 (18): 8314-8318.
- II. Zamani, A., Edebo, L., Sjöström, B., Taherzadeh, M. J. Extraction and precipitation of chitosan from cell wall of zygomycetes fungi by dilute sulfuric acid. *Biomacromolecules* 2007; 8 (12): 3786-3790.
- III. Zamani, A., Taherzadeh, M. J. Production of low molecular weight chitosan by hot dilute sulfuric acid. *BioResources* 2010; 5 (3): 1554-1564.
- IV. Zamani, A., Edebo, L., Niklasson, C., Taherzadeh, M. J. Temperature shifts for extraction and purification of zygomycetes chitosan with dilute sulfuric acid. *International Journal of Molecular Sciences* 2010; 11(8): 2976-2987.
- V. Zamani, A., Henriksson, D., Taherzadeh, M. J. A new foaming technique for production of superabsorbents from carboxymethyl chitosan. *Carbohydrate Polymers* 2010; 80 (4): 1091-1101.
- VI. Zamani, A., Taherzadeh, M. J. Effects of partial dehydration and freezing temperature on the morphology and water binding capacity of carboxymethyl chitosan-based superabsorbents. *Industrial & Engineering Chemistry Research* 2010; 49: 8094-8099.
- VII. Bidgoli, H., Zamani, A., Taherzadeh, M. J. Effect of carboxymethylation conditions on water binding capacity of chitosan-based superabsorbents. *Carbohydrate Research*, in press.
- VIII. Zamani, A., Taherzadeh, M. J. Production of biological superabsorbents from zygomycetes fungal chitosan. Manuscript.

Statement of contribution

Akram Zamani is responsible for the main part of the writing and laboratory work in all papers with some aid from supervisors and co-authors. However, in paper I, the experimental work and writing was shared between Akram Zamani and the PhD candidate Azam Jeihanipour.

Additionally, in paper VII, the experimental work and writing was mainly performed by master student Hosein Bidgoli under supervision of Akram Zamani.

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PREFACE AND SCOPE

Depletion of oil resources in conjunction with environmental problems arising from waste disposal have resulted in an increasing demand for replacement of different petroleum-based products with renewable bio-based materials. Biopolymers are one of the most important groups of renewable materials. In order to reduce the waste problems, biodegradability seems to be an essential property of the materials used in disposable products. Personal hygiene diapers used for absorption of different body fluids are examples of such products. Superabsorbent polymers (SAPs), which are hydrophilic polymer networks, are one of the most important ingredients of such diapers. In 2009, 1.63 million tons of SAPs were produced, mainly used in baby diapers and feminine hygiene and adult incontinence products [1]. Due to a significant demand for SAPs, several research projects have been granted to develop SAPs, obtained from biopolymers to replace synthetic SAPs, based on polyacrylates.

The purpose of the current thesis was to produce bio-based superabsorbents from fungal chitosan. Chitosan is a cationic biopolymer, at present mainly produced by chemical deacetylation of chitin, present in the exoskeleton of crustaceans. The cell wall of zygomycetes fungi, by having naturally occurring chitosan, may be considered as a potential replacement for crustacean shells. To be able to use fungal chitosan for production of SAPs, it was first necessary to separate the chitosan from the fungal cell wall. Furthermore, to be able to find an appropriate process for recovery and purification of chitosan, knowledge was required about other ingredients of the cell wall, which may appear as contaminants of fungal chitosan. Therefore, the research building the present thesis was carried out as several sub-projects including investigation of new methods for:

- Characterization of the cell wall of zygomycetes (Paper I)
- Purification of chitosan from the cell wall of zygomycetes fungi (Papers II-IV)
- Production of SAPs from shellfish chitosan as an established reference to pure chitosan (Papers V-VII)
- Production of SAPs from fungal chitosan (Paper VIII)

OUTLINE

The summary of the present thesis consists of three main chapters:

- In chapter 1, zygomycetes fungi are introduced and their applications discussed. After that, the fungal cell wall, as one of the most important parts of the fungal cells, is introduced and the differences between the zygomycetes cell wall and other fungal cell wall are discussed. Finally, the new method developed in the studies of this thesis and the methods already available for characterization of fungal cell wall are presented.
- Chapter 2 begins with an introduction to chitosan structure, properties, and applications, followed by a description of the technique used for production of chitosan from shellfish wastes. In the following section, the biosynthesis of fungal chitosan extracted from zygomycetes cell wall as well as its importance for industrial production of chitosan are discussed. The last part of this chapter is dedicated to recovery of chitosan from zygomycetes cell wall. In this section, the traditional method for extraction of chitosan from the cell wall is introduced and its efficiency in recovering chitosan from the cell wall is discussed. Finally, the new method of recovery and purification of fungal chitosan developed in the work of this thesis is described.
- In chapter 3, the first discussion concerns structure and application of synthetic and bio-based superabsorbents. This part is followed by a presentation of the new methods developed for production of SAPs from shellfish chitosan. Finally, the latest results, concerning production of SAPs from fungal chitosan, are presented.

Chapter 1

ZYGOMYCETES FUNGI AND THEIR CELL WALL

1.1. Introduction of zygomycetes fungi

The recent biological classifications divide cellular life types into three major domains: archaea, bacteria, and eukarya [2]. The latter domain, eukarya, is further divided into five kingdoms which are protista, plantae, chromista, fungi, and animalia [3]. Among these domains, fungi with a total number of 1.5 million species consist of a large group of organisms, extensively spread throughout the world [4]. Initially, fungi were grouped together with plants because of the lack of mobility. However, it was later discovered that from a nutrition point of view, fungi are more similar to animals than to plants in that unlike plants, which are photosynthetic autotrophs, fungi are absorbent heterotrophs [3]. The kingdom of fungi is divided into four subgroups, phyla, which are chytridiomycota, zygomycota, ascomycota, and basidiomycota [5]. Among these phyla, zygomycota is the most varied and least studied one and appears to be polyphyletic. It consists of two fungal subphyla, trichomycetes and zygomycetes [6].

Zygomycetes are probably the most ancient group of fungi and traditionally identified as the “pin molds”. They exist as extended mycelia with diverse asexual and sexual spore structures. The name of the phylum originates from the name of their sexual spores, zygospores, which have thick wall and a significant survival potential. Unlike most other groups of fungi, the vegetative mycelium of zygomycetes does not contain septa except where the presence of septa is necessary for separation of structures such as spores [5, 7]. Zygomycetes mostly live in soil and dung where they can consume a notable variety of substrates. They may also be found as parasites of other organisms such as mushrooms, amoebae, nematodes, rotifers and arthropods, or as pathogens of plants, animals, and humans [6]. Zygomycetes fungi are known as fast growers with the capability of formation of sporangiospores (or conidia) and under appropriate circumstances,

even zygospores within 1-3 weeks. The quicker growth of these fungi compared to other fungi may be due to lack of septa, leading to faster cytoplasmic movement or intra-hyphal transportation [5, 6]. In line with the differences in nutrition and morphology, zygomycetes are divided into 10 orders: Mucorales, Zoopagales, Dimargaritales, Kickxellales, Endogonales, Glomales, Entomophthorales, Basidiobolales, Mortierellales, and Geosiphonales [6]. Figure 1 illustrates the phylogenetic position of zygomycetes fungi.

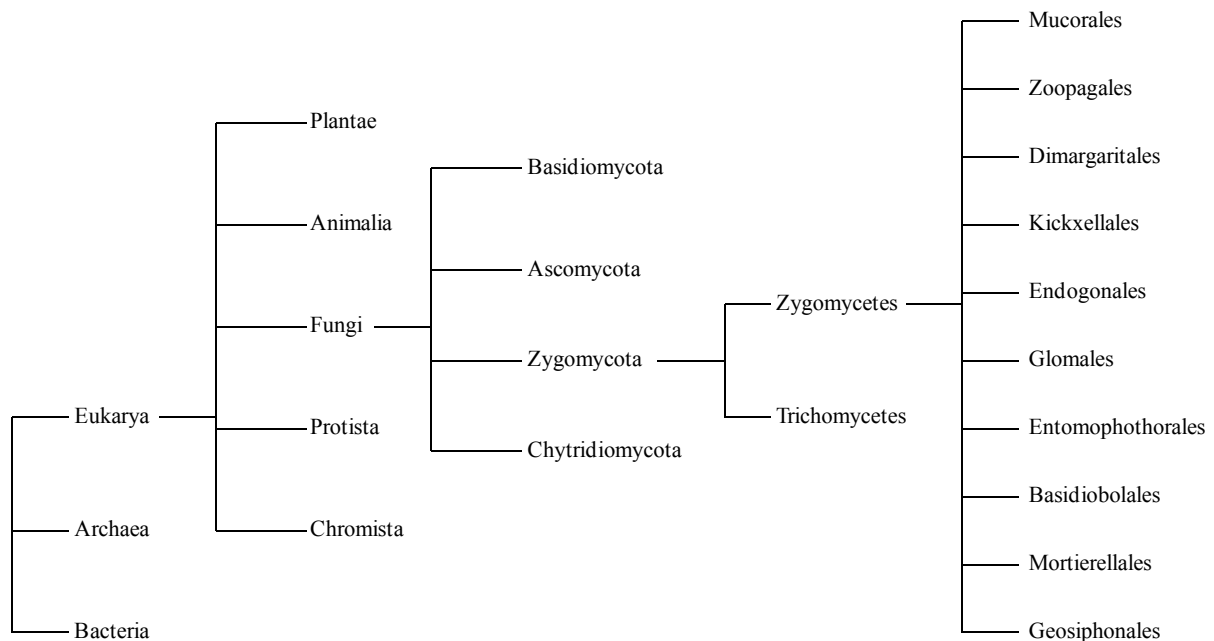


Figure 1: Diagram of relationship between zygomycetes fungi and other organisms.

1.2. Industrial importance

Zygomycetes fungi have been used for several hundred years for production of food, mostly in south Asia. Two well known examples of foods prepared from zygomycetes fungi are tempe and tofu. Tempe is a soya bean cake covered with filamentous zygomycetes growth, with a high protein content (40-50%) and containing different essential amino acids, while tofu is produced by fermentation of soya milk with some strains of zygomycetes fungi [5, 7].

Besides direct application of zygomycetes as food, these fungi are widely used for production of different organic substances such as fumarate, lactate, and poly-unsaturated fatty

acids as well as a variety of extracellular enzymes, e.g. lipases, proteases, phytase, β -glycosidase and xylanase [5, 8].

Furthermore, the ability of zygomycetes to produce ethanol from lignocellulosic materials (as a substitute feedstock for starch materials) was recently discovered. Currently, ethanol is industrially produced mainly by *Saccharomyces cerevisiae*. The main drawback of this microorganism is its inability to ferment five-carbon sugars (pentoses) which are abundant in hydrolyzates of lignocellulosic materials. In contrast, some strains of zygomycetes fungi such as *Mucor* and *Rhizopus* have shown promising results for consumption of both pentoses and hexoses and production of ethanol with almost the same productivity and yield as *Saccharomyces cerevisiae*. Therefore, zygomycetes may play an important role in the future for manufacturing this bio-fuel [8-10].

Utilization of zygomycetes fungal biomass as a source for fish feed is another newly proposed application of these fungi. At present, cultivated fish, fed with fish meal from the restricted source of wild fish, is one of the main resources of human food. Zygomycetes fungi cultivated on paper pulp wastewater, as a rich source of proteins, have been successfully tested as an alternative to these fish meals [7].

Another remarkable application of zygomycetes fungi, which is the focus of this thesis, is utilization of these fungi for production of chitosan. Amongst different types of fungi, the cell wall of zygomycetes fungi are of an unusual composition, in which fungal β -glucans (as structural components) have been replaced with chitosan [5]. Chitosan is a cationic biopolymer with several applications in biotechnology, medicine, and agriculture as well as in food, cosmetic, and hygienic industries. Structure, properties, applications, and methods of production of this polysaccharide are discussed in detail in Chapter 2.

1.3. Fungal cell wall and the cell wall of zygomycetes fungi

The fungal cell wall is undoubtedly one of the most important feature of fungal cells and plays a significant role in the particular way of living in these microorganisms [11]. The fungal cell wall determines the shape of fungal cells and protects them against a variety of environmental stresses including enzymatic attacks, dehydration, organic solvents, toxic

chemicals and ultraviolet waves [12]. Furthermore, due to the presence of the cell wall, fungal cells are able to withstand large differences in osmotic pressure and to survive under harsh conditions, when negotiating long distances searching for nutrients as well as during colonization and spore distribution [11]. Additionally, the fungal cell wall, as the outmost surface of fungal cells, displays a considerable role in all cell-environment interactions, e.g. attachment of fungi to different surfaces and nutrient uptake [13]. The fungal cell wall often makes up 20-30% of the cell dry weight and polysaccharides are the most dominant components of the cell wall (80-90%). Proteins are the second main components and represent 3-20% of the cell wall. Lipids, pigments, and inorganic salts are found in lower proportions [12-14]. Based on the architectural function, different materials of the fungal cell wall are divided into two groups, structural and cementing components. The structural materials figure the skeleton of the cell wall and are mostly composed of polysaccharides such as chitin and β -glucans. In contrast, cementing components are responsible for retaining the cell wall structure. Glucans (α and β), chitosan, polyuronids, proteins, lipids, minerals, and pigments are examples of cell wall cementing elements [11]. The whole fungal kingdom can be divided into 8 different categories based on the polysaccharide composition of the cell wall. They include three cellulose containing groups (cellulose-glycogen, cellulose-glucan, and cellulose-chitin), two mannan containing groups (mannan-glucan and mannan-chitin), and chitin-glucan, chitin-chitosan, and polygalactosamine-galactan groups. Among different taxonomic groups of fungi, zygomycetes is the only group belonging to the chitin-chitosan category [11, 14]. Chitosan is a distinctive component of the cell wall of zygomycetes fungi and its content can reach up to 3-fold of that of chitin [11, 15]. The exact role of chitosan in the zygomycetes cell wall is still under question. In the cell wall of these fungi, chitin fibers are covered and cemented by chitosan as well as proteins in a mosaic manner [16]. Therefore, fungal chitosan possibly boasts an important function in the defense system of zygomycetes by protecting the chitin against hydrolytic attack by chitinases [11, 13]. Furthermore, because of the replacement of glucan with chitosan, this biopolymer probably undertakes the role of glucans in the cell wall of zygomycetes fungi. For example, chitosan is thought to be responsible for gathering different ionic materials in the cell wall by salt or complex formation [11]. Chitosan, being a cationic polysaccharide, is in the cell wall neutralized with some anionic polymers such as polyphosphates and polyglucuronic acid [11, 12, 14].

1.3.1. Traditional methods for characterization of fungal cell wall

Since chitin and chitosan are the major polysaccharides in the cell wall of zygomycetes fungi, determination of these polymers is a major step in characterization of the cell wall of this group of fungi. Chitin and chitosan are polymers of the same structural monomers, i.e. glucosamine (GlcN) and N-acetyl glucosamine (GlcNAc) (Figure 2). When GlcN or GlcNAc is the dominant monomer (60-100%) of the polymeric chain, the polysaccharide is called chitosan or chitin respectively¹. Therefore, the measurement of the amount of GlcN and GlcNAc in the cell wall gives an estimation of the relationship between chitin and chitosan which may, however, appear as mixtures of different polymers.

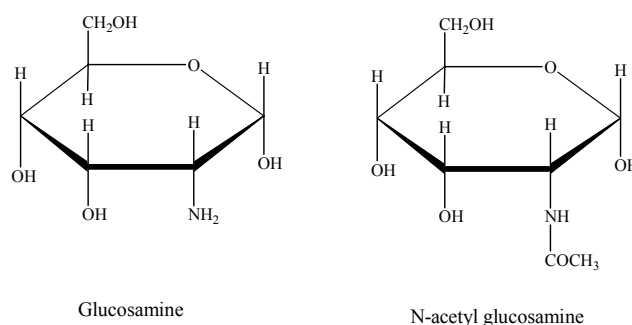


Figure 2: Structure of glucosamine and N-acetyl glucosamine.

To determine the amount of GlcN and GlcNAc, chitin and chitosan containing materials (e.g. fungal cell wall material) are usually hydrolyzed with hydrochloric acid (2.5-10 M), at elevated temperatures (80-140°C) for different periods of time (1-23 h) [17-20]. The aim of this hydrolysis step is to break the glucoside bonds between the monomers as well as deacetylation² of GlcNAc residues. At the end of this process, the amount of liberated GlcN is measured and reported as an indicator of the total amount of chitin and chitosan [Paper I]. The hydrolysis of chitin and chitosan containing materials with HCl and the determination of total GlcN, potentially have their limitations in terms of cell wall characterization. When the analysis is performed on highly crystalline materials, e.g. chitin micro-fibrils, the accessibility of acid to the

¹ More details about the structure of chitin and chitosan are presented in Chapter 2 (Section 2.1.)

² Removal of acetyl groups from GlcNAc residues and formation of GlcN residues

structure of substrate is considerably restricted. Therefore, a prolonged reaction time is required to achieve complete hydrolysis. Increasing the hydrolysis duration, leads however to degradation of the products. Consequently, the recovery, in terms of total amount of released GlcN, is somewhat low when using this method [20]. Furthermore, measurement of total GlcN content provides no additional information whether the GlcN residues in the substrate are free, as in chitosan, or in the acetylated form, as in chitin. Therefore, prediction of the amounts of chitin and chitosan in mixtures of them in e.g. a fungal cell wall is not possible using the HCl method [Paper I]. Therefore, other analytical methods should be applied to investigate the amounts of chitin and chitosan in the cell wall of zygomycetes.

1.3.2. A new method for characterization of zygomycetes cell wall

In this thesis a new method, developed for characterization of fungal cell wall and determination of their GlcN and GlcNAc contents, is presented. In this method, principles applying for hydrolysis of lignocellulosic materials [21] in conjunction with the unique behavior of chitosan in sulfuric acid [papers I-IV] and nitrous acid solutions [22], were employed for hydrolysis of chitin and chitosan prior to GlcN and GlcNAc determination.

Usually a two step sulfuric acid treatment is performed for hydrolysis and analysis of lignocellulosic materials. Firstly, these materials are treated with concentrated sulfuric acid (e.g. 72%) at low temperature (e.g. 30°C). During this step, the crystalline structure of carbohydrates is loosened and dissolution and partial hydrolysis take place. Secondly, sulfuric acid is diluted by addition of water and the materials are further treated with dilute acid at high temperatures (e.g. 120°C). At the end of this step, carbohydrates are completely hydrolyzed into sugars which can consequently be analyzed [21]. The same sulfuric acid treatment of chitin and chitosan results in a complete deacetylation and partial depolymerization of these materials (Figure 3). Therefore, at the end of the sulfuric acid hydrolysis, chitin and chitosan are converted to acetic acid and fully deacetylated low molecular weight chitosan chains. As will be discussed later in detail in Section 2.5.2., chitosan displays temperature dependant solubility in dilute sulfuric acid solutions. This polymer is totally insoluble in dilute sulfuric acid at room temperature but becomes soluble at higher temperatures (close to 100°C). Consequently, reduction of temperature of a solution of

chitosan in sulfuric acid results in precipitation of chitosan [Papers I-IV]. Hence, in order to avoid the precipitation of the product of sulfuric acid hydrolysis, i.e. fully deacetylated chitosan, it is necessary to keep the hydrolyzate heated (around 100°C) [Paper I].

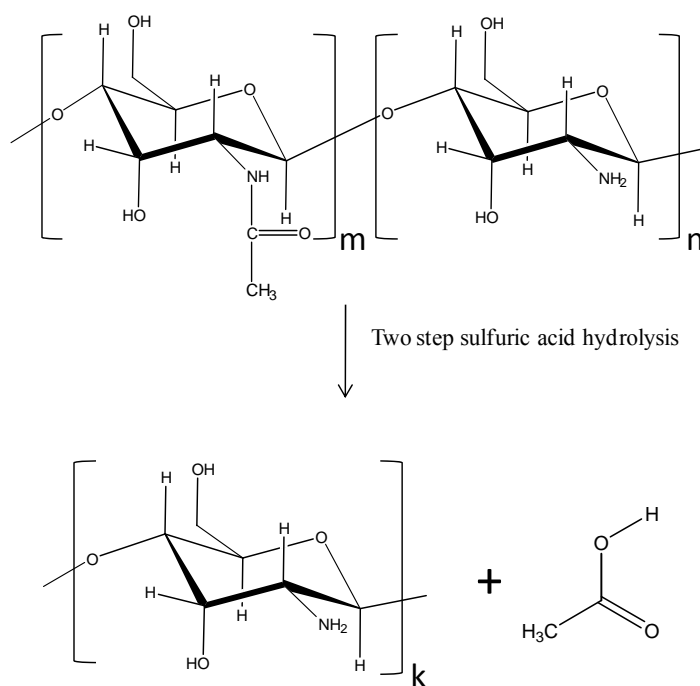


Figure 3: Full deacetylation and partial depolymerization of chitin and chitosan with sulfuric acid ($m + n > k$).

Nitrous acid has been used for several years for selective detection of chitosan [11, 22-24]. This acid depolymerizes chitosan through a deamination reaction. The reaction is performed quickly at room temperature and results in breaking the glucoside linkages between GlcN residues, discharge of N_2 , and formation of 2,5-anhydromannose (Figure 4) [24]. It should be mentioned that the deamination reaction stops when it reaches GlcN-GlcNAc bonds [22]. Therefore, if the appropriate amount of HNO_2 is added to the fully deacetylated chitosan (the product of the sulfuric acid hydrolysis), the reaction continues until the chitosan is totally converted to 2,5-anhydromannose. The number of 2,5-anhydromannose molecules at the end of the reaction, represents the total GlcN and GlcNAc residues in the initial material.

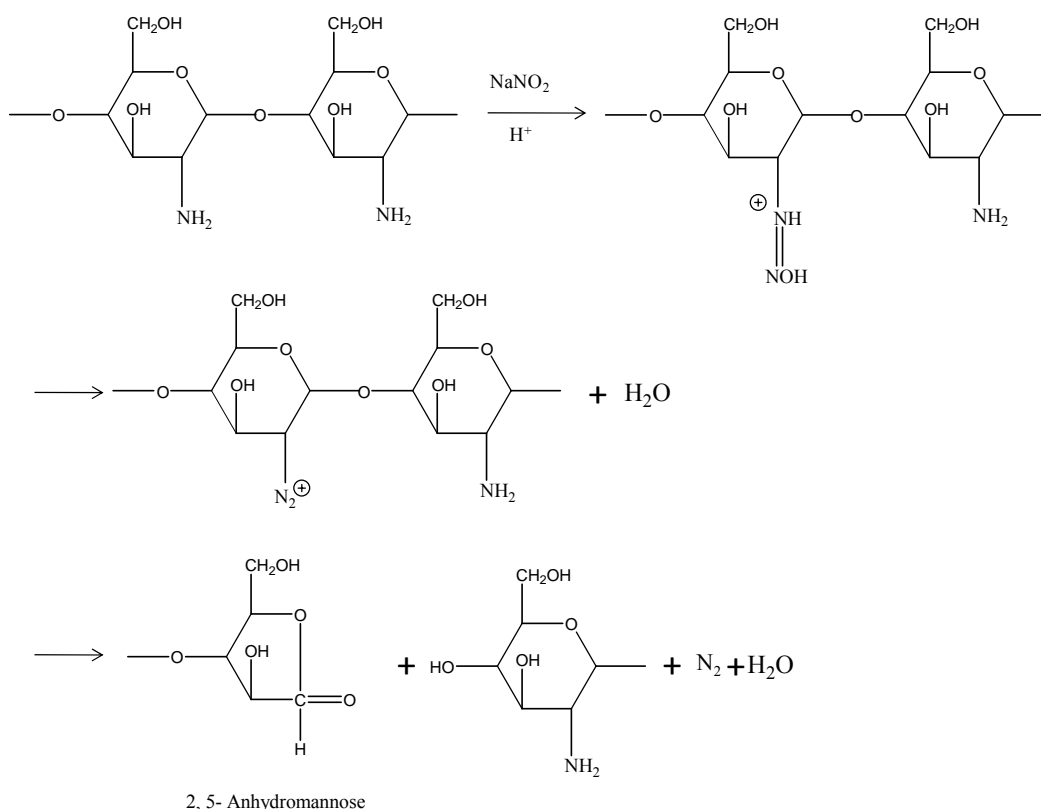


Figure 4: Deamination and depolymerization of chitosan by nitrous acid.

Characterization of the fungal cell wall in the studies of the present thesis was performed by a combination of sulfuric acid hydrolysis and nitrous acid degradation according to Figure 5. The alkali insoluble material (AIM) of fungal biomass³ was used as a representative of fungal cell wall material. Briefly, 13 M sulfuric acid was added to the AIM and mixed at room temperature for 90 min to dissolve the AIM in concentrated sulfuric acid. The sulfuric acid was then diluted to 0.45 M by addition of water and the mixture was heated in an autoclave at 120°C for 60 min. A sample was taken from the hot hydrolyzate (at 100°C). After cooling to room temperature, the sample, which contained poly-glucosamine chains, was subjected to a deamination reaction by addition of NaNO₂⁴. The unique product of deamination was 2,5-anhydromannose which was measured with the colorimetric analysis method presented by Plassard et al. [25]. 2,5-anhydromannose is an indicator of total GlcN residues, free and

³ Prepared by treatment of biomass with 2% NaOH solution at 120°C

⁴ Nitrous acid is the product of the reaction between sodium nitrite and sulfuric acid.

acetylated, in the form of chitin and chitosan in the AIM. In addition, acetic acid, the second hydrolysis product in the cold sulfuric acid hydrolyzate, was analyzed using HPLC. Acetic acid is a marker for GlcNAc residues in the AIM [Paper I].

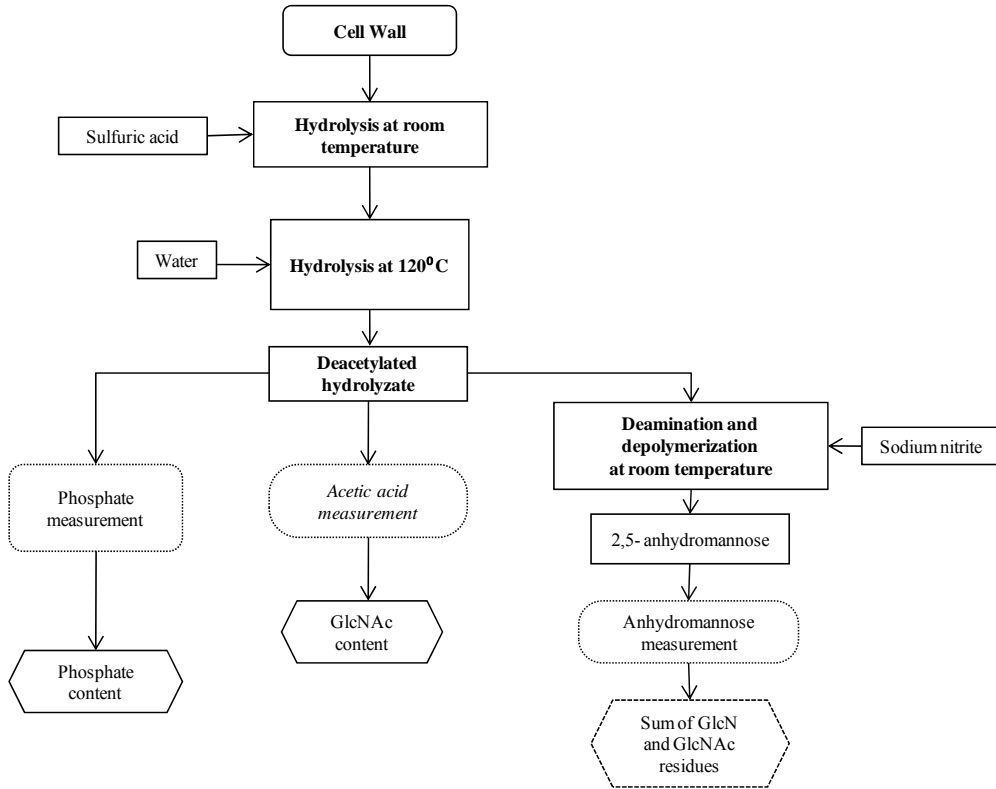


Figure 5: Procedure for the determination of GlcN, GlcNAc, and phosphate in fungal cell wall.

It was necessary, when using this method, to minimize the effect of side reactions, e.g. degradation of products, on the estimated amount of GlcN and GlcNAc content of the cell wall. Therefore, pure GlcN and GlcNAc were treated according to Figure 5 and their recoveries were calculated using equations 1 and 2.

$$r_{GlcN} = \frac{C_{2,GlcN}}{C_{1,GlcN}} \quad (1)$$

$$r_{GlcNAc} = \frac{C_{2,GlcNAc}}{C_{1,GlcNAc}} \quad (2)$$

While r_{GlcN} and r_{GlcNAc} are the recoveries of GlcN and GlcNAc (mol/mol) respectively, $C_{1,GlcN}$ and $C_{1,GlcNAc}$ are their known concentration prior to the hydrolysis, and $C_{2,GlcN}$ and $C_{2,GlcNAc}$ are

their concentration after the hydrolysis, measured by colorimetric and HPLC methods. Finally, the GlcN and GlcNAc contents of the AIM were calculated according to equations 3 and 4.

$$Y_{GlcN} = \frac{\frac{M_{GlcN}}{r_{GlcN}} - \frac{M_{Ac}}{r_{GlcNAc}}}{W} \times 161 \quad (3)$$

$$Y_{GlcNAc} = \frac{M_{Ac} \times 203}{r_{GlcNAc} \times W} \quad (4)$$

Y_{GlcN} and Y_{GlcNAc} are the GlcN and GlcNAc contents (g/g) of the AIM, respectively. W is the initial weight of the AIM (g), M_{GlcN} is the total moles of GlcN (2,5-anhydromannose), and M_{Ac} is the moles of liberated acetic acid [Paper I].

In addition to GlcN and GlcNAc contents, this method offers the possibility to determine phosphate content of the cell wall. The polyphosphates present in the cell wall of zygomycetes fungi, are depolymerized and released in the sulfuric acid hydrolyzate which consequently may be analyzed, e.g. with a spectrophotometric method [26] (Figure 5). As an example of applications of the new method, the analysis of the cell wall of the fungus *Rhizomucor pusillus* at different harvesting times disclosed that the major ingredients of the cell wall AIM were GlcN and GlcNAc (76-84%), whereas phosphates were present in lower concentrations (4-7%) (Figure 6) [Paper I].

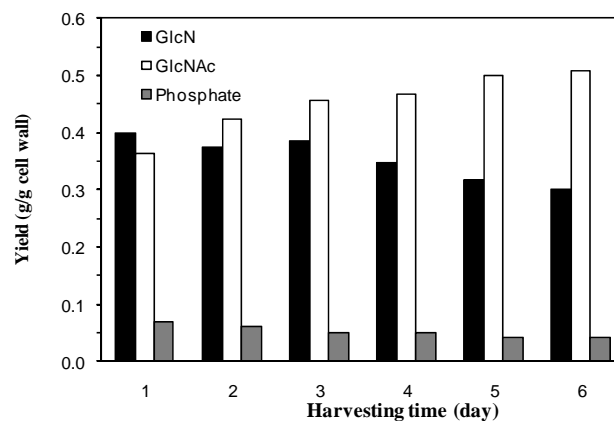


Figure 6: GlcN, GlcNAc, and phosphate content of the AIM of *R. pusillus* at different harvesting times (1-6 days).

Chapter 2

CHITOSAN: STRUCTURE, APPLICATIONS, AND PRODUCTION

2.1. Chitosan structure and properties

Chitosan is the most important derivative of chitin. The latter is the second most abundant polysaccharide in the world after cellulose [27]. The name of chitin originates from chiton, a Greek word meaning envelope. Chitin was first discovered in 1811 as one of the organic ingredients of mushrooms. This polysaccharide is the major component of exoskeletons of crustaceans and insects, fungal cell wall, radula of mollusks, and beaks of cephalopods. Altogether around 100 billion tons of chitin is produced annually [24, 28]. Despite extensive availability of chitin in the nature, extremely poor solubility of this biopolymer in aqueous solutions restricts its applications. Fortunately, the deacetylated derivative of chitin, chitosan, is readily soluble in acidic aqueous solutions and can be used in a variety of applications. Chitin and chitosan are copolymers of randomly distributed glucosamine (GlcN) and N-acetyl glucosamine (GlcNAc), where GlcNAc is dominant (60-100%) in chitin and GlcN is dominant (60-100%) in chitosan (Figure 7). In other words, chitin and chitosan are materials with similar compositions. Depending on the extent of deacetylation, a variety of chitins and chitosans are available. Conventionally, chitosan is distinguished from chitin by its solubility in dilute organic acid solutions. The proportion of glucosamine residues in chitosan and chitin is expressed as degree of deacetylation (DD). The solubility in dilute acids is usually achieved at $DD \geq 60\%$; therefore a range of chitosans with $DD = 60-100\%$ are potentially available [28-30].

Formation of strong hydrogen bonds between reactive hydroxyl and amino groups in chitin and chitosan result in crystalline structures for these materials. However, chitosan is less crystalline than chitin and hence more accessible to different reagents [24]. Chitosan is generally found in an extended two-fold helix conformation. Chitosan films can also form transient eight-fold helical structures, but will in high humidity be permanently reverted to the two-fold helix [29]. Non-bonding pairs of electrons of free amino groups, play an important role in the

physicochemical properties of chitosan. In acidic solutions ($\text{pH} < 6.5$), they are protonated (forming NH_3^+ groups) and thus responsible for solubility and basic properties of chitosan. Furthermore, due to strong nucleophilic behavior of these electrons, chitosans undergo a variety of chemical reactions, e.g. with most aldehydes and ketons. The majority of chitosan reactions are undertaken through the NH_2 group, while the hydroxyl groups attached to C_6 and C_3 of the hexosamine ring are the second most reactive groups of chitosan. A number of modification reactions including O-carboxymethylation, O-acylation, sulfonation, and cyanoethylation may be instigated through these groups [24, 31-33].

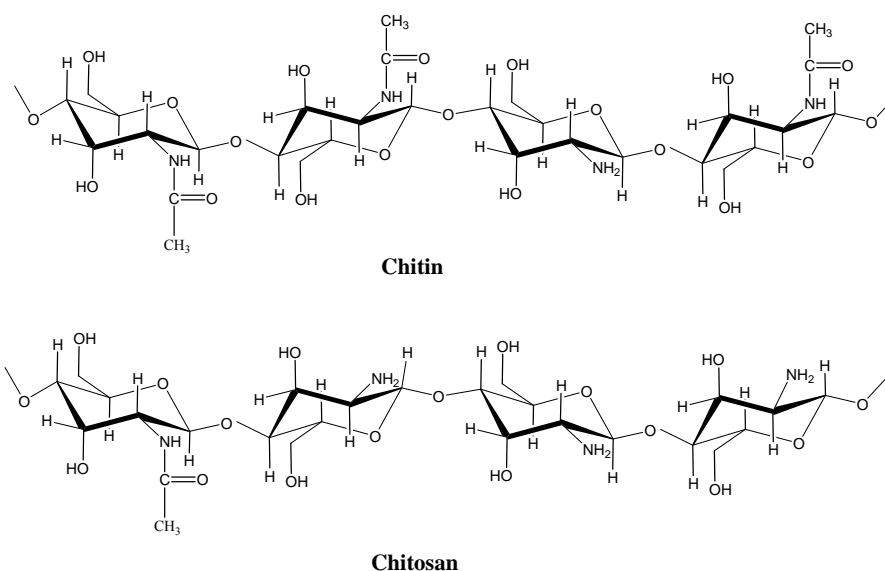


Figure 7: Structure of chitin and chitosan.

2.2. Applications of chitosan

Biocompatibility, biodegradability, reproducibility, non-toxicity, and an easily changeable molecular structure are the most important features of chitosan that make this biopolymer valuable and a unique choice for many different applications [24, 31]. Most commonly, chitosan is used for removal and recovery of toxic heavy metals (e.g. Pb^{2+} , Cu^{2+} , and Cd^{2+}) from drinking and wastewater. The metal removal is performed through the formation of coordinate covalent bonds between NH_2 groups of chitosan and metal ions [34]. Moreover, the

positively charged chitosan in aqueous solutions, is used as a polycationic flocculant for removal of negatively charged materials such as proteins from food processing wastes [35]. Recently, chitosan has shown promising potential in agricultural applications, in that coating of wheat seeds with chitosan enhanced the antifungal activity of the plant and consequently, higher crop yields were obtained. Chitosan can also be employed in the form of hydrogels for controlled release of pesticides and herbicides [24]. In pharmaceutical industry moreover, chitosan is used as a controlled drug release agent and it has shown significant wound healing and anti-cancer properties as well [36]. In the food industry, chitosan is utilized as packaging and coating films, thus protecting the food from being deteriorated. Furthermore, chitosan can be used for clarification of different fruit juices. The antioxidant activity of chitosan can be applied to restrict off-flavor and rancidity progress in meat products. In addition, chitosan has shown strong activity against growth of different microorganisms and may therefore be used as an antimicrobial agent. Another interesting property of chitosan which may be employed in dietary foods is its cholesterol lowering ability [24, 35]. In cosmetics, chitosan can be used in a variety of products including creams and permanent waving lotions. In biotechnology, chitosan gels can be used for immobilization and encapsulation of cells and enzymes [24]. Production of superabsorbent materials used in hygienic industry is another new application of chitosan [Papers V-VIII] which will be discussed in detail in Chapter 3.

2.3. Production of chitosan

Chitosan can be produced from chitin through the chemical or enzymatic deacetylation reaction.

2.3.1. Production of chitosan by chemical deacetylation of chitin

Chitosan is currently industrially produced by chemical deacetylation of chitin. In the exoskeletons of crustaceans (e.g. crab and shrimp), the major source of industrial chitosan, chitin is strictly connected to proteins, inorganic materials (mostly CaCO_3), pigments, and lipids. The process of production of chitosan from shellfish wastes contains the following two major steps [28, 31, 35], purification and deacetylation of chitin:

a. Purification of chitin

In this step, proteins and minerals are removed through the treatment of shells with dilute NaOH and HCl, respectively. Pigments and lipids are then further removed by extraction with organic solvents (acetone or ethanol), or by destroying with bleaching agents (e.g. KMnO₄ and H₂O₂), and finally a purified chitin is obtained.

b. Deacetylation of chitin

Deacetylation of chitin is usually performed in the presence of a strong sodium hydroxide solution (30-50%), at elevated temperatures (100-140°C), and under N₂ atmosphere.

This chemical approach for deacetylation of chitin and production of chitosan has several drawbacks. The harsh conditions of the deacetylation step necessitate large quantities of heat and sodium hydroxide and in addition, create a high volume of wastewater with very high chemical oxygen demand (COD). Moreover, the strong alkali and high temperatures result in inconsistent physicochemical properties of shellfish chitosans. Furthermore, the supply of raw material of this process is seasonally limited and a stable run of the process is often not achievable [37-40]. Besides shellfish chitosan, the cell wall of zygomycetes fungi, which naturally contain chitosan are an alternative resource for this biopolymer [41].

2.3.2. Biosynthesis of chitosan in the cell wall of zygomycetes fungi

Biosynthesis of fungal chitosan is hypothetically believed to be a result of tandem action of two enzymes, i.e. chitin synthase and chitin deacetylase. The former is responsible for biosynthesis of chitin while the latter catalyses the deacetylation reaction of chitin and produces chitosan. Uridine-diphospho-N-acetyl glucosamine (UDP-GlcNAc) is the dominating glucosyl donor for chitin formation. This reaction in fungal cells is thought to occur in the vicinity of the plasma membrane where chitin synthases admit UDP-GlcNAc from cytoplasm and add them to the chitin chain which is simultaneously sent to the cell wall [11, 12, 14]. In the fungal cells, chitin synthase is kept in some unique sub-cellular organelles called chitosomes. Around two thirds of the chitosomes are made of protein (chitin synthase) and the remaining part is lipid. In

the chitosomes, chitin synthase is in its zymogenic⁵ form and partial proteolysis is required to activate it. Synthesis of chitin in cells is delayed due to this zymogenicity until the chitosomes reach the specific location on the cell surface where the cell wall will grow [42, 43]. As soon as chitosomes reach the plasma membrane, the chitin synthase becomes activated and each chitosomal subunit starts to synthesize a new chitin chain. The chitosomal chitin synthases (subunits) that reach the cell surface can either remain tightly linked in a complex or alternatively disperse. When the chitin synthases remain associated, the growing chitin chains are located closely to each other and can therefore directly crystallize into long micro-fibrils. However, when the chitin synthases turn into a dispersed form on the cell surface, the chitin chains need longer time to crystallize and therefore their nascent stage will be prolonged. After the synthesis of chitin, chitin deacetylase located in the cell wall attack the newly prepared chitin to produce chitosan. However, this enzyme is ineffective on crystalline chitin and hence, only the chitin chains produced from dispersed chitin synthase would be susceptible to deacetylation into chitosan. Since chitosan is more abundant than chitin in the cell wall of zygomycetes fungi, it appears therefore, that a major portion of the chitin synthase operates in the dissociated form at the plasma membrane of these fungi [15, 42-45].

2.4. Industrial aspects of production of chitosan from zygomycetes cell wall

The biological production of chitosan in the cell wall of zygomycetes fungi apparently eliminates the expensive chemical deacetylation step which presently is used in industrial production of chitosan from shellfish wastes [46, 47]. With the progress of fermentation technology, zygomycetes fungi are today widely applied for production of different biotechnological products such as enzymes and metabolites. The chitosan containing mycelia of these fungi are therefore, produced in large scale as a by-product of such processes. On the other hand, unlike shellfish wastes, the fungal biomass is easily obtainable by cultivation, regardless of geographical location and season [37, 38]. Additionally, zygomycetes fungi are able to grow on different waste materials. Hence, biological production of chitosan in the cell wall of zygomycetes seems to be environmentally acceptable [46]. Furthermore, in the fungal cell wall, enzymatic deacetylation of chitin occurs under much milder conditions than what is used in chemical deacetylation of chitin. Therefore, fungal chitosans have a more consistent quality and

⁵ Inactive

higher biological activities compared to shellfish chitosans [46, 48]. Consequently, chitosan obtained from fungal cell wall appears to be a good candidate for replacement of shellfish chitosan in the future.

Among the different types of waste materials, spent sulfite liquor (SSL), was chosen for production of fungal biomass containing chitosan in this thesis. Spent sulfite liquor is the by-product of the sulfite process for production of cellulose from wood in the paper pulp industry. In this process, around 50% of the wood is dissolved in the cooking sulfite liquor. Depending on the severity of the cooking process, SSL contains 8-15% dissolved solids. The major ingredients of these dissolved solids are lignosulfonate (50-60%) and sugars (15-22%) [10, 49-51]. The lignosulfonate part of SSL can be utilized as plasticizer in concrete technology. However, contamination of the lignosulfonate with sugars hinders the hardening process of the concrete. Therefore, it is often preferable to remove the sugars prior to lignosulfonate usage [10]. Then again, SSL has a high biological oxygen demand (BOD), and hence its disposal into nature raises serious environmental concerns [10, 49, 51]. Sugar removal from SSL can be carried out in fermentation processes with *Saccharomyces cerevisiae* during e.g. ethanol production. SSL contains various types of sugars such as arabinose, galactose, glucose, mannose, and xylose. The inability of *S.cerevisiae* to absorb pentoses is one of the major drawbacks of the industrial production of ethanol from SSL. In contrast, zygomycetes fungi which are able to assimilate hexoses as well as pentoses are good candidates for sugar removal from SSL either directly or after ethanol production by *S.cerevisiae* [24, 49]. In this work (paper II, IV, and VIII), a xylose-rich SSL obtained from a paper pulp factory (Domsjö AB, Sweden) after ethanol production by *S.cerevisiae* (SSL-AE), was used as the carbon source for fungal cultivation. This liquor was further supplemented with $\text{NH}_4\text{H}_2\text{PO}_4$ and NH_4OH . The cultivation was performed in a 60-70 l airlift fermentor at $37\pm 1^\circ\text{C}$ and $\text{pH } 6.0\pm 0.3$ [Papers II, IV, and VIII].

2.5. Recovery of chitosan from the fungal cell wall

The recovery process of chitosan from fungal biomass usually contains two major steps: separation of the chitosan/chitin-rich part of the cell wall from the biomass, followed by extraction of acid soluble chitosan from this fraction of the cell wall. The first step is usually

carried out under a mild alkali treatment, where proteins, lipids, and alkali soluble carbohydrates are dissolved in 2-4% NaOH at 90-120°C for 15-120 min. At the end of this step, the cell wall is recovered as alkali insoluble material (AIM) [46, 52, 53]. The second step, i.e. separation of chitosan from the cell wall, is usually performed by dissolution of chitosan in an acid solution. Different types of acids can be utilized in this acid treatment, which may lead to different recovery and also purity of the extracted fungal chitosan [Papers II, IV]. When choosing the appropriate acid for extraction of chitosan from the cell wall, the following three questions should be addressed:

- 1- How much chitosan is available in the cell wall?
- 2- How effective is the acid for selective extraction of chitosan from other materials of the cell wall?
- 3- How high is the purity of the chitosan recovered by this acid?

The standard method described in section 1.3.2., for characterization of the zygomycetes cell wall [Paper I] can be utilized as an effective tool to answer these questions.

2.5.1. Purification of fungal chitosan

The results of our study on the characterization of the zygomycetes cell wall, confirmed that the major component in the AIM of these fungi are glucosamine, N-acetyl glucosamine, and phosphate. The first two components are indicators for fungal chitosan and chitin [Paper I]. The second goal of the present work was to accomplish separation of chitosan from the mixture in the cell wall, as well as its recovery and purification. The foundation of almost all chitosan separation methods is the solubility of chitosan in aqueous acid solutions. Since chitin is defined as being insoluble in acid solutions, treatment of AIM with acid results in extraction of chitosan from fungal chitin [52-56]. Hitherto, however, attentions have not been paid to the cell wall phosphates, and it is not clear whether phosphates are extracted along with the fungal chitosan or remain insoluble. Traditionally, acetic, formic, and hydrochloric acid solutions are utilized for extraction of fungal chitosan [38, 54-56], whereas the former has been the one most frequently used in many studies [37, 39, 40, 46, 52, 53, 57-65].

2.5.2. Solubility of chitosan in dilute sulfuric acid

To obtain fungal chitosan, the AIM of fungal biomass is usually treated with 2-10% acetic acid solutions at 25-95°C for 1-24 h, in order to extract the acetic acid soluble material (AcSM). AcSM, which is generally accepted as “fungal chitosan”, is in this step separated from the residue of AIM, which is labeled alkali-and acid-insoluble material (AAIM). Finally, the fungal chitosan (AcSM) is recovered from the acid solution by precipitation at elevated pH (8-10) [46, 52, 53]. Our preliminary goal in this part of the project was to investigate the effectiveness of acetic acid for recovery of fungal chitosan (Paper II). Around that time, we became aware of the unique behavior of chitosan in dilute sulfuric acid solutions. Unlike many acids, dilute sulfuric acid does not dissolve chitosan at room temperature. However, in boiling solutions of this acid [66], chitosan is easily dissolved. Interestingly, the dissolved chitosan can be precipitated and recovered from sulfuric acid solution by lowering the temperature. Furthermore, the experiments indicated that this temperature dependant solubility of chitosan in dilute sulfuric acid is not shared by other fungal cell wall ingredients such as chitin and glucans. Therefore, this unique property of chitosan was exploited to develop a new method for separation of fungal chitosan. Preliminary experiments established that pure shellfish chitosans, regardless of the molecular weight, can be dissolved in 1% (v/v) sulfuric acid solution by 20 min heating at 120°C in an autoclave. Therefore, fungal cell wall AIM was treated under similar conditions to dissolve the chitosan-rich material. Then, in order to avoid precipitation of chitosan, the mixture was filtered at high temperature (100°C), to separate the chitosan from hot-alkali and acid-insoluble material (HAAIM). Finally, the solution was cooled down in an ice bath to precipitate and recover the chitosan-rich material (Figure 8) [Paper II].

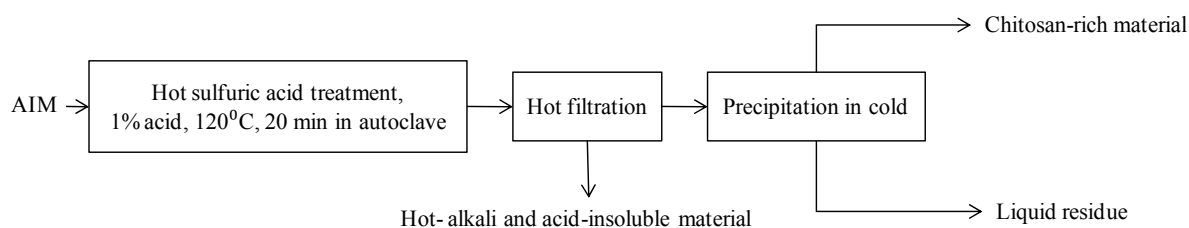


Figure 8: One step hot dilute sulfuric acid treatment for separation of chitosan from the AIM.

The material extracted from the AIM with hot acid and precipitated in cold had a FTIR spectrum similar to the spectrum of pure chitosan, extracted and precipitated under similar conditions (c.f. Figure 2 of Paper II). Furthermore, this material was easily degraded with nitrous acid, which is an indicator of chitosan. Therefore, we dared to name this cell wall derivative as “chitosan-rich material”. Subsequently, the efficiency of the acetic acid extraction was compared with this new method. Acetic acid treatment was performed on the AIM of *R. pusillus* with 10% acid at 60°C for 6 h. This treatment resulted in the recovery of the AIM as 16.5% AcSM and 79.0% AAIM. After that, the AIM was treated according to the new sulfuric acid method which resulted in 45.3% of the AIM as soluble chitosan-rich material and 29.0% as HAAIM. Thus, the yield of sulfuric acid-soluble chitosan-rich material was significantly higher than the yield of AcSM. Furthermore, sulfuric acid extraction of AAIM resulted in 43.7% of this cell wall derivative being chitosan-rich material. Therefore, the ability of acetic acid to extract chitosan from fungal cell wall was significantly lower than that of sulfuric acid. The AcSM was also treated with sulfuric acid at 120°C and only 38.5% of it was recovered as chitosan-rich material, whereas 31.7% of AcSM was released as soluble phosphates in the liquid residue after this treatment. This revealed that AcSM, which often is referred to as purified fungal chitosan, contains major phosphate impurity (c.f. Table 3 of paper II).

Hot dilute sulfuric acid treatment, undoubtedly showed promising results for extraction and recovery of fungal chitosan [Paper II]. However, the purity of the chitosan-rich material needed to be evaluated by other techniques than FTIR analysis and HNO₂ degradation.

2.5.3. Effect of dilute sulfuric acid on pure shellfish chitosan

The solubility of chitosan in aqueous acid solutions is one of the most important features of chitosan, making it more preferable than chitin. In this work [Paper III], the acetic acid solubility of pure shellfish chitosan, extracted with hot sulfuric acid and recovered in cold, was examined. The results of this study indicated that dissolution and precipitation of chitosan in dilute sulfuric acid solution, ends with formation of an acetic acid insoluble derivative of chitosan. It has previously been reported that, cooling the solution of chitosan in multivalent acids, such as oxalic acid and sulfuric acid at low pH, leads to formation of ionic cross-linked

chitosan hydrogels. Interaction of chitosan with dilute sulfuric acid may consist of two major reactions, namely protonation of amino groups of chitosan at high temperature and formation of sulfate bridges between NH_3^+ groups of two chitosan chains at lowered temperature (Figure 9) [66, 67]. The sulfate cross-linking of chitosan reduces the free amino groups which will react with acetic acid and consequently, sulfuric acid precipitated chitosan is insoluble in acetic acid solutions.

The most common way of recovery of chitosan from its dissolved form in different acids, is elevation of pH to 8-10. Similarly, simultaneous cooling and raising the pH of a solution of chitosan in hot sulfuric acid, results in precipitation of an acetic acid soluble chitosan. The pH elevation (to 8-10), may avoid formation of sulfate cross-linked chitosan [Paper III].

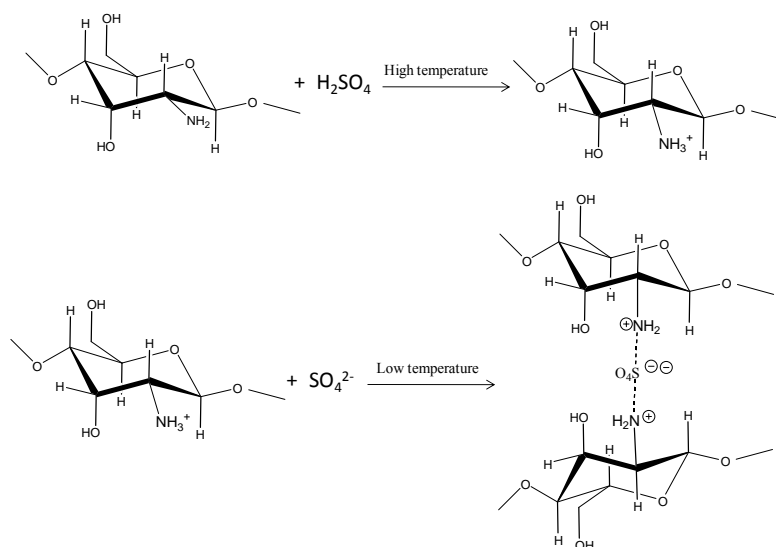


Figure 9: Protonation and ionic cross-linking of chitosan in dilute sulfuric acid.

In addition, the effect of the concentration of sulfuric acid on the solubility of different preparations of shellfish chitosan was investigated. With a concentration of sulfuric acid of 72 mM or higher, different chitosans, with low, medium, and high molecular weights, were dissolved during 5 min heating at 120°C (Figure 10).

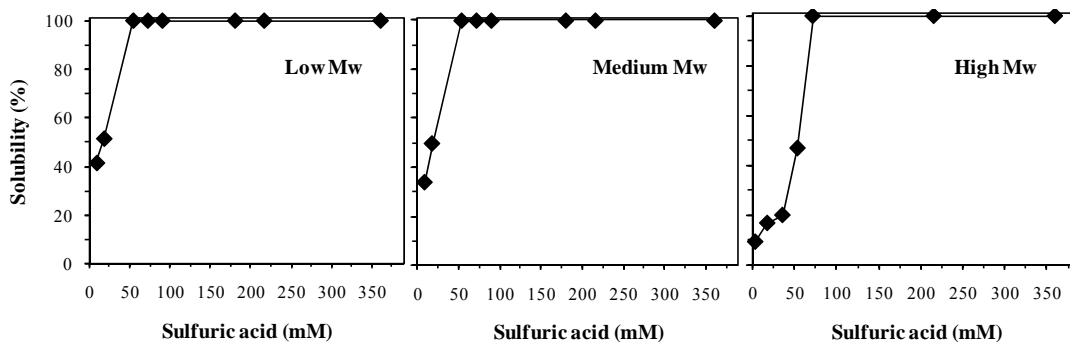


Figure 10: Solubility of shellfish chitosans in dilute sulfuric acid at 120°C during 5 min.

Furthermore, the effect of duration of sulfuric acid treatment of a high molecular weight shellfish chitosan on the recovery and its molecular weight at 120°C was studied. The results indicated that the dissolution of chitosan in hot dilute sulfuric acid solution is accompanied by partial depolymerization of chitosan. As shown in Figure 11, more than 80% of the high molecular weight chitosan may be recovered after 30 min treatment with 72 mM sulfuric acid upon cooling and pH alteration. During this time, the intrinsic viscosity of chitosan was reduced more than 3-fold (Figure 11) and consequently, the viscosity average molecular weight (M_v) of chitosan was reduced from $1,388 \times 10^3$ to 174×10^3 (Table 1). Further increasing of the treatment duration to 120 min did not decrease the Mw significantly. However, the chitosan recovery was reduced to 64% during this time period and by further doubling the reaction time to 240 min, a considerable reduction of both M_v and recovery was observed ⁶ (Figure 11 and Table 1) [Paper III].

⁶ The ability of dilute sulfuric acid for depolymerization of chitosan at elevated temperatures became the basis for development of a new method for production of low molecular weight chitosan. For further details please refer to paper III.

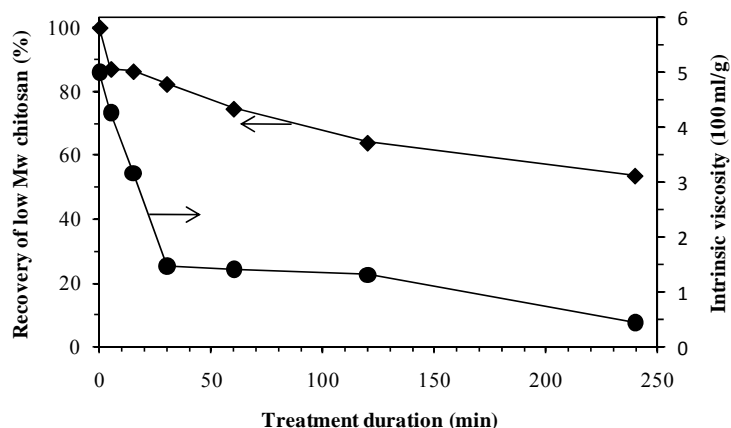


Figure 11: Recovery and intrinsic viscosity of high Mw chitosan after treatment with 72 mM sulfuric acid at 120°C.

Table 1: Viscosity average molecular weight of high Mw chitosan after treatment with 72 mM sulfuric acid at 120°C.

Treatment duration (min)	$M_v \times 10^{-3}$
0	1,388
5	1,063
15	642
30	174
60	160
120	145
240	24

2.5.4. Recovery and purity of fungal chitosan obtained by dilute sulfuric acid treatment

The results of treatment of shellfish chitosan with hot dilute sulfuric acid solutions indicated that a pH elevation is crucial to precipitate an acetic acid soluble chitosan [Paper III]. Correspondingly, a pH increasing step was added to the original sulfuric acid treatment for precipitation of chitosan from fungal cell wall extracts (Figure 12). The next aim of this thesis was to evaluate the purity of the material extracted from AIM by hot sulfuric acid and precipitated in cold alkaline solution (cold alkali precipitate, CAIP). Moreover, to achieve

maximum recovery, optimization of extraction conditions and minimization of chitosan loss, were included in the objectives. Accordingly, the recovery and purity of CAIP after 5-90 min treatment of AIM of *R. pusillus* with dilute sulfuric acid⁷ at 120°C was investigated.

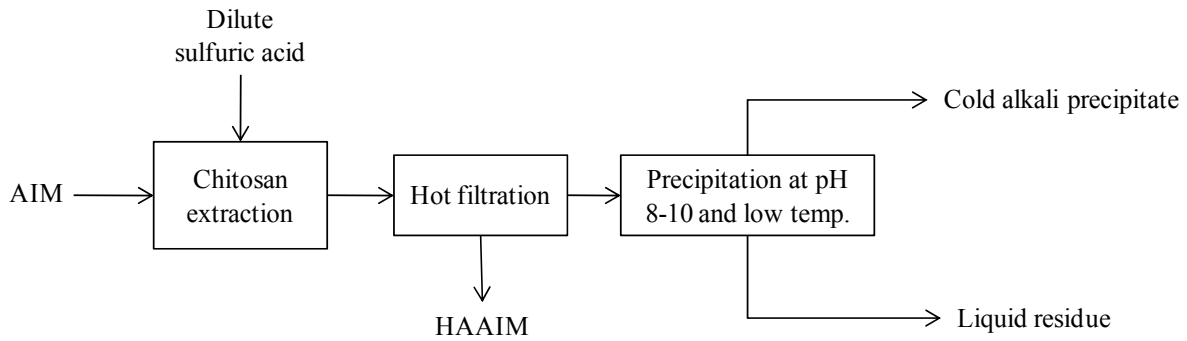


Figure 12: Extraction of fungal chitosan with hot dilute sulfuric acid and its precipitation and recovery as CAIP at elevated pH and lowered temperature.

The results presented in this thesis indicate that maximum amounts of total GlcN and GlcNAc (as indicators for chitosan) in CAIP was achieved when the treatment time was 45 min, while the CAIP was not adequately recovered when the treatment time was shorter than that (Figure 13). Furthermore, the CAIP which was expected to be pure chitosan contained a main phosphate impurity (Figure 13). Figure 13 illustrates that after 5-10 min, the major ingredient of CAIP was phosphate, while GlcN and GlcNAc appeared as minor components. Significant presence of phosphate in the CAIP obtained after short time intervals indicates that polyphosphates may readily be extracted from the AIM and separated from the rest of the cell wall (HAAIM). During the cooling and pH elevation step, the dissolved phosphates probably form insoluble salts with extracted chitosan (GlcN + GlcNAc) and are recovered as CAIP. Prolonging the treatment to 20 min, however, reduced the recovery of phosphates. This may be due to depolymerization of extracted polyphosphates during longer time intervals which cannot be precipitated along with small portions of extracted chitosan (Figure 13).

⁷ The minimal concentration of acid in which high, medium and low molecular weight shellfish chitosans were dissolved in 5 min was 72 mM. This concentration was chosen for extraction of chitosan from cell wall.

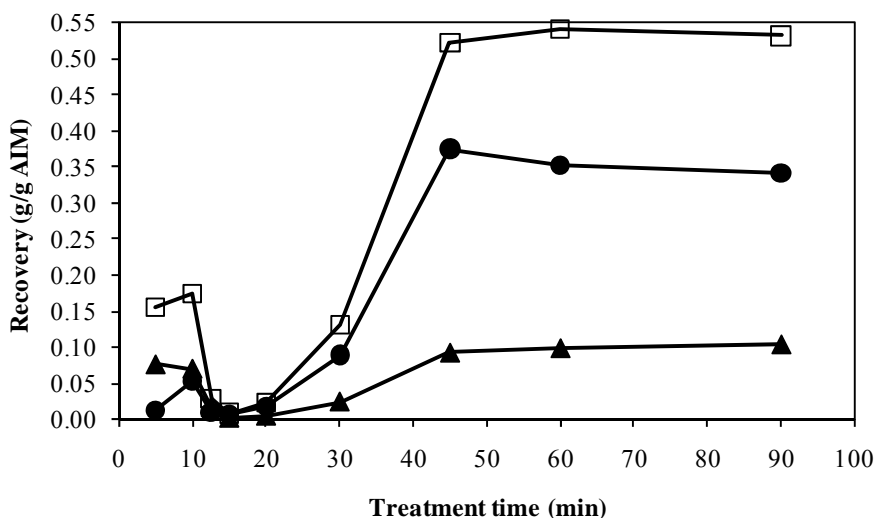


Figure 13: The yield of CAIP (□) and the recoveries of (GlcN + GlcNAc) (●) and phosphates (▲) in CAIP (as g/g AIM) after 5-90 min treatment of AIM with 72 mM sulfuric acid at 120°C.

Further increase of the treatment duration increased the recovery of chitosan (GlcN + GlcNAc). The improvement of chitosan recovery was accompanied by increased phosphate recovery. This may be due to formation of insoluble salts of chitosan with soluble phosphates. In other words, during very short treatments, presence of minor amounts of chitosan probably resulted in precipitation of polyphosphates, while after a prolonged treatments, higher concentration of chitosan, non-soluble in cold alkali solution, caused co-precipitation of soluble phosphates. Presence of phosphate in the chitosan extracted with acetic acid, AcSM⁸, may be caused by similar simultaneous extraction and precipitation phenomena. Therefore, existence of phosphate in the cell wall of zygomycetes fungi seemed to be the bottleneck for purification of fungal chitosan (Figure 13) [Paper IV].

2.5.5. Phosphate removal from the AIM and purification of fungal chitosan

In order to obtain purified fungal chitosan it was necessary to prevent simultaneous precipitation of phosphate and chitosan after the acid extraction. The preliminary investigations indicated that the key point to achieve this was lying in the difference between the behavior of polyphosphates and chitosan in dilute sulfuric acid solutions. Unlike chitosan, polyphosphates

⁸ C.F. section 2.5.2

dissolve in dilute sulfuric acid not only at high but also at room temperature. The release and dissolution of polyphosphates from the AIM at room temperature thus offered a possibility of separating it from fungal chitosan and the treatment of zygomycetes AIM with dilute sulfuric acid at room temperature resulted in separation of phosphates from the residue of the chitosan-rich cell wall. Extraction of chitosan from this cell wall residue and its precipitation in cold alkali solution resulted in a purified phosphate-free fungal chitosan (Figure 14) [Paper IV].

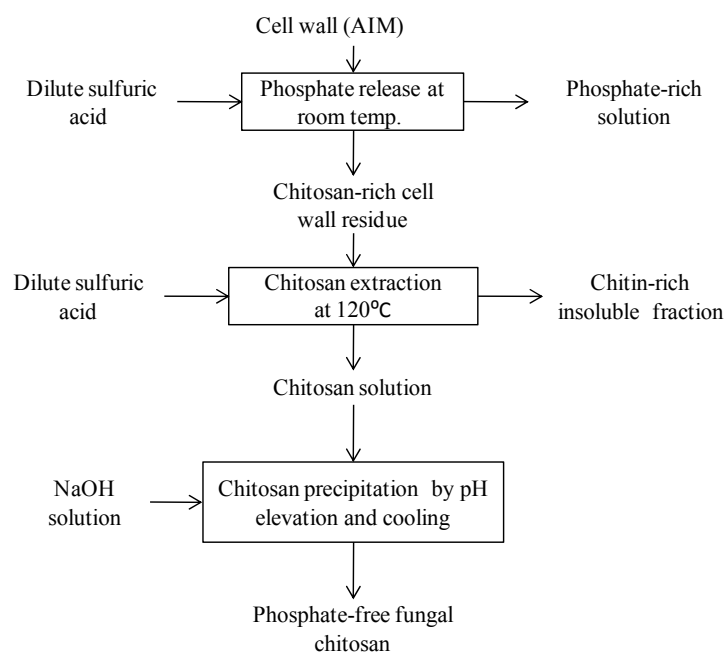


Figure 14: The process of extraction and purification of fungal chitosan by dilute sulfuric acid.

To summarize part of the work in this thesis, the traditional methods for separation and recovery of chitosan from the fungal cell wall were replaced by a more efficient method using dilute sulfuric acid. This method employed the differences in solubility of cell wall ingredients (i.e. chitosan, chitin, and polyphosphates) in dilute sulfuric acid solutions for purification of fungal chitosan. Phosphates were in this method first separated from the cell wall by dissolution in dilute sulfuric acid at room temperature. Subsequently, chitosan was dissolved in hot dilute sulfuric acid and separated from fungal chitin. The dissolved chitosan was finally recovered by precipitation at pH 8-10 and lowered temperatures (Figure 14) [Paper IV].

Chapter 3

PRODUCTION OF SUPERABSORBENT POLYMERS FROM CHITOSAN

3.1. Introduction of superabsorbent polymers

Superabsorbent polymers (SAPs) are highly hydrophilic polymers which are fairly cross-linked in three dimensions and able to absorb considerable amounts of aqueous liquids [68, 69]. In 1978, the cross-linked starch-grafted polyacrylate was introduced as SAP to feminine hygienic industry in Japan for the first time. Since then, a variety of synthetic and biological polymers have been utilized for production of SAPs. Presently, cross-linked polymers of acrylic acid and corresponding salts are the most commonly used materials for production of SAPs [70]. These types of SAPs are produced by simultaneous polymerization and cross-linking of acrylic acid and its salts. The polymerization is a free-radical initiated reaction which may be performed in bulk aqueous solutions of 20-40% monomers or in suspensions of drops of aqueous solutions in a hydrocarbon solvent. In bulk solution polymerization, the monomers may be either in acidic (pH 2-4) or partially neutralized (pH 5-7) salt forms. However, in suspension polymerization, in order to avoid the phase separation of monomers and solvent, acrylic acid needs to be converted to sodium acrylate. Addition of small portions of co-polymerizable cross-linkers, results in a cross-linking-polymerization reaction. The outcome of this reaction, also called gelation reaction, is a polymeric gel. Superabsorbent polymers are obtained by consequent drying and grinding of this gel [71, 72]. Figure 15 demonstrates an example of cross-linking polymerization of partially neutralized acrylic acid. In this reaction, ethylene glycol dimetacrylate is used as a cross-linking agent [71].

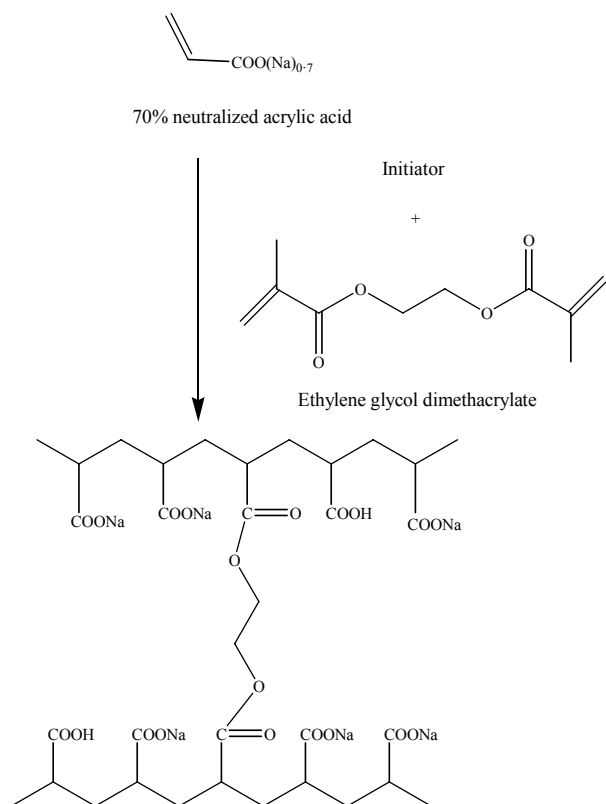


Figure 15: Cross-linking polymerization of sodium acrylate.

Water absorbency by SAPs is carried out through a diffusive mechanism. The non-ionic SAPs prepared directly from acrylic acid, without neutralization, contain hydrophilic $-\text{COOH}$ groups, which become hydrated in water, forming strong hydrogen bonds with water molecules. In other words, the hydrophilic polymer chains tend to dissolve in water, but presence of cross-links between the chains inhibits dissolution of SAPs. In contrast, water diffuses into the network of cross-linked polymers and the polymers move in the opposite direction of water diffusion in order to lodge the coming water molecules. These polymer movements result in formation of a swollen gel from SAP particles. The mechanism of water absorbency in ionic SAPs, e.g. SAPs prepared from (partially) neutralized acrylic acid, is different from that of non-ionic SAPs. In ionic polymers, the neutralized building blocks of polymer are similarly charged, thereby repelling each other. In dry SAPs however, the negative carboxylate groups are neutralized with positive sodium ions. Once water is added to dry SAPs, the sodium ions become hydrated, resulting in a reduced interaction with carboxylate ions. The reduced interaction brings about free

movement of the Na^+ ions inside the SAP and consequently an osmotic pressure is created between the SAP and the surrounding water. However, due to the weak attraction of sodium ions to COO^- groups, they are trapped and cannot migrate from the SAP network. The osmotic pressure is the driving force for diffusion of water into the SAPs and water absorption by SAPs. Swelling of SAPs, as a result of water diffusion, continues up to the point when the forces generated from elongation of polymer chains are equal to the forces resulting from osmotic pressure. At this point, the equilibrium water binding capacity of the SAP is obtained, and the water uptake stops. Previous studies suggest that superabsorbent polymers are able to absorb up to 1000 g pure water per g polymer at the equilibrium point [69, 73].

3.2. Applications of superabsorbent polymers

Today, close to 90% of the total production of SAPs is used to produce infant diapers for urine absorption. These disposable diapers generally contain an absorbent core between two sheets of non-woven materials. Absorbent cores are usually a mixture of SAP (10-50%) and cellulose fluff pulp. The higher concentration of SAP in modern diapers has resulted in production of extremely thin absorbent cores. The urine flows quickly through the top sheet layer of the diaper, made of porous material, reaching the absorbent core, where it must be absorbed. The back sheet of the diaper is made of an impermeable material, preventing urine leakage from the diaper [74, 75]. Superabsorbent polymers are also widely used in other personal hygiene goods such as feminine hygiene and adult incontinence products [70, 75]. In addition to hygienic products, SAPs may also be used as soil additives (0.1%) to improve the soil moisture retention [75, 76]. Furthermore, these polymers are utilized as controlled release agents of different materials such as medicines, fertilizers, and pesticides [75, 77]. Additionally, SAPs may be used to protect construction materials as well as electrical cables from water damage. In such applications, SAPs form gels upon contact with water, thus preventing further water flow. SAPs may also be utilized in food packaging systems to absorb drained liquids. Production of artificial snow for indoor ski arenas is another interesting application of SAPs. In cosmetic industry, SAPs may be used to improve the moisturizing effects of different products [75].

3.3. Production of superabsorbents from biopolymers

Due to widespread application of SAPs in disposable personal care products, the use of synthetic polymers, e.g. polyacrylates, for production of SAPs have raised ecological and sustainability awareness and concerns. These polymers are generally classified as non-biodegradable materials and, therefore, disposal of them in large amounts sustains environmental pollution. Furthermore, land-filling using these plastic-type materials may be accompanied with leaching of toxic chemicals and the subsequent pollution of ground water. Hence, synthetic SAPs are a potential threat to human health. Additionally, polyacrylates are mostly petroleum-based materials. Due to the limited natural gas and oil resources and the increasing global oil price, production of SAPs from polyacrylates may face serious economical problems in the future [78-81]. As a result, there has been a growing interest in recent years, for production of SAPs from non petroleum-based renewable resources. Natural polymers such as cellulose, starch, chitin, and chitosan are possible candidates for production of bio-based SAPs. These widely available renewable polymers are able to undergo a biological decomposition after usage, thus escaping the environmental concerns of synthetic materials. Moreover, they are generally considered as biocompatible and non-toxic materials and may therefore be safely utilized in different applications such as biomedical and personal care products [78-83]. However, the natural polymers usually exhibit poor or limited water solubility and therefore, they lack the essential water-binding hydrophilic characteristics of polymers used for production of SAPs. Nevertheless, these materials are easily modified chemically and converted to their hydrophilic derivatives by covalent attachment of hydrophilic groups. These derivatives, natural-based water-friendly polymers, have shown promising capacity for production of biological SAPs, such as carboxymethyl cellulose (CMC) and carboxymethyl starch, and have been successfully utilized for production of superabsorbents [84, 85].

3.3.1. Superabsorbent polymers based on chitosan

It has been reported that SAPs can be produced by cross-linking of chitosan in acid solutions, e.g. by addition of glutaraldehyde or a heat-curing process, and drying the resultant hydrogel. These chitosan-based SAPs were able to absorb up to 85 g distilled water or 25 g saline solution per g SAP under a load of 2.1 KPa [82]. The major benefit of chitosan over other biopolymers, as a substrate for production of SAPs, is probably the polycationic nature of this

biopolymer. The human skin is slightly acidic (pH 4.3-6.6) and the water binding capacity (WBC) of cationic SAPs at this range of pH is to some extent higher than that of anionic SAPs such as carboxymethyl cellulose or carboxymethyl starch based SAPs [82, 86-89]. Additionally, chitosan exhibits strong antimicrobial activity [90] and hence may inhibit the growth of different bacteria on the surface of diapers containing chitosan-based SAP. Restriction of microbial growth avoids generation of off-odors from the diapers and increases the functional life-time of these products. Therefore, chitosan seems to be a good candidate for production of bio-based SAPs. However, the insolubility of chitosan in water at neutral pH is considered as a major drawback of this biopolymer, restricting its utilization in different applications [91].

In contrast, water-soluble derivatives of chitosan are easily produced and used in a variety of applications. Different chemical modification reactions such as carboxymethylation, hydroxylation, partial N-acetylation, quaternarization, and sulfonation may be used for production of water soluble derivatives of chitosan [92].

3.4. Carboxymethylation of chitosan

Carboxymethyl chitosan (CMCS) is one of the most well known water soluble derivatives of chitosan. The carboxymethylation reaction is usually accomplished by the reaction of chitosan with monochloroacetic acid (MCAA) in presence of sodium hydroxide. Most commonly, chitosan (1g) is mixed with sodium hydroxide (0.5-2.5 g) in a 2-propanol-water⁹ solvent system (10 ml) to obtain alkalized chitosan. The alkalization step results in swelling of the chitosan, which facilitates penetration of MCAA¹⁰ (1.2-2.5 g) into the polymer structure, thereby enhancing the carboxymethylation yield. The carboxymethylation reaction is usually carried out at 20-50°C [92-95]. In the chitosan chains of the glucosamine residues, the hydroxyl groups at C₃ and C₆ and the amino group at C₂ positions are the most reactive sites for reaction with MCAA. When the carboxymethylation reaction is brought about at hydroxyl groups, the product is called O-carboxymethyl chitosan (O-CMCS), while the product of the reaction occurring at amino groups is called N-carboxymethyl chitosan (N-CMCS) [96]. After the appropriate time, the reaction is stopped by addition of 70% ethanol, allowing CMCS to be separated from the reaction

⁹ The water: 2-propanol quota is usually chosen as 2:8 ml/ml

¹⁰ MCAA is usually dissolved in 2 ml 2-propanol and added to the reaction mixture

mixture by filtration. The CMCS is then repeatedly washed with 70% ethanol followed by filtration, to acquire a filtrate with neutral pH. The final product after drying is sodium salt of CMCS (Figure 16). CMCS is a highly hydrophilic biopolymer with the ability to form gel, film as well as fiber and may be used as moisture-retention, antimicrobial, wound healing, and controlled drug release agents [97]. Additionally, graft polymerization of acrylic acid into CMCS chains has been successfully used for production of SAPs [98]. However, direct production of SAP from CMCS has not been reported before. In a part of the present thesis, the possibility of production of SAP from CMCS was tested by using commercially available pure shellfish chitosans. Then, different parameters affecting the water binding capacity of CMCS-based SAP were investigated and optimized. Finally, the principles applied for producing SAP from shellfish chitosan were applied for producing SAP from fungal chitosan.

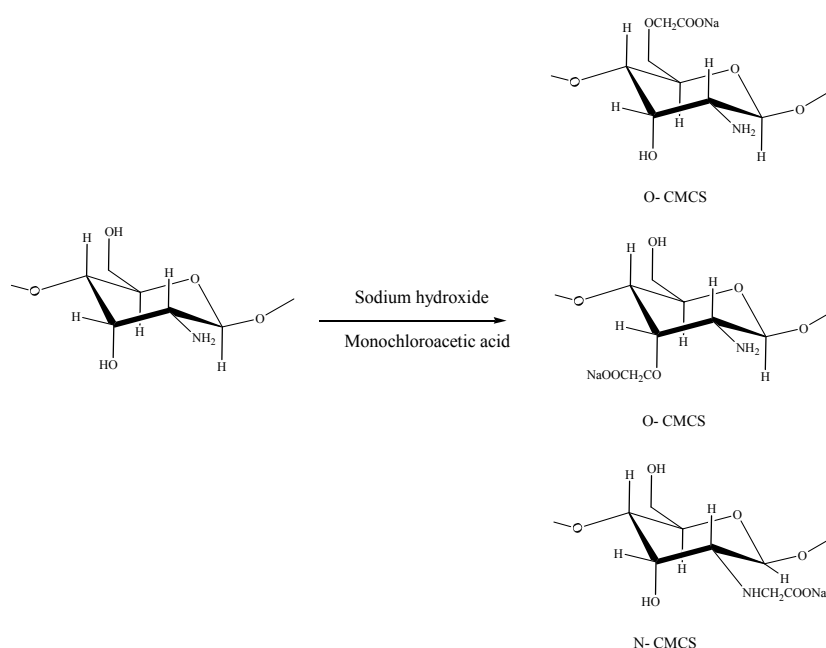


Figure 16: Mechanism of formation of sodium salt of O- and N-carboxymethyl chitosan.

3.5. Production of superabsorbent polymers from carboxymethyl chitosan

Carboxymethylation of chitosan results in formation of a hydrophilic polymer which potentially may be cross-linked to form SAP. We tested the possibility of SAP formation from

CMCS by cross-linking CMCS in aqueous solutions with glutaraldehyde. Glutaraldehyde is a dialdehyde molecule which reacts with amino groups of chitosan, forming covalent imine bonds. Since glutaraldehyde is a bi-functional agent, it reacts with two amino groups of two different molecules and forms a hydrocarbon bridge between the chitosan chains (Figure 17). Consequently, the dissolved chitosan is cross-linked and converted into a gel [99]. The same reaction can occur between the amino groups of CMCS. In this work, three different shellfish chitosans with low, medium, and high molecular weight were carboxymethylated [100] and 1% aqueous solutions of the obtained CMCSs were cross-linked with glutaraldehyde to form gels. The resultant gels were then freeze-dried and shredded to small particles (<1 mm). The water binding capacity of the products in water and 0.9% NaCl solution was measured and compared with the WBC of two commercially available synthetic SAPs [Paper V].

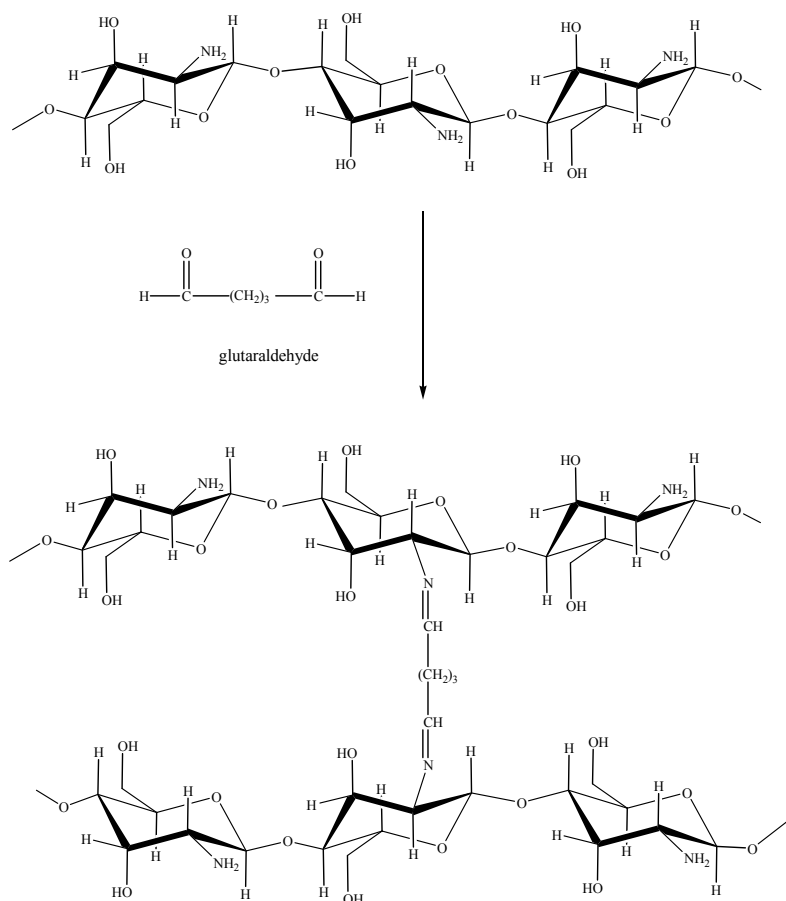


Figure 17: Mechanism of cross-linking of chitosan with glutaraldehyde.

The freeze-dried cross-linked CMCSs were able to absorb 44-81 and 83-176 g water per g SAP after 1 and 60 min exposure, respectively. The respective WBC of the tested synthetic SAPs was 63-158 and 285-579 g water per g SAPs. Evidently, in pure water the WBC of CMCS-based SAP was lower than that of synthetic SAP. However, addition of salt into the water reduced the WBC of synthetic SAPs significantly (23-28 g/g after 1 min) (Table 2). The reduction of WBC in salt solutions is a result of the salt lowering the water potential, which consequently reduces the diffusion of water into the structure of SAPs, driven by the osmotic pressure [69, 101]. The CMCS-based SAPs exhibited a higher salt solution uptake (32-45 g/g) under identical conditions than the commercially accepted SAPs (Table 2) [Paper V]. CMCS-based SAPs may therefore be considered as good potential candidates for application in personal care products where the swelling agents are salty liquids such as urine and blood. In the present study, the molecular weight of CMCS as well as the amount of cross-linking agent (glutaraldehyde) had a significant effect on the WBC of CMCS-based SAP. For high and medium molecular weight CMCSs, higher WBC, after 1 min, was achieved by using lower amount of glutaraldehyde. However, the WBCs after 60 min for these SAPs were enhanced by utilization of higher amount of glutaraldehyde. In contrast, increasing the amount of glutaraldehyde resulted in an increasing of the WBC of low molecular weight CMCS, after short as well as long exposure times (Table 2) [Paper V].

Table 2: WBC of freeze-dried cross-linked CMCSs and two commercial SAPs.

SAP type	Glutaraldehyde (g/g) ¹	WBC after different exposure times (g/g)		
		Water 1 min	0.9% saline 1 min	Water 60 min
High Mw-CMCS	0.04	67	35	173
High Mw-CMCS	0.02	73	45	143
Medium Mw-CMCS	0.04	54	45	176
Medium Mw-CMCS	0.02	81	43	134
Low Mw-CMCS	0.08	58	38	108
Low Mw-CMCS	0.02	44	32	83
L520 ²	---	63	23	285
5A70 ³	---	158	28	579

¹Amount of glutaraldehyde used as cross-linking agent (g per g CMCS)

²Sumitomo SEIKA (5A70), a commercial synthetic superabsorbent.

³Aqualic ca. (L520), a commercial synthetic superabsorbent.

3.5.1. Effect of drying method on the water binding capacity of CMCS-based SAPs

Although the freeze-dried CMCS gels showed promising WBC, the high cost of freeze-drying was recognized as a limiting factor for industrial acceptance of CMCS-based SAPs. Therefore, it was necessary to dry the CMCS gel with more cost-efficient methods. In this case, the effect of air-drying was first investigated which resulted in products with significantly lower WBC compared to that of the freeze-dried products (c.f. Table 1 of Paper V). The reduction of WBC may be the consequence of considerable shrinkage of the CMCS gel during air-drying. Unlike freeze-dried gels, these air-dried materials did not have a porous structure [Paper VI].

Creation of a porous structure that facilitates water diffusion by creation of capillary forces is an essential step in production of modern synthetic SAPs [68, 72, 102]. The porous structure is generally formed by generation of gas bubbles and subsequent foaming in the polymerization reaction medium. Different types of blowing agents can be utilized for creation of foam and among them sodium carbonate is the most recognized one. This foaming agent releases CO₂ bubbles in the presence of acidic foaming aids, such as acetic and acrylic acids. In other words, production of modern SAPs involves two simultaneous steps of cross-linking polymerization (i.e. gelation) and foaming. During this process, the progress of the polymerization reaction results in an increase of the viscosity of the reaction mixture over the time. At the same time, decomposition of sodium carbonate produces CO₂ bubbles, ending with foaming of the reaction mixture. Finally, the foamed gel is dried and mechanically ground to obtain SAP [72]. In addition to sodium carbonate, acetone and light alkanes with 5-7 carbon atoms such as n-pentane have been successfully utilized in the foaming process. All of these foaming agents are low boiling point liquids that quickly can be evaporated in the polymerization reaction mixture and lead to foam formation [68, 102-104].

We tested the effectiveness of different blowing agents for generation of foam in CMCS gel. Sodium carbonate did not seem to be a good porosity generator in this system, since the pH of the CMCS solution was higher than was required for generation of CO₂ bubbles. Neither did acetone show any promising results, because CMCS was precipitated from its water solution by addition of acetone. In contrast, n-pentane, which is a liquid totally immiscible with water and with a boiling point at 36°C, showed to be a suitable porosity generator in CMCS gels.

3.5.1.1. Drying by a foaming technique

In order to prevent shrinkage of the CMCS gel during the air-drying process, a foaming technique was developed by using n-pentane as a blowing agent in this study. In this method, CMCS was first dissolved in water and heated to 60°C. Then, n-pentane was added and the mixture was stirred vigorously to thoroughly disperse the n-pentane in the CMCS solution. Since the initial temperature of this solution was higher than the boiling point of n-pentane, this foaming agent started to boil upon mixing. In the next step, a water solution of glutaraldehyde was added and mixed. Addition of glutaraldehyde resulted in cross-linking of CMCS and formation of a gel. Consequently, the dispersed n-pentane droplets were captured inside this gel. Therefore, further evaporation of n-pentane generated a foamed CMCS gel. The foamed gel was then placed in an oven to air-dry at 60°C. The air-drying of the gel was accompanied with further evaporation of n-pentane, which stabilized the foam and prevented collapsing of the gel during the air-drying procedure. The product of this step was a SAP with a porous structure (Figure 18) [Paper V].

In this foaming process, molecular weight and concentration of CMCS as well as amount of glutaraldehyde and amount of n-pentane, significantly affected the WBC of the final SAP. For high as well as medium molecular weights of CMCS, the highest WBCs were obtained when the concentration of CMCS was 2% and the solution was added with glutaraldehyde, 0.02 g/g CMCS, and 0.5 ml n-pentane/ml CMCS solution. The WBC of the SAP obtained under this condition was 52-54 g/g in pure water and 32-33 g/g in saline solution, after 1 min exposure. Longer exposure time (60 min) increased the WBC of these SAPs to 85-91 g/g. These SAPs were able to retain 73-78% of the absorbed water under the load exerted by centrifugation¹¹ (Figure 19). A comparison between these foam-dried products and the different freeze-dried products indicates that in average, 72% of the WBC of the freeze-dried CMCS SAP in salty water was reached after air-drying combined with n-pentane foaming. The low molecular weight CMCS, however, displayed different characteristics during the foaming process in that a higher amount of glutaraldehyde was required to create a stable foamed gel. The highest WBC of SAP of this CMCS was achieved when 2% CMCS solution (20 ml) was mixed with 10 ml n-pentane and

¹¹ Centrifuge retention capacity (CRC)

cross-linked with 0.04 g glutaraldehyde/g CMCS, but the best WBC of the low molecular weight CMCS was lower than the ones obtained from high and medium molecular weight CMCSs (Figure 19).

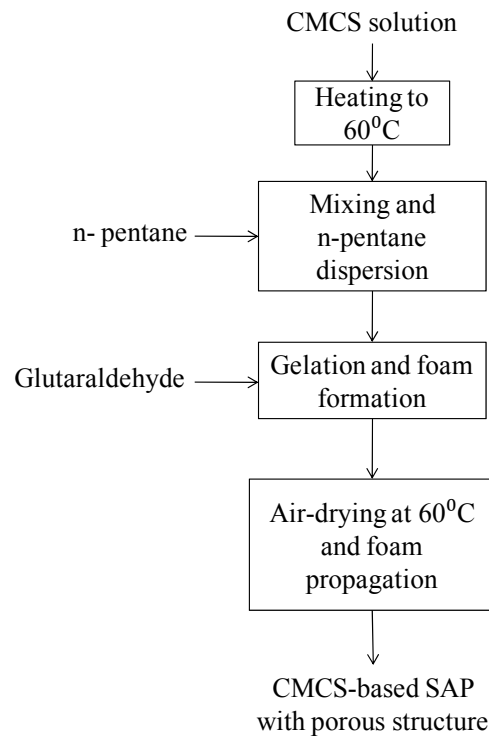


Figure 18: Production of CMCS-based SAP with the n-pentane foaming technique.

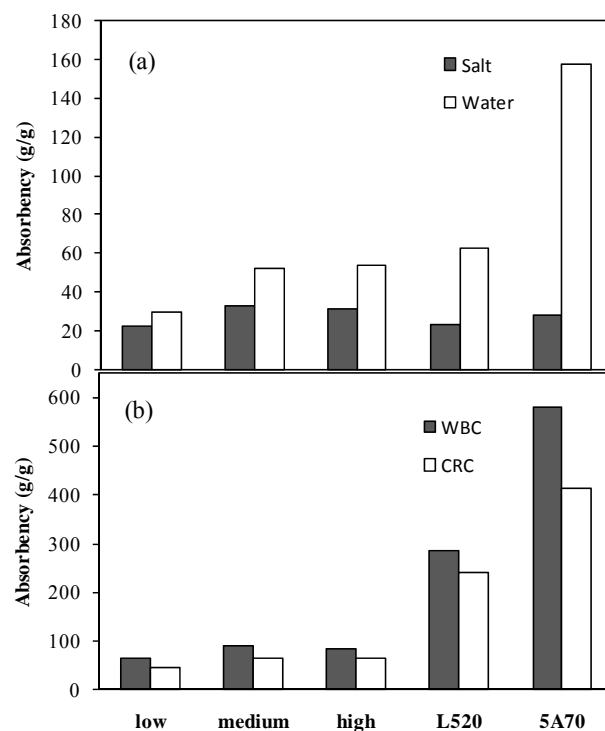


Figure 19: Water absorbency of the best low, medium, and high molecular weight CMCS-based SAPs and commercial SAPs. (a) WBC in pure water and in 0.9% NaCl solution after 1 min and (b) WBC and CRC in pure water after 60 min.

3.5.1.2. *Partial thermal dehydration and subsequent freeze-drying*

Although the foam-drying method resulted in a porous structure of cross-linked CMCS, utilization of n-pentane, which is a highly flammable liquid, may raise some concerns about the process from the safety point of view. Therefore, this project also investigated other solutions for reduction of freeze-drying costs. Among the different possibilities, partial dehydration of CMCS gel prior to the freeze-drying step resulted in SAPs with WBC close to the WBC of directly freeze-dried CMCS. In this method, CMCS prepared from a shellfish chitosan was dissolved in water (0.9%) and cross-linked with glutaraldehyde (0.02 g/g CMCS) to form a gel. The obtained gel was then partially dehydrated in a vacuum rotary evaporator at 70°C for 120-300 min, 85°C for 45-135 min, or 100°C for 15-60 min. The partially dehydrated gels were then frozen at -196, -

20, or -5°C and freeze-dried (Figure 20). The WBCs of the final dried products are presented in Figure 21 [Paper VI].

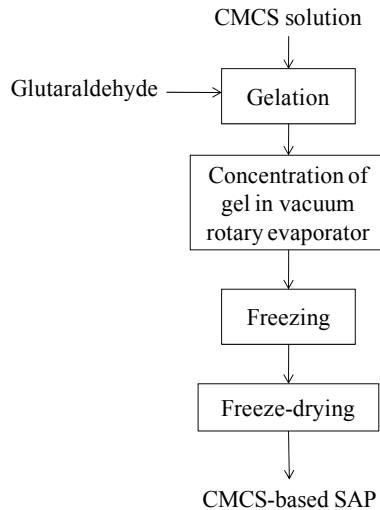


Figure 20: Production of CMCS-based SAP by partial dehydration of the CMCS gel followed by freeze-drying.

Generally, dehydration of gels at 70-100°C and freezing at -20 or -5°C resulted in reduction of WBC in water (after 10 min exposure). The 32-95% dehydrated gels frozen at -20°C displayed 25-84% of the WBC of the fresh gel (0.9%) subjected directly to identical freezing and freeze-drying steps. Performing the freezing step at -5°C resulted in lower WBC of both fresh and dehydrated gels. Freezing the CMCS gels in liquid nitrogen (-196°C) on the other hand, resulted in SAPs with totally different properties. The WBC of the fresh gel frozen at -196°C prior to freeze-drying was to some extent lower than WBC of the one frozen at -20°C. However, 32-85% dehydrated gels frozen in liquid nitrogen showed enhanced WBC compared to the different fresh freeze-dried gels (Figure 21) [Paper VI].

The highest WBC was obtained by 81% dehydration of the gel at 100°C, followed by freezing the concentrated gel (4.7%) at -196°C, and freeze-drying (Figure 21). This SAP was able to absorb 59-79 g water per g SAP after 1-100 min exposure in pure water. The WBC of this SAP in urine and saline solution was 32 and 40 g per g SAP respectively, which falls within the range of WBC of commercially acceptable SAP (Table 3) [Paper VI].

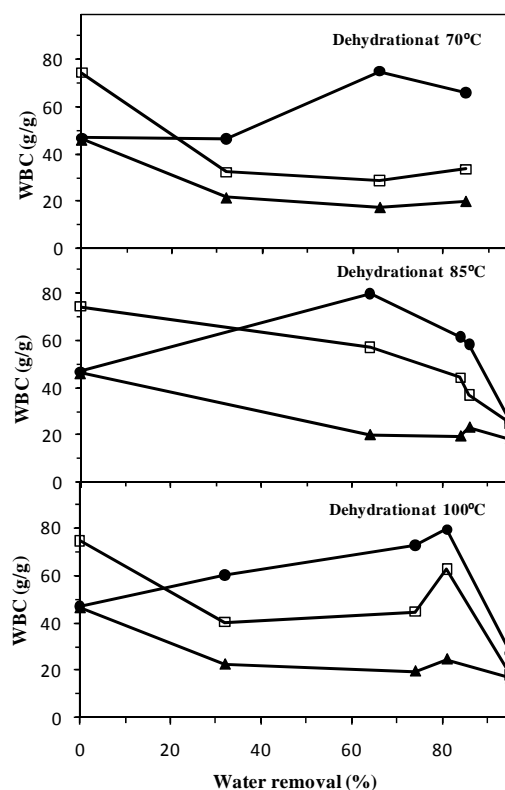


Figure 21: WBC of partially dehydrated gels, frozen at -196 (●), -20 (□), and -5 (▲) °C, and freeze-dried after 10 min exposure in pure water.

Table 3: WBC of the best CMCS-based SAPs and commercially available SAPs.

	WBC (g/g)		
	Best CMCS-based SAP	5A70	L520
Water-1 min	59	151	43
Water-10 min	79	463	213
Water-100 min	128	498	273
Saline-10 min	40	57	34
Urine-10 min	32	37	30

In summary, CMCS gel was successfully converted to SAP by applying freeze-drying, foam-drying, and a combination of partial thermal dehydration and freeze-drying. In the next

step, the effect of carboxymethylation condition on the properties of CMCS-based SAP was investigated.

3.5.2. Effect of carboxymethylation condition on the water binding capacity

Earlier in section 3.4., it was put forward that CMCS is produced by the reaction of alkaline chitosan with MCAA. The alkaline chitosan is obtained by mixing of chitosan with NaOH in the presence of a water-2-propanol solvent. It has been reported that the progress of the carboxymethylation reaction is significantly affected by the reaction conditions [105-107]. The progress of this reaction is usually described by the degree of substitution (Ds) which is the relative fraction of carboxymethylated chitosan monomers.

The objective of this step was to investigate the effects of three carboxymethylation reaction parameters, i.e. the reaction time and amounts of NaOH and MCAA, firstly on the progress of the reaction and secondly on the WBC of the SAP prepared from CMCS. CMCSs were prepared through the reaction of 1 g chitosan with 0.49-3.01 g MCAA in the presence of 0.49-3.01 g NaOH for 38-442 min at room temperature. A mixture of water-2-propanol (2:10) was used as a solvent. A response surface design was used for optimization of the level of each of the three parameters.

The results indicated that the Ds¹², as a measure of progress of the carboxymethylation reaction, was highest when chitosan (1 g) reacted with 1.75 g MCAA in the presence of 1.75 g NaOH for 4 h. These levels of reaction parameters correspond to the center point of the contour plots presented in Figure 22. As shown in Figure 16, a carboxymethylation reaction can occur on both O- and N-sites. Consequently, CMCS with Ds of 0-3 can theoretically be obtained. However, an FTIR analysis of differently prepared CMCSs revealed that, under the conditions used in this study, N-CMCS was not noticeably formed (c.f. Figure 2 of Paper VII)¹³. The free amino groups of chitosan are responsible for most of its unique characteristics, such as solubility in acid and antimicrobial properties [90]. Thus, performance of O-carboxymethylation, in which

¹² Measured by conductimetric titration

¹³ For details, please see paper VII.

amino groups remain intact, creates the possibility of benefitting from these properties of the amino groups in CMCS [Paper VII].

The differently prepared CMCSs were converted to SAPs and the WBC was measured after 10 min exposure in pure water (Figure 22). Generally, the CMCS with the highest D_s resulted in SAP with the highest WBC. Therefore, the center point of contour plots in Figure 22 represents the optimal carboxymethylation conditions to obtain SAP with highest WBC. Consequently, the optimum levels of reaction parameters (1.75 g MCAA and 1.75 g NaOH per g chitosan for 4 h), were used for carboxymethylation of chitosan prior to production of SAP from fungal chitosan [Paper VII].

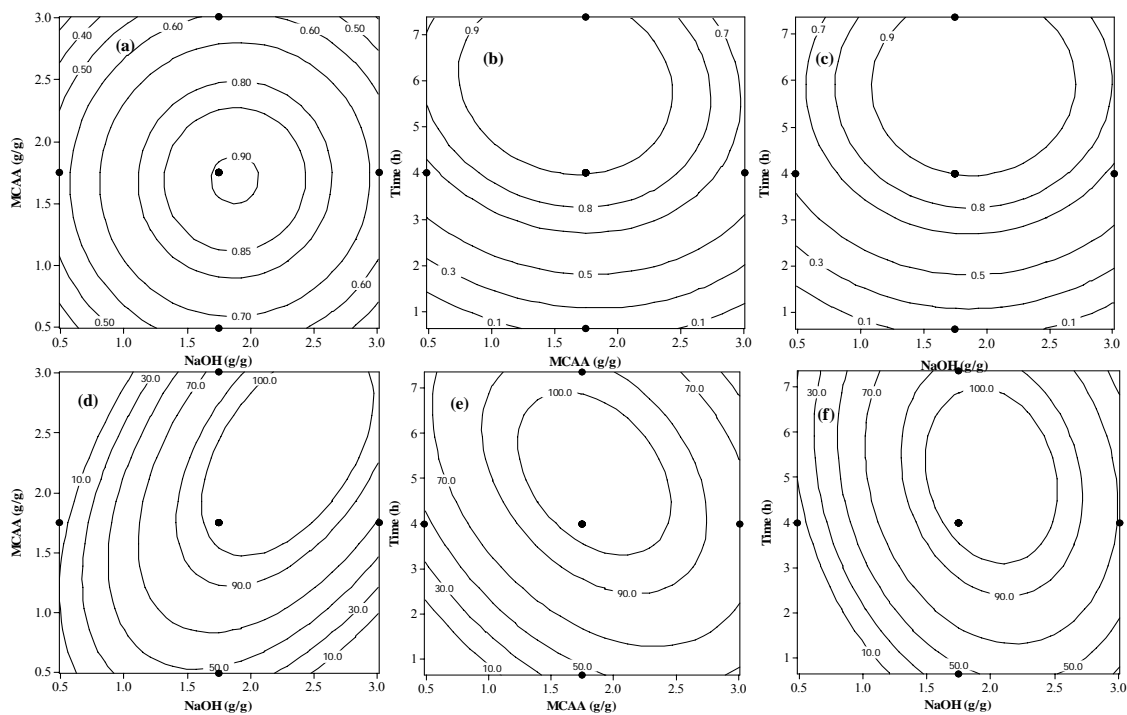


Figure 22: Effects of amount of NaOH and MCAA (g/g chitosan) and the reaction time (h) on D_s of CMCS (a-c) and the WBC of CMCS-based SAP in pure water after 10 min (d-f).

3.5.3. Production of superabsorbent polymers from the fungal chitosan

In this part of the thesis the knowledge obtained in previous parts, concerning purification of fungal chitosan and production of SAP from shellfish chitosan, was combined to produce a biological SAP from fungal chitosan.

In short, the alkali insoluble material (AIM) of biomass of *R. pusillus*, cultivated on SSL, was treated with sulfuric acid in two steps at room temperature to remove phosphate from AIM and at 120°C to dissolve the fungal chitosan. After that, the chitosan was precipitated at elevated pH (8-10) and lowered temperature (in an ice bath), washed with water and freeze-dried. The purified chitosan was subjected to carboxymethylation reaction to produce carboxymethyl fungal chitosan (CM-f-CS). This was then dissolved in water (1%) and cross-linked with glutaraldehyde (0.025 g/g CM-f-CS) to form a gel. The gel was further frozen at -20°C and freeze-dried. The WBC of the dried product was measured after 10 min exposure in distilled water and urine, saline, and blood solutions (77, 30, 33, and 45 g/g respectively) [Paper VIII].

3.5.4. Production of superabsorbent polymers from the AIM

In the last section of the thesis, the ambition was to remove the chitosan extraction step and produce SAP directly from the AIM. Fungal phosphates were released from the chitosan phosphate complex by treatment of the AIM with dilute sulfuric acid at room temperature [Paper IV]. The phosphate-free AIM residue was subsequently subjected to a carboxymethylation step to improve the hydrophilic properties of this cell wall derivative. This reaction resulted in 50% water solubility of the carboxymethylated derivative of AIM. The water soluble fraction was cross-linked by addition of glutaraldehyde to an aqueous mixture of the carboxymethyl AIM. The mixture was frozen at -20°C and freeze-dried. The WBC of the freeze-dried product in distilled water was comparable to that of SAP from CM-f-CS (73 g/g). However, it showed a lower WBC in saline, urine, and blood solutions under identical conditions (24, 22, and 37 g/g respectively). On the other hand, the yield of SAP obtained from AIM was 3-fold higher than the yield of SAP from CM-f-CS (0.9 vs. 0.3 g/g AIM). Therefore, on the whole, a higher water uptake was achieved by direct carboxymethylation of phosphate free residue of AIM [Paper VIII].

4. CONCLUSIONS

- 1- The methods described and used in the present thesis have established that chitosan/chitin (45-85%) and polyphosphate (4-20%) are major components of alkali insoluble material (AIM) of zygomycetes cell wall.
- 2- Polyphosphates may be released from the zygomycetes cell wall when treated with dilute sulfuric acid at room temperature. During this treatment, chitosan/chitin does not dissolve.
- 3- Chitosan may be extracted from the phosphate-free fungal cell wall residue by treatment with dilute sulfuric acid at 120°C. During this process, chitosan is separated from chitin, which is insoluble in the hot acid.
- 4- Fungal chitosan may be recovered from its solution in hot dilute sulfuric acid by raising the pH to 8-10 at lowered temperatures.
- 5- Superabsorbent polymers may be produced from shellfish and fungal chitosan during a process entailing carboxymethylation of chitosan, gelation of carboxymethyl chitosan in aqueous solution with glutaraldehyde, and drying the resultant gel.
- 6- The water binding capacity of CMCS-based SAPs (up to 200 g/g) is significantly affected by the carboxymethylation and gelation conditions as well as the drying method.

5. FUTURE WORK

The current study can be continued within different fields. The following subjects are suggested:

- In this thesis the downstream processing during the production of fungal chitosan, i.e., the extraction and purification of fungal chitosan, was studied. However, further experiments in the field of physicochemical characterization and biological activities of the purified chitosan are needed. Furthermore, in the work of this thesis, the purification process was performed on alkali insoluble material of fungal biomass. It would be interesting to investigate the possibility of chitosan purification directly from biomass, by using a combination of dilute sulfuric acid treatments. Such a process would practically and economically be more preferable since it would omit the alkali treatment step.
- The results of this study indicate that the composition of the fungal cell wall may be significantly affected by growing conditions. Experimental study for optimization of the fungal growth conditions (i.e., nutrients, pH, and temperature) to acquire the highest chitosan production possible in the cell wall, would be of interest.
- This work showed promising results for production of superabsorbents from carboxymethyl chitosan. These superabsorbents however, should be tested under real conditions, e.g. in combination with non-woven materials in diapers. Furthermore, the economical aspects of the process should be investigated.
- In addition to production of SAPs, fungal chitosan could be tested in other applications such as for medical purposes, i.e. wound healing and controlled drug release, where shellfish chitosans already showed promising results.

6. NOMENCLATURE

AAIM	Alkali and acid insoluble material
AcSM	Acetic acid soluble material
AIM	Alkali insoluble material
CAIP	Cold alkali precipitate
CMCS	Carboxymethyl chitosan
CM-f-CS	Carboxymethyl fungal chitosan
CRC	Centrifuge retention capacity
DD	Degree of deacetylation
Ds	Degree of substitution
GlcN	Glucosamine
GlcNAc	N-acetyl glucosamine
HAAIM	Hot alkali- and acid- insoluble material
MCAA	Monochloroacetic acid
M_v	Viscosity average molecular weight
SAP	Superabsorbent polymers
SSL	Spent sulfite liquor
WBC	Water binding capacity

7. ACKNOWLEDGMENTS

A PhD study is a long journey with serious challenges and complexities. The onset of this journey was accompanied with big changes in my life, leaving my country, being away from family, and facing a totally new world with different language, traditions, and climate. When I look back at those days, I remember some great people who kindly helped me to get a good start and who continued to give their support throughout these years. I believe that completing this thesis would never have been possible without their support. Although words will never suffice to show my deep gratitude, I here take the opportunity to express my sincere thanks to all of them.

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