Characterization and Application of Chitosan Extracted from Local Fungal Sources

By

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CERTIFICATION OF THESIS WORK

We the undersigned, glad to certify that **Mr. Md. Masirul Afroz,** candidate for the degree of Master of Science in Engineering (Chemical) has presented his thesis work on the subject **"Characterization and Application of Chitosan Extracted from Local Fungal Sources"**. The thesis is acceptable in form and content. The student demonstrated a satisfactory knowledge of the field covered by this thesis in an oral examination held on June 04, 2018.

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Candidate's Declaration

I do hereby, declare that this thesis work or any part of it has not been submitted for the purpose of any other diploma or degree.

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ABSTRACT

Chitosan is an adaptable and multifunctional biopolymer which can be applied in various fields such as food preservation, medical, agricultural, water treatment etc. The main sources of industrially produced chitosan nowadays are the crustaceans including shrimps, crabs and squids. Production of chitosan from fungal sources, however, can be advantageous because of high degree of deacetylation, homogeneity in polymer length and better antimicrobial activity over crustacean sources. In this study, two locally obtained fungal species *Aspergillus niger* and *Saccharomyces cerevisiae* were investigated as alternative sources of chitosan. *A. niger* was isolated from moldy onions and cultured in potato dextrose broth. *S. cerevisiae* was bought from local market and kept in sporulating medium. Chitosan was extracted from the mycelia of *A. niger* and the sporulated yeast cells through treatment with sodium hydroxide and acetic acid. The maximum yields of chitosan were 16.15 ± 0.95 mg/g dry biomass from *A. niger* at 12th day and 20.85 ± 0.35 mg/g dry biomass from *S. cerevisiae* at 4th day. The extracted fungal chitosan was characterized using FTIR (Fourier Transform Infrared Spectroscopy) and acid base titration method to determine degree of deacetylation (DD%). Using FTIR method, DD% of chitosan from *A. niger* and *S. cerevisiae* were found to be 61.39% and 63.41% respectively. Using acid base titration method, DD% of chitosan from *A. niger* and *S. cerevisiae* were found to be 59.61% and 53.28% respectively. Chitosan obtained from *S. cerevisiae* was further studied to test antimicrobial efficacy of fungal chitosan. Agar well diffusion method was utilized to find zone of inhibition. At a concentration of 2 g/L, fungal chitosan had shown the maximum inhibition zone diameter of 15.48 ± 0.07 mm. The degree of deacetylation and antimicrobial activity of the fungal chitosan was comparable with data obtained from literature and the results indicate that these fungal species have potential to be alternative chitosan sources.

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All the praise be to Allah, who made all the attempts and efforts fruitful.

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CHAPTER 1

INTRODUCTION

INTRODUCTION

In this chapter, background information and present state of this research work has been presented. Based on the existing challenges and research gap, purpose and scope of work has been addressed. A research hypothesis has been developed so that the experiments can fulfill the specific aims of this study. The main objectives and subobjectives of this work have been discussed. Finally, an outline of this thesis has been given.

1.1. Background of the Study

Chitosan is a partially deacetylated derivative of chitin. Chitin is the second most abundant polysaccharide after cellulose on earth and it is found naturally in the shells of shrimp, crab, in the cuticles of insects and in the cell wall of fungi. Chitosan has versatile applications. It is used in biomedical field because it has a strong antimicrobial activity and biocompatibility [1]. Chitosan is biodegradable and used in agricultural sectors as well as pharmaceutical, food and in some fields of biotechnology such as enzyme immobilization [2]. Due to adsorption capacity, it can also be used in water treatment plant as flocculent or to remove metal ions from wastewater [3].

Industrially, chitin is extracted from the shrimp or crab shells which also contain carbonates, proteins, pigments and lipids [4]. The chitin is then treated with hot and concentrated sodium hydroxide for the deacetylation of chitin into chitosan [5]. To maintain a consistent product quality, the treatments need to be adapted according to the chitin source [6], which is a challenge in the traditional chitosan production process. Studies are being carried out extensively for other sources of chitosan and increased attention is now being given to chitosan production from fungal sources [7]. Chitosan from fungal sources can be advantageous, compared to that from the

crustacean sources, because the fermentation of fungal biomass is simple and does not depend upon the time of the year or location, while the supply of crustacean shell may vary depending on the season. Fungal chitosan extraction can also be comparatively cost reducing because it does not require some of the steps such as demineralization [8].

Chitosan is a major structural component of the fungal cell walls in the class of Zygomycetes like *Aspergillus*, *Rhizopus*, *Absidia*, *Gongronella* [9]. The amount of chitosan present in the cell wall of mycelial fungi varies widely depending on the species and strains of fungi [8]. Chitosan is also found in sporulated *Saccharomyces cerevisiae* also known as baker's yeast. Chitosan does not exist in the vegetative cell wall, but it is a component of the spore cell wall in the budding yeast when it undergoes meiosis cell division [12]. Sporulation of yeast takes place in a nutrition starvation condition [13]. Yeast spores contain four layers in their spore walls. The outermost layer is a dityrosine layer and the second one is a chitosan layer followed by β-glucan and mannoprotein layers [14]. The morphology of the yeast spore that contains chitosan is like a bead and has a spherical diameter of around 3 μm [15].

Chitosan can also be a valuable industrial byproduct. For example, citric acid is produced industrially by submerged culture of *Aspergillus niger*. Citric acid industries around the world produce around 80,000 tons of *Aspergillus niger* as waste mycelia [10]. This waste can be used to extract chitosan instead of burning the mycelia [11].

1.2. Purpose and Scope of the Study

The present study would have an impact on current understandings of using fungi as a chitosan source in terms of extraction from locally obtained and cultured biomass and important properties of the extracted chitosan. Although previous studies have shown that some fungal

species have potentiality to produce chitosan, availability of studies on important properties such as degree of deacetylation and antimicrobial activity of chitosan from fungal species are limited. To evaluate fungi as alternative sources of good quality chitosan, proper knowledge of these important physicochemical and functional properties along with optimized culture conditions for biomass growth is necessary. The present study is an attempt to address this research gap.

1.3. Research Hypothesis

Two fungal species can be selected as representatives of mycelial and unicellular fungal types respectively. For this study, *Aspergillus niger* and *Saccharomyces cerevisiae* are good options. The former one might be chosen because it is one of the most abundant mycelial fungal species and easy to isolate from the environment. Use of *Aspergillus niger* would be advantageous in several ways. Firstly, it has a unique morphology that can be identified by visual inspection. Secondly, if the fungal culture gets contaminated, it would be clearly observed and taken care of. On the other hand, *Saccharomyces cerevisiae* is readily available in the local market as baker's yeast. That no published data has been found that reported production of chitosan using it, is another reason to choose this fungus.

The experimental work can be divided into four parts. The first one includes isolation and culture of *A. niger* in nutrient medium. This part also includes study of *S. cerevisiae* in sporulating medium to produce fungal biomass for the sole purpose of chitosan extraction. In the second part, chitosan extraction is carried out from the fungal biomass to determine yields from each species. Fungal chitosan is characterized to determine degree of deacetylation (DD%) in the third part that would represents its quality. The fourth part contains study of antimicrobial activity of fungal chitosan using zone of inhibition method.

1.4. Objectives of the Study

The main objective of this study is to evaluate the potential of locally obtained fungal species as alternative sources of good quality chitosan. Characterizing the extracted fungal chitosan and determination of antimicrobial activity are the subobjectives of this work. To fulfill the abovementioned objectives, followings can be done.

1. Isolation of *Aspergillus niger* from black molded onion and repeated culture to obtain pure strain
2. Biomass growth of *A. niger* using laboratory made Potato Dextrose Broth (PDB) culture media
3. Biomass preparation of *Saccharomyces cerevisiae* using sodium acetate solution as sporulation media
4. Extraction of chitosan from fungal biomass using hot alkali and acid treatment
5. Characterization of the extracted chitosan to determine degree of deacetylation (DD%) using Fourier Transform Infrared Spectroscopy (FTIR) and acid base titration
6. Evaluation of antimicrobial property of fungal chitosan against *Staphylococcus aureus* isolated from human nasal specimen using zone of inhibition method

1.5. Thesis Organization

Chapter 1 contains background, scope of work, research hypothesis and objectives of this study.

Chapter 2 includes reviews of previous works on fungal chitosan. Chitosan definition, structural properties, major sources, extraction methods, characterization techniques and applications have also been discussed thoroughly.

Chapter 3 presents the detailed experimental methodology used in this study. Isolation technique and culture of fungal species has been discussed. Besides, chitosan extraction and characterization methods have been described. Isolation of *Staphylococcus aureus* and the procedure to test its susceptibility towards fungal chitosan have been presented.

Chapter 4 shows the results obtained from this study. Biomass growth and chitosan yields obtained from the fungal species have been presented. FTIR spectra and titration results of the extracted fungal chitosan and the obtained degree of deacetylation have been shown. Images of zone of inhibition and zone diameters found using fungal chitosan is also presented in this chapter.

Chapter 5 includes detailed discussion of results obtained in this work. Explanations of the graphical trends along with limitations and conflicting results have been discussed. The obtained results have also been compared with literature.

Chapter 6 presents the major findings of this research work. It also includes recommendations and scope of future work.

CHAPTER 2

LITERATURE REVIEW

LITERATURE REVIEW

2.1. Chitosan

Chitosan is one of the most important and valuable biopolymers found in biomass resources. It is a partially deacetylated form of chitin. Chitin is the second most abundant organic compounds on earth after cellulose. Chitin has a wide range of availability among different organisms. Main sources of chitin are the shells of a variety of marine organisms such as shrimps, crabs, squids and other shellfishes [16]. The crustacean shells may commonly contain 20-30 % chitin [19]. It is also found in the shells of insects and molluscan organisms. Chitin and chitosan are also observed as one of the cell wall constituents of several fungi, molds and yeasts [17]. However, chitin does not exist in higher plants and vertebrates [18]. In higher plants, cellulose is synthesized instead. Table 2.1 shows common sources of chitin.

Table 2.1: Chitin content found in different organisms [21]

Type of Organisms	**Chitin Content (%)**	**Type of Organisms**	**Chitin Content (%)**
Crustaceans		**Insects**	
Callinectes (blue crab)	14.0[a]	Blatella (cockroach)	18.4[c]
Carcinus (crab)	64.2[b]	Colcoptera (beetle)	27-35[c]
Paralithodes (king crab)	35.0[b]	Diptera (truefly)	54.8[c]
Cancer (crab)	72.1[c]	Pieris (sulfur butterfly)	64.0[c]
Crangon (shrimp)	69.1[c]	Bombyx (Silk worm)	44.2[c]
Nephrops (lobster)	69.8[c]	Calleria (Wax worm)	33.7[c]
Homarus (lobster)	60-75[c]	Periplaneta (cockroach)	2.0[d]
Lepas (barnacles)	58.3[c]		
Alasakan shrimp	28.0[d]		

Type of Organisms	Chitin Content (%)	Type of Organisms	Chitin Content (%)
Fungi		**Molluscan Organs**	
Aspergillus niger	42.0[e]	Clamshell	6.1
Penicillium notatum	18.5[e]	Oyster shell	3.6
Penicillium chrysogenum	20.1[e]	Squid, Skeletal pen	41.0
Saccharomyces cerevisiae	2.9[e]	Krill, deproteinized shell	40.2
Mucor rouxii	44.5		
Lactarius vellereus (mushroom)	19.0		
a = wet body weight, b = dry body weight, c = organic weight of cuticle d = total dry weight of cuticles, e = dry weight of cell wall			

Molecular structure of chitin, chitosan and cellulose has significant similarities. Figure 2.1 shows the structures of these three naturally occurring polymers.

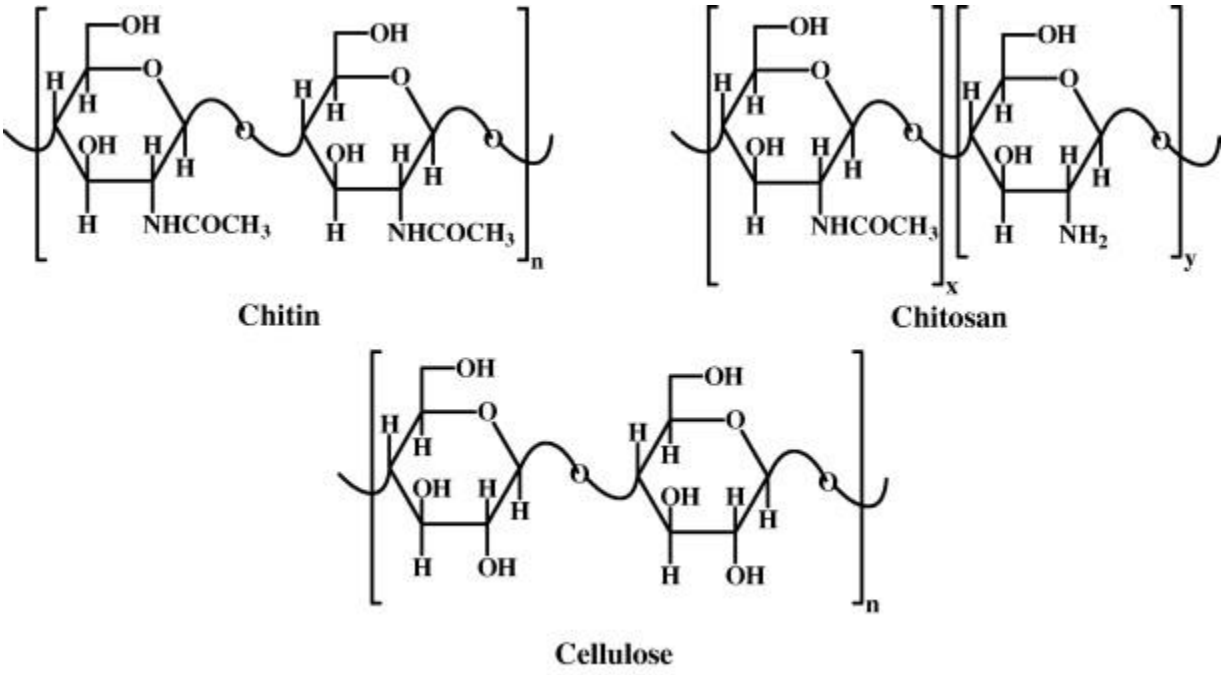


Figure 2.1: Chemical Structures of chitin, chitosan and cellulose [20]

Chitin consists of polymeric chain of *N*-acetyl glucosamine units, which are connected to each other by oxygen linking C1 of one unit to C4 of an adjacent unit. Chitosan is formed when the acetyl (CH_3CO-) groups are removed from chitin. The process of removing acetyl groups from chitin is called deacetylation. A fully deacetylated chitosan contains only glucosamine units in their chemical structure. However, in real life, chitosan contains both glucosamine and *N*-acetyl glucosamine units at different ratios.

On the other hand, chitosan and cellulose structures shown in Figure 2.1 implies that the main difference between these two is the presence of amine ($-NH_2$) groups. At C2 position, hydroxyl (-OH) groups of cellulose are replaced by amine groups in chitosan. Chitosan consists of carbon (44.1%), hydrogen (6.84%), and nitrogen (7.97%) [75]. Since it has a high percentage of nitrogen compared to synthetically substituted cellulose, they have gained great commercial interests [76].

2.1.1. History of Chitosan

In 1811, chitin was first discovered by Henri Braconnot, who was a French professor of natural history. He identified and isolated it from mushrooms and the substance was named fungine by him [26]. However, the name chitin came later at 1830 when it was isolated for the first time from insects [27]. After the discovery of chitin, Rouget discussed a deacetylated form of chitosan in 1859 [28]. After boiling chitin in a solution of concentrated potassium hydroxide, he discovered a fraction of chitin that is soluble in acid. This report is considered as the first one to discuss about chitosan. The chronological history of chitosan is depicted in Table 2.2.

Table 2.2: History of chitosan [30]

Year	Important Figures	Description
1811	Henri Braconnot, Director of the Botanical Garden in Nancy, France Professor of Natural History	Conducted research on mushrooms and extracted chitin Hypothesis: chitin did not dissolve in sulfuric acid
1823	Ojer	Named “chitin”, based on Greek word “khiton” meaning “envelope”
1832	Opperman	Chitin was extracted from insects; Similar substances as chitin can also be found in the structure of insects
1843	Lassaigne	Demonstrated the presence of nitrogen in chitin
1859	Rougeut	Discovered chitosan Observed that the substance in chitin could be manipulated through chemical and temperature treatments for it to become soluble Treated chitin with hydro potassium concentrated at higher temperature
1878	Ladderhose	Identified chitin as made of glucosamine and acetic acid
1894	Hopper-Seyler (German scientist and physiologist)	Proposed the name of chitosan
1930	Rammelburg	Identified more chitin sources from insects and fungi Chitosan can be extracted from marine arthropods e.g., crab, shrimp, lobster Detected that chitin is a polysaccharide of glucosamine
1950	Darmon and Rudall	Structure of chitosan discovered X-ray analysis advanced the study on the discoveries of chitin and chitosan X-ray, the most advanced technology at that period, recorded the existence of the chitin and cellulose in the cell wall The absorption spectra of chitin, chitosan nitrate and wood cellulose have

Year	Important Figures	Description
		been recorded in the region 3600-750 cm^{-1} using polarized radiation-
1951	First book was published 140 years after the initial observation of Braconnot, which was then confirmations were done by many researchers on the discovery of chitosan biomaterials	
1960 Till Present	Many researchers have conducted research using modified and unmodified chitosan derivatives in the biomedical field	

2.1.2. Chitosan Characteristics

Chitosan is an example of highly basic polymers, whereas most other naturally occurring polymers such as cellulose, dextran, pectin, agar, agarose and carragenas are neutral or acidic ones [31]. Properties of chitosan can be described in two groups: physicochemical and functional properties.

One of the most important physicochemical characteristics of chitosan is degree of deacetylation and molecular weight. Other characteristics such as crystallinity, inorganic matter content, water content and purity among other properties can be relevant depending upon application of chitosan [33].

Chitosan has distinctive functional and chemical characteristics assembled in it. The hydrophobic interaction and hydrogen bonds between *N*-acetyl glucosamine units in the molecule provides it with reinforced and rigid structural property [33]. Chitosan has a linear structural formation at acidic pH. Both the amino and hydroxyl groups in chitosan can be chemically modified which eventually enhance the functional properties of chitosan.

In acidic solutions, protonation occurs at the amine groups of chitosan and exhibits a high positive charge of $-NH_3^+$ to the chitosan chains. This charge allows chitosan to bind with negatively charged proteins, lipids, metal ions, cholesterols and macromolecules [5.7]. Chelating property of chitosan towards metal ion is due to NH group activity and both sorption and flocculation can take place simultaneously [32]. Figure 2.2 shows the positive charged $-NH_3^+$ in chitosan molecules.

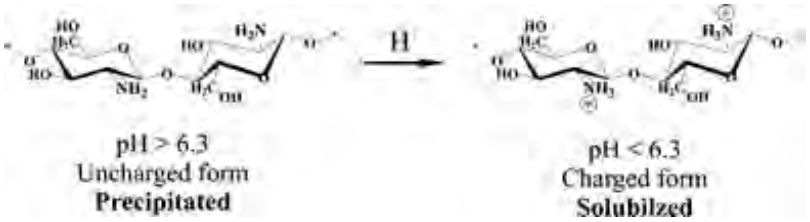


Figure 2.2: Positive charge in chitosan at lower pH [23]

Chitosan is also non-toxic and biodegradable. Several enzymes that are naturally found in a wide range of sources, starting from prokaryotes to higher animals such as chitinases and chitobiases, can degrade chitosan [34].

Another important functional property of chitosan is its biological activities. Chitosan has shown antimicrobial activity against several bacteria, fungi, and viral infections [35]. Although bioactivity has been found on both gram positive and gram negative bacteria, apparently gram positive ones are more susceptible to chitosan [34]. Functional properties of chitosan on that can be used in different fields are shown in Table 2.3.

Table 2.3: Functional properties of chitosan [34]

Field	**Chitosan Functional Features**
Agricultural	Plant stimulant, Antimicrobial, Structural capacity, Biocompatibility
Food	Antimicrobial, Structural capacity, Biocompatibility, Adsorption
Environmental Engineering	Adsorption, Structural capacity, Reactivity

2.1.3. Chitosan Biosynthesis

2.1.3.1. Chitin Biosynthesis in Crustaceans

A possible pathway for the biosynthesis of chitin is shown in Figure 2.3. Trehalose is a nonreducing sugar which is the most common disaccharide found in insects. A hydrolyzing enzyme called trehalase is responsible for hydrolysis of this sugar which and consequently UDP-GlcNAc (uridine diphospho-N-acetylglucosamine) are produced. UDP-GlcNAc works as a precursor that can synthesize oligosaccharides. These oligosaccharides are soluble and linked to polyprenol lipids. They get attached to specific residues on a receptor protein after being transported outside the cell. A primer is produced as a result (attached to receptor protein) and gets extended by addition of GlcNAc residues sequentially in presence of chitin synthetase [21].

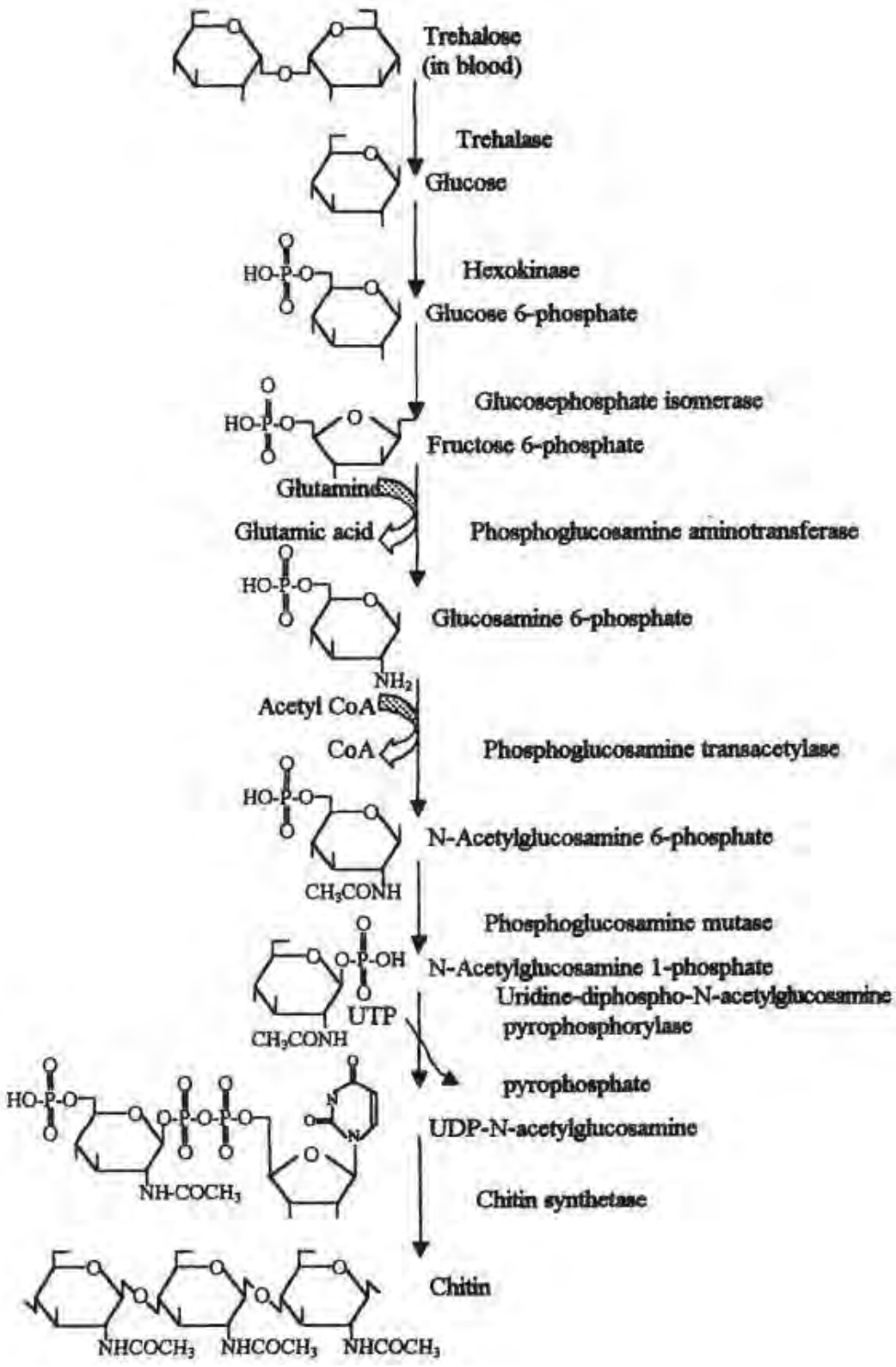


Figure 2.3: Chitosan biosynthesis in crustaceans [21]

2.1.3.2. Chitosan Biosynthesis in Fungi

In fungi, glucose is first converted into *N*-acetylglucosamine-1-phosphate. A reactions series is involved in this process [36]. "The *N*-acetylglucosamine-1-phosphate then reacts with UTP (the nucleotide uridine triphosphate) to form UDP-*N*-acetylglucosamine, which is the nucleotide diphosphate sugar. Finally, this sugar transfers the *N*-acetylglucosamine moiety to the growing chitin chain, functioning as a primer, and becomes one of its subunits. The enzyme chitin synthetase and Mg^{2+} ions are required for this polymerization step. The completed chitin molecule is a long chain of sugar subunits that are joined by β-1,4 links" [37]. Chitin deacetylase then converts chitin into chitosan by hydrolyzing acetic groups form *N*-acetylglucosamine. A possible pathway for chitosan biosynthesis is shown in Figure 2.4.

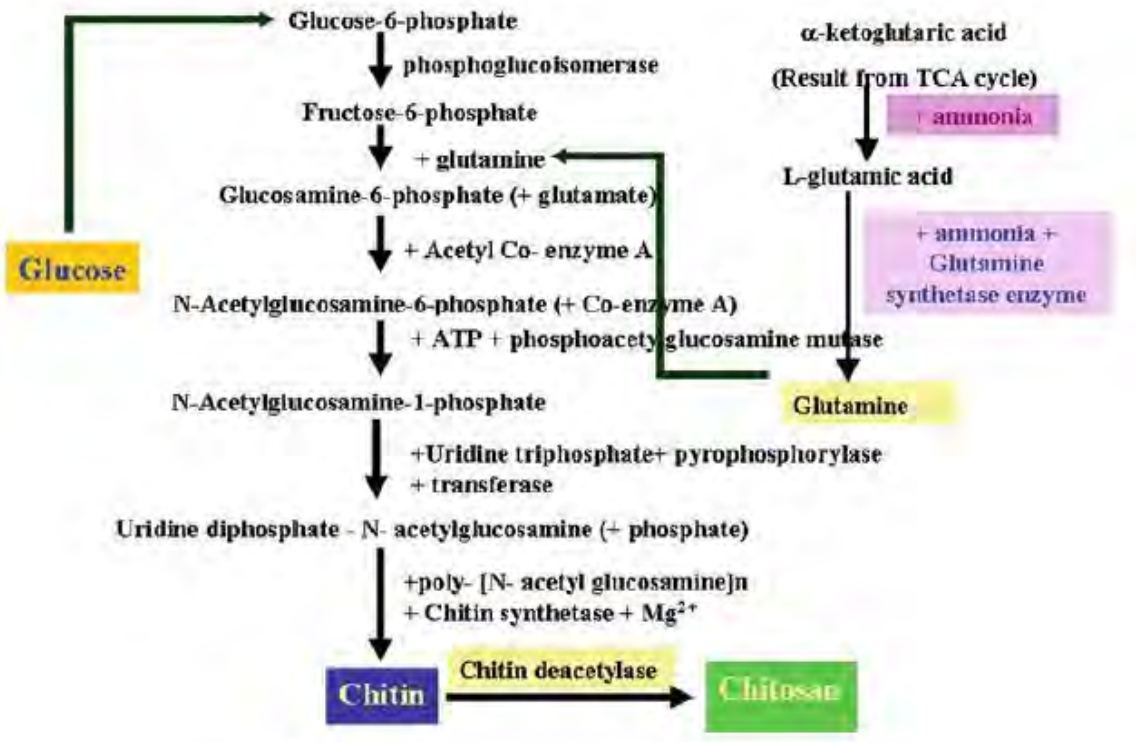


Figure 2.4: Pathway of chitosan biosynthesis in fungi [36]

Content of *N*-acetylglucosamine which can also refer to degree of deacetylation is dependent upon chitin deacetylase activity in the fungal cell wall. Properties of the synthesized chitosan thus depend on the activity of this enzyme [37].

2.1.4. Life Cycle of Mycelial Fungi

Life cycle of fungi can be discussed by starting with spores. In appropriate conditions, the spores germinate to produce filamentous hyphae. Vertical and horizontal extension of these hyphae produce mycelium. Mature hyphae can then create sporangiophore. On top of the sporangiophore, a sporangium is found where sporangiospores are formed. Both sexual and asexual reproduction is common for fungal species. Fungi reproduce asexually by spores which can withstand harsh situations. Types of asexual spores of fungi include conidia and sporangiospores [36].

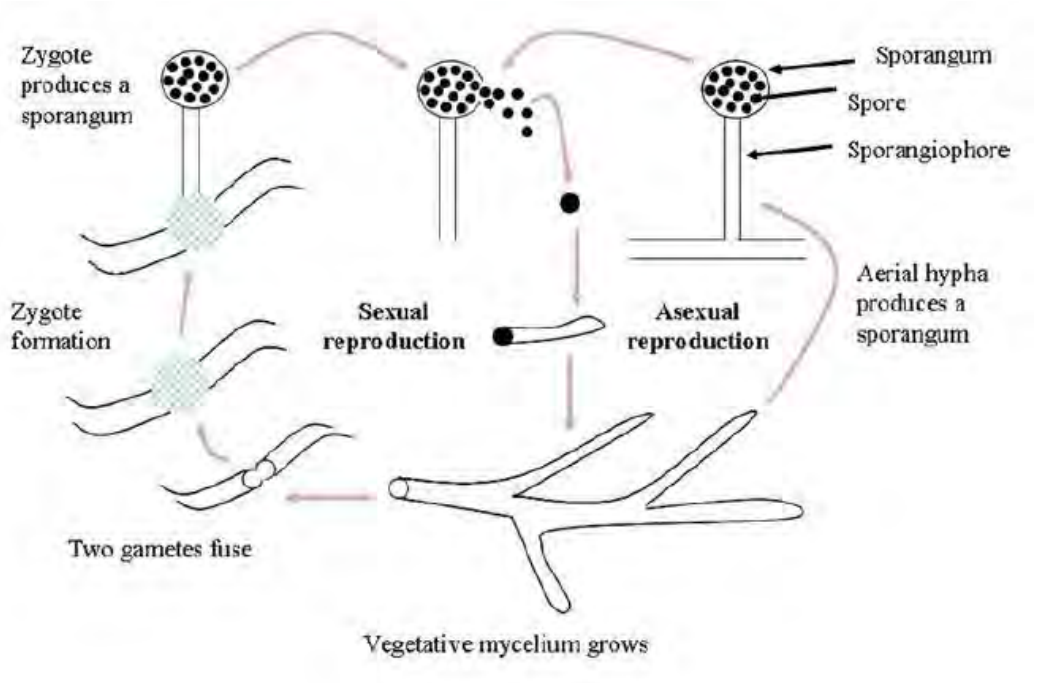
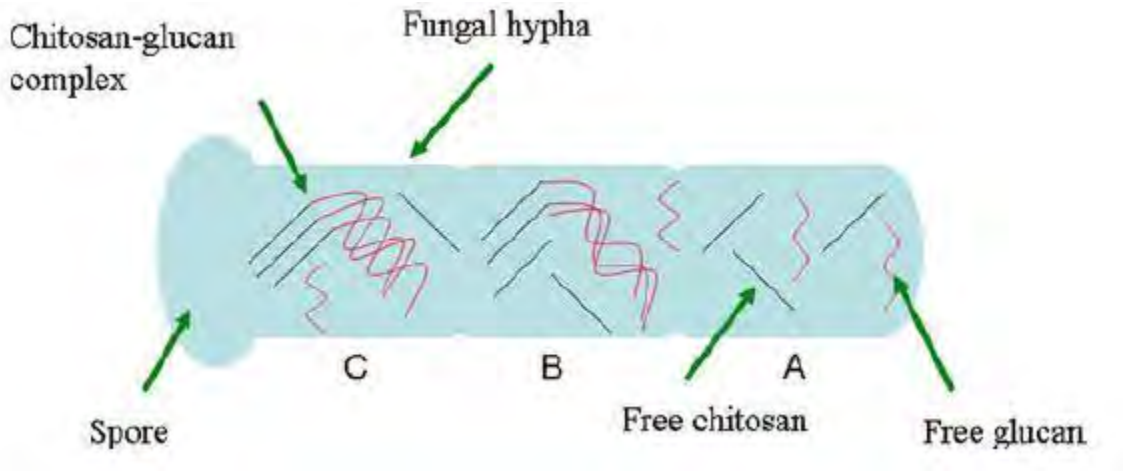


Figure 2.5: Stages of life cycle in mycelial fungi [36]

Possible chitosan formation at different stages of fungal growth is shown in Figure 2.6.



A = free chitosan and free glucan formation in hyphae at early stages of growth, B and C = link formation between chitosan and glucan chains at later stages of hyphae growth

Figure 2.6: Possible chitosan synthesis at different stages of fungal growth [36]

Matured cell wall of mycelial fungi contains chitosan as one of the structural components. A mature cell wall is shown in Figure 2.7.

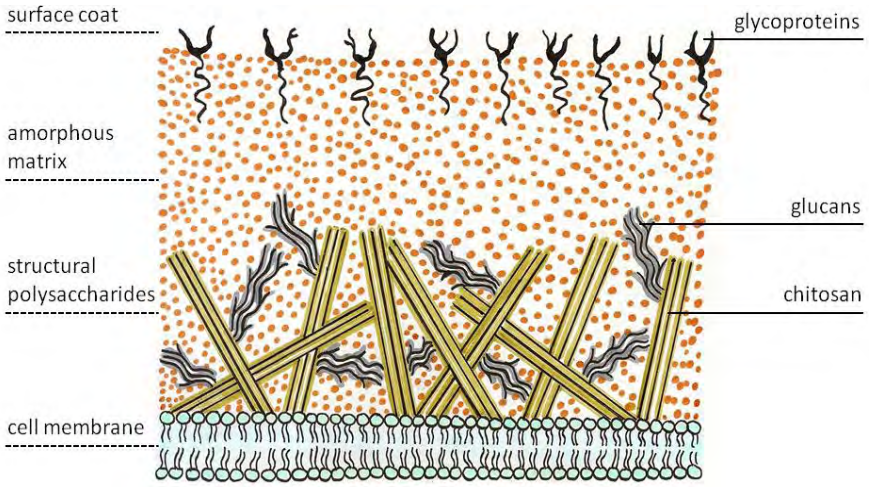


Figure 2.7: Cell wall structure of a matured fungal mycelia

(url: https://commons.wikimedia.org/wiki/File:Cell_wall_structure_of_Fungi.png)

2.1.5. Life Cycle of *Saccharomyces cerevisiae*

Two common life cycles are found in *S. cerevisiae*: a) an asexual one and b) a sexual one with an alternation of haploid/diploid phases by meiosis and conjugation. Yeast can grow in both forms dividing by mitosis: either as haploid or diploid. If the diploid yeast cells are kept into media that lacks nitrogen and carbon sources, meiosis is induced which is also known as sporulation [38].

The life cycle of yeast is given in Figure 2.8.

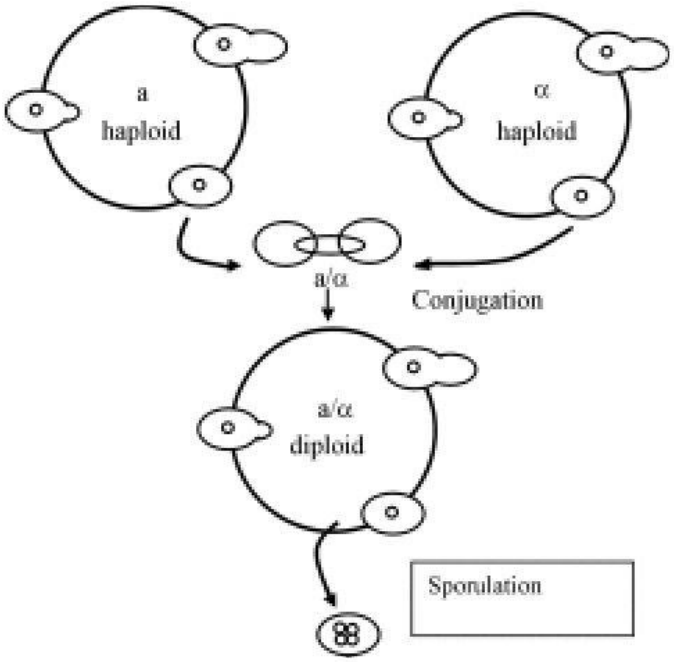


Figure 2.8: Life cycle of *S. cerevisiae* [38]

Sporulated cell wall of yeast contains chitosan whereas vegetative cell wall does not [12]. A comparison of yeast spore and vegetative cell wall is depicted in Figure 2.9.

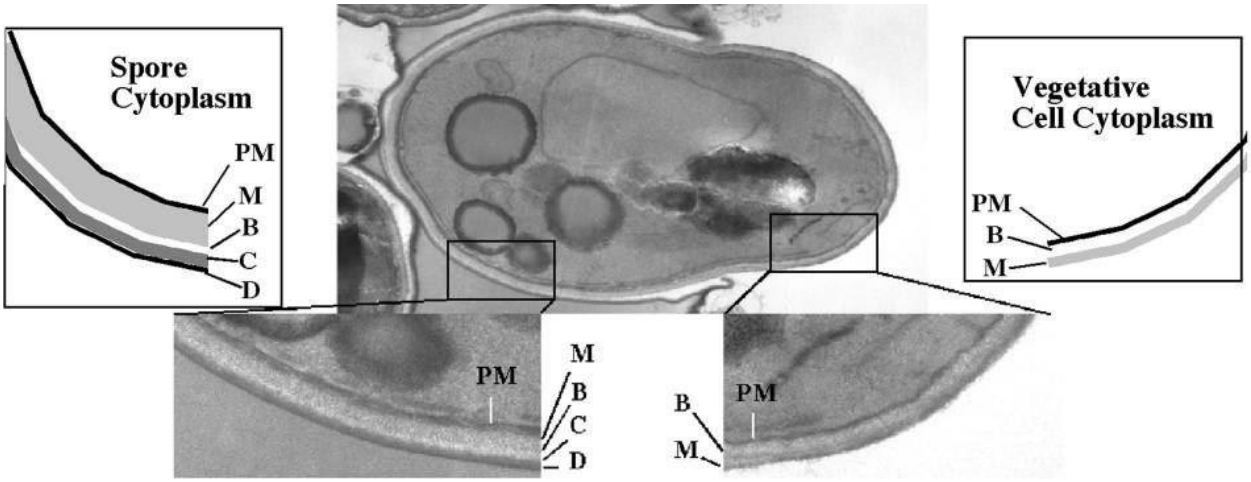


Figure 2.9: Cell wall composition of a yeast spore [39]

In this figure, a germinating ascospore is shown. Left side of the yeast cell is covered by spore wall. The wall consists of four layers: mannan (M), beta-glucan (B), chitosan (C), and dityrosine (D). On the other hand, the portion of the germinating cell that is bounded by a vegetative cell wall has only a beta-glucan (B) and mannan layers (M). The same mannan layer appears to be continuous throughout the cell wall. PM represents plasma membrane here [39].

2.1.5.1. Sporulation Pathway of *S. cerevisiae*

S. cerevisiae sporulates in three major phases. On a basis of lack of nitrogen source, carbon source and mating type, yeast cells decide to create spores [57]. This is the beginning of the first phase.

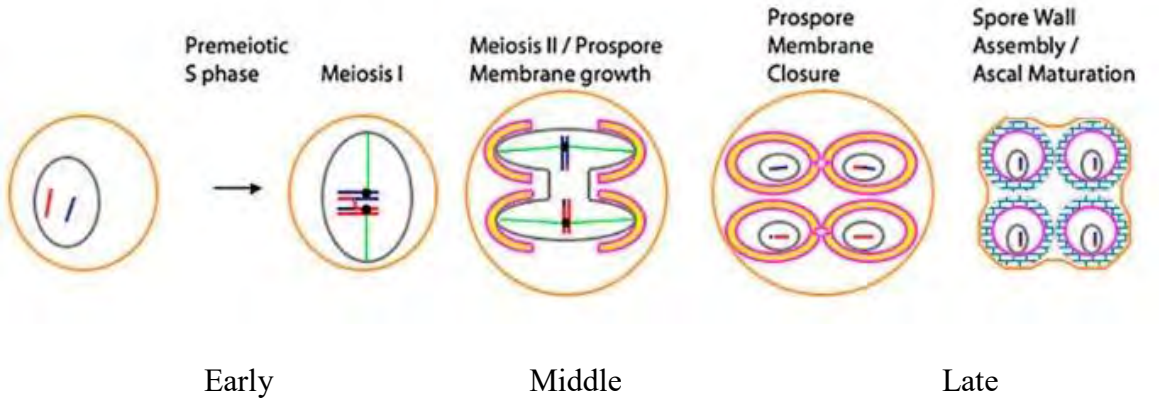


Figure 2.10: *S. cerevisiae* sporulation stages [39]

Actual sporulation takes place at the middle phase. In this phase, the yeast cell divides its nucleus into four haploid nuclei following the meiotic division path and then they get packed into daughter cells (Figure 2.10). A host of changes is required by the package in cytoplasm. At the beginning, the spindle pole bodies (SPBs) are modified in such a way that they work as the formation sites for new membrane compartments. These new membranes are called prospore membranes [58]. Formation of prospore also requires changes in the late stages of the secretory pathway. Therefore, post-Golgi secretory vesicles get redirected from the plasma membrane to the prospore membranes which eventually generate and expand these membrane compartments [58]. As they grow, the prospore membranes try to cover up the forming haploid nucleus adjacent to it (Figure 2.10). After karyokinesis gives rise to the daughter nuclei, each prospore membrane surrounds each nucleus. Cytokinesis separates that nucleus from the cytoplasm of mother cell and ascus is formed (Figure 2.10).

At the late phase, the closure of the prospore membrane takes place. A spore wall gets assembled around each spore begins shortly after the membrane gets closed [59] (Figure 2.10). Moreover, compaction of the chromatin in the spore nucleus as well as regeneration of certain organelles

occurs after closure [60]. When the spore wall becomes fully assembled, the mother cell collapses and tetrahedral mature ascus is formed.

2.1.5.2. Sporulation Conditions for *S. cerevisiae*

Two culture steps are applied to sporulate yeast cells in vitro; culture of the vegetative cells using a presporulation medium (PSM) and then transfer the cells into a sporulation medium (SM) to initiate sporulation [53]. The PSM is a nutrient-rich medium and the SM is a starvation medium that contains a nonfermentable carbon source such as acetate and lacks nitrogen and fermentable carbon sources [54]. The culture of yeast cells in a presporulation medium requires 2 days of cultivation. The SM media is usually 1% potassium acetate [55]. Sodium acetate can also be used as sporulation media [56].

2.1.6. Applications of Chitosan

Chitosan has a wide range of characteristic features such as biocompatibility, biodegradability, antimicrobial activity, chelating property, non toxicity and reactivity [33]. These features allow chitosan to be adopted in versatile area of applications. Chitosan has applications in biomedical sectors, food industry, agriculture, pharmaceuticals, water purification, and many more. Table 2.4 summarizes some applications of chitosan related to the different sectors.

Table 2.4: Applications of chitosan in various sectors [2.14, 2.26]

Area of Application	**Examples**
Antimicrobial Agent	Bactericidal Fungicidal Measure of mold contamination in agricultural commodities

Area of Application	**Examples**
Edible Films	Controlled moisture transfer between food and surrounding environment Controlled release of antioxidants Controlled release of nutrients, flavors, and drugs Reduction of oxygen partial pressure Controlled rate of respiration Temperature control Controlled enzymatic browning in fruits Reverse osmosis membranes
Additives	Clarification and deacidification in fruits and beverages Natural flavor extender Texture controlling agent Emulsifying agent Food mimetic Thickening and stabilizing agent Color stabilization
Nutritional Quality	Dietary fiber Hypocholesterolemic effect Livestock and fish feed additive Reduction of lipid absorption Production of single cell protein Antigastric agent Infant feed ingredient
Recovery of Solid Materials	Affinity flocculation
Food Processing Wastes	Fractionation of agar

Area of Application	**Examples**
Purification of Water	Recovery of metal ions, pepsticides, phenols, and PCBs Flocculation/Coagulation of proteins, dyes, amino acids Filtration
Pulp and Paper	Surface treatment Photographic paper Carbonless copy paper
Medical	Bandages, Sponges Artificial blood vessels Blood cholesterol control Tumour inhibition Membranes Dental/Plaque inhibition Skin burns/artificial skin Eye humour fluid Contact lens Controlled release of drugs Bone disease treatment
Cosmetics	Make-up powder Nail polish Moisturizers Fixtures Bath lotion Face, hand and body creams Toothpaste Foam enhancing

Area of Application	Examples
Biotechnology	Enzyme immobilization Protein separation Chromatography Cell recovery Cell immobilization Glucose electrode
Agriculture	Seed coating Leaf coating Hydroponic/Fertilizers Controlled agrochemical release Increased crop production Animal feed
Membranes	Reverse osmosis Permeability Solvent separation

2.2. Production of Chitosan

Chitosan can be produced from crustacean shells and from fungal sources. Chitosan extraction from crustaceans involves chemical deacetylation of chitin. This process is a more traditional way of producing chitosan. On the other hand, fungal biomass can be either fermented in bioreactors or collected from natural or industrial sources to extract chitosan from the cell wall materials.

2.2.1. Chitosan Production from Crustaceans

Figure 2.11 shows a typical way of chemical synthesis of chitosan from crustacean shells. Proteins are first removed from the raw shells of the crustaceans using sodium hydroxide. This process is called deproteination. Acetone is used to remove color from the shells. Demineralization is done afterwards using hydrochloric acid to obtain chitin. Deacetylation reaction involves treatment of chitin with concentrated sodium hydroxide to produce chitosan.

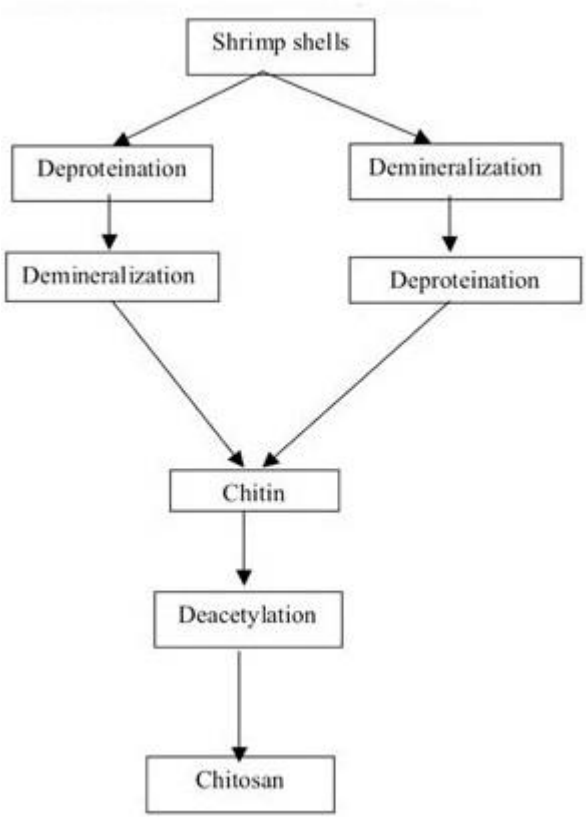


Figure 2.11: Chemical synthesis of chitosan from crustaceans [73]

2.2.2. Chitosan Production from Fungal Sources

Fungal sources for extraction of chitosan are gaining more attention these days. Chitosan from fungi can be advantageous, compared to that from the crustacean sources, because the fermentation of fungal biomass is simple, and it does not depend upon the time of the year or location. On the other hand, raw material supply of crustacean shell may vary depending on the season. Chitosan extracted from waste shells of shrimp would also depend upon the food habits of people in a certain area. Production consistency in terms of quality and quantity, fungal can be a great advantage of fungal chitosan over the traditional way. Fungal chitosan extraction can also be comparatively cost reducing because it does not require some of the steps such as demineralization [9].

2.2.2.1. Chitosan Producing Fungi

The yields of chitosan from several fungi (*Absidia, Aspergillus, Cunninghame, Gongronella, Mucor, Penicillium, Phycomyces, Rhizopus* and *Zygorhyncus*) have been evaluated in a lot of studies [36]. *Gongronella butleri* and *Cunninghamella echinulata* produced the highest amount of chitosan compared to total extractable chitosan from a number of *Zygomycetes* fungi species [44]. Chitosan was also extractable from *Ascomycetes* strains (e.g. *A. gossypii* and *G. fujikuroi* var.), *Deuteromycetes* strains (e.g. *Aspergillus* spp.) and *Basidiomycetes* strains (e.g. *A. bisporus*) [49]. Chitosan was also extractable from waste biomass collected from citric acid industry [45]. *Rhizomucor miehei* and *Mucor racemosus* produced high quality chitosan in relatively simple steps compared to shrimp shells [46]. Different strains of *Aspergillus niger* had shown good growth and chitosan yield [47].

2.2.2.2. Culture Media

One of the prerequisites to study microorganisms is to prepare culture media that is suitable for them. They have a wide range of growth requirements as they live in different environmental conditions. To grow fungi, significant amount of carbon and nitrogen source must be present. The pH range of 5 to 6, and a temperature range from 15 to 37˚C is good for fungal culture [48].

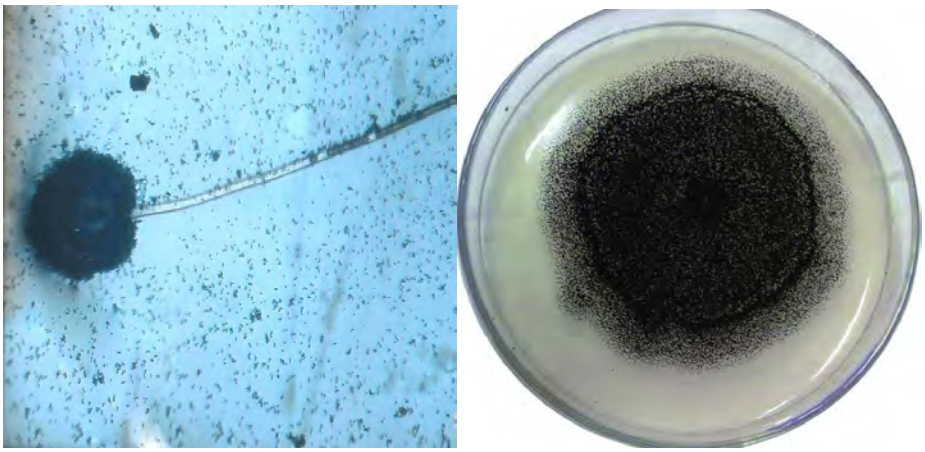
Four strains of *Aspergillus niger* were grown in media containing Potato Dextrose Broth and D-glucose as carbon source, different concentrations of L-Asparagine and Thiamine as nitrogen sources. Nitrogen sources are also used as ammonium salts. For example, PGY salt broth was used to culture four groups of fungi (Zygomycetes, Ascomycetes and Deuteromycetes and Basidiomycetes) consisted of 20 g glucose, 10 g peptone, 1 g yeast extract, 5 g ammonium sulfate, 1 g di-potassium hydrogen orthophosphate, 1 g sodium chloride, 5 g magnesium sulphate-7-hydrate, 0.1 g calcium chloride-2-hydrate and 1 L of distilled water. The pH of PGY salt broth was adjusted to 4.5 using hydrochloric acid [49].

Sabouraud Dextrose Broth having different concentrations of glucose was used to grow *Aspergillus niger* [50]. Besides, corn steep liquor for *Absidia corymbifera* [74], potato dextrose broth [76] and soybean residue [75] to grow *A. niger*, sugar cane juice and molasses to culture *Cunninghamella bertholletiae* [77] etc. has been reported.

2.2.2.3. Aspergillus niger Identification

Aspergillus niger is a cosmopolitan fungus and the most abundant species of the genus *Aspergillus*. It is found in a large variety of substances in the environment. Sources of *Aspergillus niger* include apples, onions, peanuts, grapes, plants, dust, soil, damp walls of houses

etc. The surface colony color of this fungal species is initially white and turns deep brown to black with the growth of conidia while the bottom part is pale yellow or colorless. This species is easily identifiable by visual inspection.



(a) (b)

Figure 2.12: (a) *A. niger* under microscope [51], (b) *A. niger* on petri dish [52]

2.2.2.4. Fungal Culture Techniques

Surface Culture

The liquid surface culture (LSC) was the first large scale process for microbial citric acid production. The technique was introduced in 1919 by Société des Produits Organiques in Belgium, and in 1923 by Chas Pfizer & Co. in US [41]. Submerged fermentation was developed afterwards. Even though submerged culture is more sophisticated, surface culture method requires less effort in operation and installation and energy cost [42]. Although citric acid production plant utilizes this method to culture *Aspergillus niger*, no study has yet been found that cultured fungi using this method to extract chitosan.

Submerged Culture

In submerged fermentation (SMF), fungi are grown inside the liquid. Continuous agitation or aeration is required in this process. SMF has three main approaches: batch, fed-batch, and continuous cultivation [36]. There are some problems associated with SMF. In every cultivation approach, mycelia grow on baffles, impellers, fermenter lid, and fermenter wall. Varying the inoculum size, changing the agitation speed, varying aeration, making the fermenter with hydrophobic materials, and changing the pH and medium composition, this problem can be solved [36].

Solid State Culture

Solid state culture generally utilizes solid substrates on which fungal species can grow. As the substrates are consumed slowly, they can be used for a long period of time. It involves solid, liquid and gaseous phases for fungal growth. Agitation is not required here. There are some limiting factors in this technique. It has limitations in unique sterilized conditions for fungal growth, limited accessibility and availability of substrate, unique conditions for mass transfer of oxygen, and the difficult regulation of physical factors such as mixing, pH, temperature, and moisture content [43].

2.2.3. Extraction of Chitosan

Chitosan extraction from fungi is relatively simple and quick compared to crustaceans. The process has two basic steps: deproteination using NaOH and extraction of chitosan using acetic acid. Table 2.4 summarizes the extraction procedure of fungal chitosan.

Table 2.5: Fungal chitosan extraction procedure

Strain	**Deproteination using NaOH solution**			**Extraction of chitosan using acetic acid**			**Chitosan yield**
	Conc. (M)	**Temp. (ºC)**	**Time (min)**	**Conc. (%)**	**Temp. (ºC)**	**Time (h)**	
Absidia coerulea	0.5	121	20	10	60	6	[a]6.12 g/kg of potato pieces
Gongronella butleri	11	45	780	2	95	5	[a]4 g/kg of sweet potato
Gongronella butleri	1	45	780	2	95	5	[a]0.8 g/kg of sweet potato
Rhizopus oryzae	1	121	15	2	95	8	[a]4.3 g/kg of soybean residue
Rhizopus oryzae	1	121	30	2	95	8	[a]5.63 g/kg of rice straw
Aspergillus niger	1	121	15	2	95	8	[b]963 mg/L
Absidia atrospora	0.5	115	60	2	RT	0.5	[b]296 mg/L
Absidia blakesleeana	1	121	15	2	95	12	[b]280 mg/L
Absidia coerulea	11	45	780	2	95	5	[b]500 mg/L
Absidia coerulea	1	45	780	2	95	5	[b]450 mg/L
Absidia coerulea	1	121	15	2	95	12	[b]480 mg/L
Absidia coerulea	0.5	-	60	2	RT	24	[b]10 g/100 g mycelia
Absidia glauca	1	121	15	2	25	1	[b]326 mg/L
Absidia glauca	1	121	15	2	121	0.25	[b]7 g/100 g mycelia
Absidia orchidis	1	121	10	1	-	-	[b]1.79 g/L

Strain	Deproteination using NaOH solution			Extraction of chitosan using acetic acid			Chitosan yield
	Conc. (M)	Temp. (°C)	Time (min)	Conc. (%)	Temp. (°C)	Time (h)	
Absidia repens	1	121	-	2	100	17	[b]2.8 g/L
Cunninghamella echinulata	1	121	-	2	100	17	[b]398 mg/L
Gongronella butleri	11	45	780	2	95	5	[b]800 mg/L
Gongronella butleri	1	45	780	2	95	5	[b]360 mg/L
Gongronella butleri	1	121	15	2	25	1	[b]467 mg/L
Gongronella butleri	1	121	15	2	95	12	[b]250 mg/L
Gongronella butleri	0.5	115	60	2	RT	0.5	[b]352 mg/L
Gongronella butleri	0.5	90	120	10	60	6	[b]1.19 g/L
Mucor hiemalis	1	121	15	2	25	1	[b]256 mg/L
Mucor rouxii	1	121	15	2	95	12	[b]256 mg/L
Mucor rouxii	1	121	15	2	95	12	[b]370 mg/L
Mucor rouxii	1	121	15	2	95	-	[b]250 mg/L
Mucor rouxii	1	121	15	1 M HCl	95	24	[b]4–8 g/100 g mycelia
Mucor spp.	1	121	15	2	25	1	[b]252 mg/L
Penicillium chrysogenum	0.5	70	3	2	100	5	[b]0.37 g/100 g mycelia
Phycomyces blakesleeanus	1	121	15	2	95	12	[b]67 mg/L

Strain	Deproteination using NaOH solution			Extraction of chitosan using acetic acid			Chitosan yield
	Conc. (M)	Temp. (°C)	Time (min)	Conc. (%)	Temp. (°C)	Time (h)	
Rhizopus arrhizus	1	121	15	2	25	1	[b]234 mg/L
Rhizopus microsporus	1	121	15	2	25	1	[b]149 mg/L
Rhizopus oligosporus	1	121	15	2	25	1	[b]278 mg/L
Rhizopus oryzae	0.5	121	15	2	95	12	[b]700 mg/L
Rhizopus oryzae	1	121	15	2	95	8	[b]345 mg/L
Rhizopus oryzae	1	121	15	2	95	8	[b]345 mg/L
Zygorhyncus moelleri	1	121	15	2	25	1	[b]238 mg/L

- Not mentioned in the paper; RT = Room Temperature
[a] Solid State Fermentation
[b] Submerged Fermentation

2.3. Characterization of Chitosan

2.3.1. Degree of Deacetylation

Chitosan is a partially deacetylated form of chitin. It is a copolymer of N-acetyl glucosamine units and D-glucosamine units. Degree of deacetylation (DD%) is defined as molar fraction of N- acetyl glucosamine units in the chitosan polymer chain, shown in Equation 2.1 [78]:

$$DD\% = n_{GlcN} / (n_{GlcN} + n_{GlcNAc}) \quad \text{.....(2.1)}$$

Where, n_{GlcN} = glucosamine unit, n_{GlcNAc} = *N*-acetyl glucosamine unit

Sometimes degree of acetylation is also used. The formula is shown in Equation 2.2 [78]:

$$DA = 100 - DD \quad \text{.....(2.2)}$$

Several methods are available to measure degree of deacetylation. The simplest and low cost method among them is the pH-metric titration. Other methods include FTIR (Fourier Transform Infrared Spectroscopy), UV-vis Spectroscopy, X-ray Spectroscopy and NMR (Nuclear Magnetic Resonance) Spectroscopy. NMR is the most accurate, but it is very expensive.

Titration methods are cheap, but they have some disadvantages. Sample preparation time is long and titration itself is lengthy. At higher pH, mixing becomes difficult and as chitosan precipitates during the titration, errors might occur [78].

Spectroscopic techniques, however, may contain some problems such as broadening of a peak and overlapping of two or more peaks [83]. Consequently, it may give incorrect results. For qualitative study, this method is reliable. But it is required to obtain precise quantitative analysis, some complex procedures must be followed; for example, statistical analysis of several absorption ratios [83]. Selecting suitable reference and characteristic bands along with drawing a good baseline is necessary for getting a reliable result.

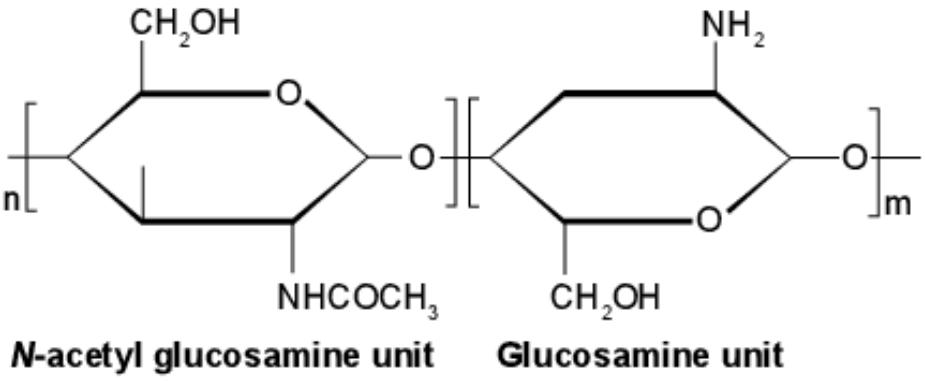


Figure 2.13: Glucosamine units in chitosan chain [61]

Deacetylation of chitin exposes amino groups along the molecule chain. Properties of the molecule undergo several changes depending upon the extent and distribution of these amino groups. Since the amino groups can be ionized, chitosan becomes polycationic in acidic media. This particular quality allows chitosan to form solutions and interact with a wide diversity of molecules. Thus, degree of deacetylation can determine most of the properties of chitosan.

2.3.1.1. Fourier Transform Infrared Spectroscopy

To characterize chitin and chitosan, Infrared (IR) spectroscopy is one of the most widely used analytical techniques because of its simplicity and availability. Principle of FTIR is related to the vibrations of the atoms in a molecule or polymer. The infrared zone of the electromagnetic radiation is utilized to determining what fraction of the incident radiation is transmitted or absorbed at a particular energy [62]. Each peak in the absorption spectrum corresponds to the vibration of a molecule part. Thus, it gives a qualitative identification of certain functional groups and bonds in the polymer sample.

Table 2.6: Absorbance band used to estimate degree of decetylation [33]

Bond	**Vibration Type**	**Wavenumber (cm^{-1})**	**Use**
O─H	Stretching	3450	Reference
N─H	Stretching	3360	Reference
C─H	Stretching	2878	Reference
C─H	Deformation (pyranose ring)	1420	Reference
C─O─C	Stretching in glycosidic linkage	1150-1040	Reference
N─H	Bending in secondary amide (amide II)	1560	Characteristic

Bond	Vibration Type	Wavenumber (cm^{-1})	Use
C═O	Stretching in secondary acetoamide (amide I)	1660	Characteristic
NH_2	Bending in amino group	1590	Characteristic
C─N	Stretching in secondary amide (amide III)	1320	Characteristic
CH_3	Rocking in acetoamide group	1380	Characteristic

To calculate DD%, the measurement of a characteristic band and an internal reference band is necessary. Several characteristic and reference bands such as: A_{1560}/A_{2875}, A_{1655}/A_{2875}, A_{1655}/A_{3450}, A_{1320}/A_{3450}, A_{1655}/A_{1070}, A_{1655}/A_{1030}, A_{1560}/A_{1160}, A_{1560}/A_{897} and A_{1320}/A_{1420} have been proposed to effectively determine the DD% of chitin and chitosan using FTIR.

2.3.1.2. Acid Base Titration Method

This is the simplest method of determining degree of deacetylation in terms of performance and calculation. Although its accuracy is lower than that of the spectroscopic methods, it is an easy and low cost method compared to others.

In this method, 0.2 g dried chitosan was dissolved in 20 mL 0.1 M HCl and 25 mL distilled water was added to it. After 30 min of continuous stirring, next portion of 25 mL distilled water was added. The whole solution was stirred for another 30 min. It was then titrated using 0.1 M NaOH. Degree of Deacetylation (DD%) was determined using the Equation 2.3 [78]:

$$DD\% = 2.03 \times (V_2 - V_1) / [m + 0.0042 \times (V_2 - V_1)] \quad \text{.....(2.3)}$$

Here,

m is the amount of chitosan used in the titration (0.2 g for these experiments); V_1 and V_2 are the deflection points in the pH curve; coefficient 2.03 results from molecular weight of chitin monomer unit; coefficient 0.0042 results from difference between molecular weights of chitin and chitosan monomer units.

The difference of the volumes of these two points (V_1 and V_2) corresponds to the acid consumed by the amine groups and allows to calculate degree deacetylation. Determination of the first derivative helps in precise reading of V_1 and V_2.

2.3.2. Molecular Weight and Viscosity

Molecular weight of chitin and chitosan are expressed using Da (Daltons; 1 Da is equivalent to 1 g/mol). It can range from several to more than thousands of kDa. The variation of molecular weight depends upon initial source (shrimp, crabs, fungi etc.) of chitosan [80]. Usually, molecular weight of fungal chitosan is lower compared to chitosan from marine sources. For example, a previous study has reported that molecular weight of chitosan extracted from *Aspergillus niger*, *Rhizopus oryzae*, *Lentinus edodes* and *Pleurotus sajo-caju*, and two yeast strains, *Zygosaccharomyces rouxii* and *Candida albicans* varied from 27 kDa to 190 kDa [76]. On the other hand, chitosan from waste shrimp shells had molecular weight of 23. kDa to 280 kDa [81].

Viscosity of chitosan also varies widely. For fungal chitosan, viscosity was found to range between 3.1 to 6.2 centipoises [76]. Chitosan obtained from shrimp shells showed a viscosity of ranging between 106 ± 5 centipoise to 6370 ± 254 centipoise depending on chemical treatments used [82]

2.4. Antimicrobial Property of Chitosan

Studies have been done regarding the antimicrobial activity of chitosan and its derivatives against a wide variety of microorganisms. Several factors influence the antimicrobial effect including molecular weight of chitosan. Antimicrobial activity of low molecular weight chitosan is higher compared to high molecular weight chitosan [118]. It is also influenced by degree of deacetylation of chitosan [63] and pH of the solution [1].

Numerous possibilities for the mode of action for chitosan have been proposed starting from binding to DNA of bacteria which causes inhibition of mRNA, to activity on bacterial surface [64]. If chitosan had the ability to bind to bacterial DNA, it would be applied in gene delivery. But such activity of chitosan is not clear in terms of antimicrobial activity. Studies have shown that chitosan cannot enter a cytoplasm to reach a target [65]. It was proposed that chitosan might bind to the bacterial membrane to disrupt it [66]. However, the size of hydrated chitosan is bigger compared to that of the cell wall. Therefore, it cannot cross the cell wall and interact with the cell membrane directly [67].

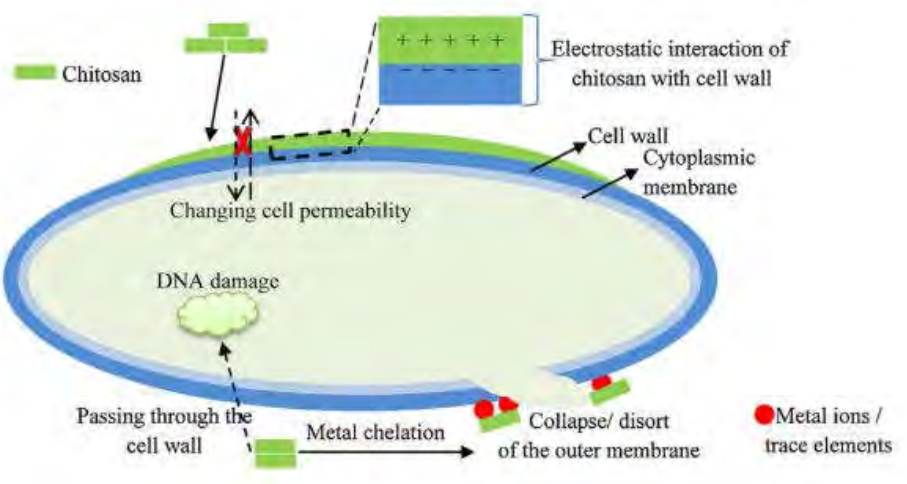


Figure 2.14: Antimicrobial mechanism of chitosan and its derivatives [79]

The hypothesis that is accepted widely says that the presence of amine groups (NH_3^+) in chitosan results in the olycationic nature of chitosan and it might allow chitosan to interact with surface molecules of microorganisms that are negatively charged. It eventually leads to leakage of cell components through the ruptured cell wall and therefore leads to cell death [68].

2.4.1. Antimicrobial Susceptibility Testing

2.4.1.1. Disk Diffusion Method

Kirby Bauer disk method or disk diffusion method is a simple way in which antibiotic loaded wafers or disks are used to test whether a certain bacterial species is susceptible to the antibiotic or not [69]. Figure 2.15 shows antibiotic susceptibility of S. aureus against methicillin, ampicillin and vancomycin [70].

The antibiotic diffuses from the disc into the agar in a decreasing manner. If the organisms were killed or inhibited by the concentration of the antibiotic, there will be no growth in the immediate area around the disks represented as zone of growth inhibition. The diameter of the zone of inhibition indicates the activity of the antimicrobial agent on the target microorganism. The higher the zone of inhibition, better the antimicrobial activity.

Figure 2.15: Disk diffusion method of antimicrobial susceptibility testing

2.4.1.2. Agar Well Diffusion Method

In this method, wells are dug in the bacteria mounted agar medium and antimicrobial agents are poured into it as a solution. As it diffuses through the agar, a circular zone of inhibition is observed if the target bacterial species is susceptible to the antimicrobial agent. Zone diameter qualitatively indicates the antimicrobial activity. Figure 2.16 shows antimicrobial effect of plant extracts such as neem and aloe vera against *C. albicans* [71].

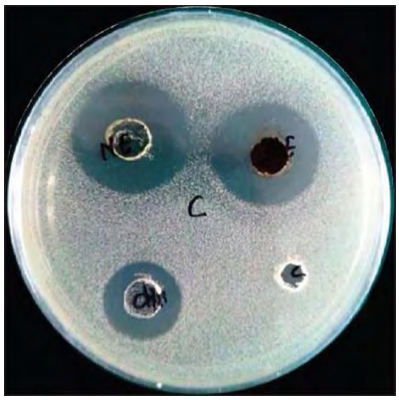


Figure 2.16: Agar well diffusion method

Agar well diffusion method has also been used to test antifungal susceptibility of 158 clinical isolates of *Candida* spp. using amphotericin B, caspofungin, posaconazole, itraconazole, and fluconazole [72]

CHAPTER 3

MATERIALS AND METHODS

MATERIALS AND METHODS

3.1. Materials

3.1.1. Microorganisms

Microorganisms used in this study were *Saccharomyces cerevisiae, Aspergillus niger* and *Staphylococcus aureus*. Former one was bought from local market as baker's yeast. *A. niger* was isolated from a random black molded onion. *S. aureus* was isolated from human nasal specimen.

3.1.2. Chemicals

Dextrose Monohydrate and fresh potatoes were bought from market. Potato Dextrose Broth (PDB) was prepared in the laboratory. Deionized and distilled water was used in all preparations. Laboratory grade ethanol was obtained from a local source. Other commercial grade chemicals that have been used throughout the experiments are listed in Table 3.1.

Table 3.1. Chemicals and Company Names

Product Name	**Company**
Acetic Acid	Merck
Hydrochloric Acid (fuming)	Merck
Mannitol Salt Agar Base	HiMedia
Mueller Hinton Agar	Lab M
Potato Dextrose Agar	Oxoid
Sodium Acetate	Qualikems
Sodium Chloride	Merck
Sodium Hydroxide Pellets	Merck
Sodium Nitrate	Merck
Sulfuric Acid	Merck

3.2. Methods

3.2.1. Isolation of the Microorganisms

3.2.1.1. Preparation of Agar Plates

Agar plates for *Aspergillus niger* were prepared using commercial Potato Dextrose Agar (PDA) medium. Maintaining the ratio recommended by the manufacturer, nutrient agar solution was autoclaved for 20 min. The solution was poured into gas sterilized plastic petri plates inside a biosafety hood (level 2) and left to cool down and solidify for 4-5 min. Plates were also prepared using commercial Mannitol Salt Agar Base (for *Staphylococcus aureus* culture) and Mueller Hinton Agar (for antimicrobial efficacy testing) following the same procedure. The plates were kept upside down until using them in the experiments.

3.2.1.2. Isolation of *Aspergillus niger*

Onions with black molds were collected from local market. Spores of *Aspergillus niger* from the black mold were taken aseptically using a sterile inoculum loop and transferred into a 1.5 mL centrifuge tube which contained 1 mL sterilized solution of previously prepared 0.85% NaCl. The spore suspension was homogenized using a vortex mixer. 100 μL of the suspension was poured and spread onto a previously prepared PDA plate. After 3 days of incubation at 37ºC, colonies with black conidia became clearly visible. A single colony of *Aspergillus niger* was scratched out using a sterile inoculum loop and transferred onto another PDA plate. The procedure was repeated to make sure that the species strain becomes pure. The pure culture of the species was maintained at 37ºC and fresh culture plates were prepared after every 7 days of incubation. Old cultures were labeled and disposed off after 10 min exposure to UV light.

3.2.1.3. Isolation of *Staphylococcus aureus*

A cotton swab containing human nasal specimen was placed into 2 mL sterilized 0.85% NaCl solution and homogenized using vortex mixer. 1 drop of the suspension was added to each MSA plate and streaked with an inoculum loop. The plates were incubated at 37ºC for 24 h. After the incubation, *S. aureus* appeared as yellow clusters or colonies. One of the colonies were picked using an inoculum loop and transferred into 2 mL sterilized 0.85% NaCl. It was then streaked on to MSA plates and incubated at 37ºC. The *S. aureus* cultures were maintained in MSA plates and new culture plates were made at an interval of 2 days.

3.2.2. Culture Conditions

3.2.2.1. Preparation of the PDB Media

300 g of fresh potato was washed and cut into small pieces without peeling. The pieces were boiled with 500 mL of distilled water for 30 min. The potato infused liquid was collected by filtration and a 1 L solution was prepared with 100 g glucose and 0.4 g sodium nitrate. Sodium nitrate was used as the nitrogen source for *Aspergillus niger*. Sulfuric acid was added to bring the pH level to 4.5. The culture media was stored at 4ºC.

3.2.2.2. Surface Culture of *Aspergillus niger*

A seven-day-old agar plate of the *Aspergillus niger* was taken inside the biosafety hood. 15 mL of sterilized 0.85% NaCl solution was poured into it. The surface of the agar plate was scratched gently using an inoculum loop to release the spores into the saline solution. The spore suspension was filtered using a filter cloth to remove mycelial fragments. The suspension was homogenized using vortex mixer. The suspension was used as inoculum for surface culture. 1 mL of the

inoculum was poured into each 250 mL Erlenmeyer flask containing 50 mL of sterilized PDB medium. The flasks were kept at room temperature (around 28ºC). After desired culture periods the fungal mats were taken out from the flasks, washed with distilled water and dried at 60ºC.

3.2.2.3. Sporulation of *Saccharomyces cerevisiae*

1 g of dry *Saccharomyces cerevisiae* was washed with 1% sodium acetate solution, centrifuged and re-suspended in 200 mL of 1% sodium acetate. For the control experiment, the dry yeast cells were washed with distilled water, centrifuged and re-suspended in 200 mL distilled water. Both the samples were incubated at room temperature on an orbital shaker at 150 rpm. The biomass at different days were centrifuged and dried at 60ºC.

3.2.3. Fungal Chitosan Extraction

The dried biomass was grinded to fine powder using a mortar-pestle and mixed with 1 M NaOH at 1:50 (w/v) ratio. The mixture was homogenized using a commercial waring blender. It was then autoclaved at 121ºC for 30 min. The autoclaved content was centrifuged at 5000 rpm for 10 min and washed with distilled water by re-suspending and centrifugation. The washing step was repeated 4 times to bring the pH around 7.0. The material was dried in an oven at 40ºC and collected as the Alkali Insoluble Material (AIM).

The AIM was grinded and homogenized with 2% acetic acid at 1:50 (w/v) ratio. Afterwards, the solution was heated at 95ºC for 6 h in an oven. After cooling down, it was centrifuged at 5000 rpm for 20 min. The supernatant liquid was collected as the dissolved chitosan solution. 4 M NaOH was added dropwise until the pH of the solution reached around 10.0 and the chitosan precipitation were visible. The chitosan was centrifuged at 5000 rpm for 10 min and washed four times with distilled water to bring the pH close to 7.0. Finally, the sample was rinsed with

ethanol, centrifuged and dried at 40ºC. Figure 3.1. shows the block diagram of extraction of chitosan from fungal biomass.

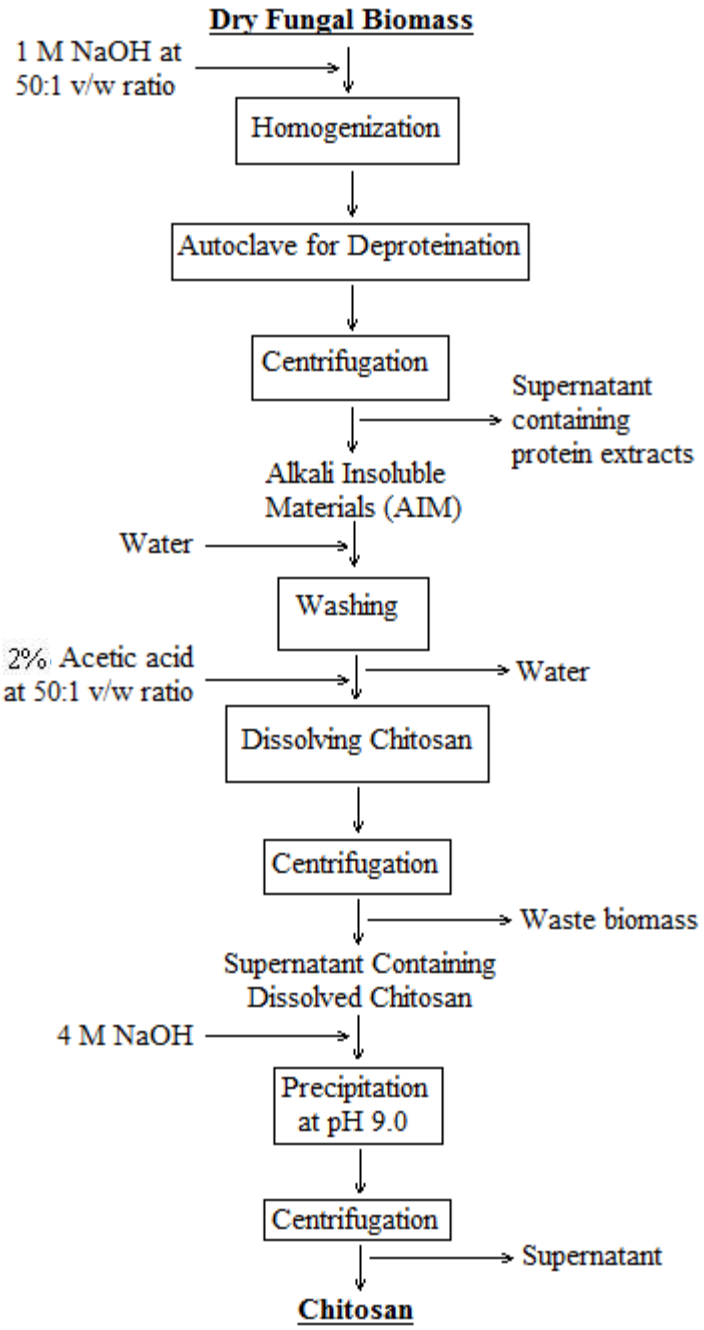


Figure 3.1: Block Diagram for Chitosan Extraction

3.2.4. Characterization for Degree of Deacetylation

3.2.4.1. Fourier Transform Infrared Spectroscopy (FTIR)

Infrared spectroscopy analysis of the chitosan samples was done using a Shimadzu FTIR spectrophotometer by the Department of Chemistry, BUET. Potassium bromide (KBr) disk method was utilized to obtain the spectra.

The process involved mixing KBr with chitosan samples at nearly 100:1 ratio using a mortar. The mixture was then pressed into a disk and mounting in the spectrophotometer. Frequency range of the spectra was from 4000 to 400 cm^{-1} with 45 scans. The resolution was 2 cm^{-1}. Degree of Deacetylation (DD%) was determined using Equation 3.1 [5.34]:

$$DD\% = 100 - (A_{1655}/A_{3450}) \times (100/1.33) \quad \text{.....(3.1)}$$

Here, A_{1655} is the value of absorbance at peak near 1655 cm^{-1},

A_{3450} is the value of absorbance at peak near 3450 cm^{-1}.

3.2.4.2. Acid Base Titration

0.2 g dried chitosan was dissolved in 20 mL 0.1 M HCl and 25 mL distilled water was added to it. After 30 min of continuous stirring, next portion of 25 mL distilled water was added. The whole solution was stirred for another 30 min. It was then titrated using 0.1 M NaOH. Degree of Deacetylation (DD%) was determined using Equation 3.2 [5.34]:

$$DD\% = 2.03 \times (V_2 - V_1) / [m + 0.0042 \times (V_2 - V_1)] \quad \text{..... (3.2)}$$

Here,

m is the amount of chitosan used in the titration (0.2 g for these experiments),

V_1 and V_2 are the deflection points in the pH curve,

Coefficient 2.03 results from molecular weight of chitin monomer unit,

Coefficient 0.0042 results from difference between molecular weights of chitin and chitosan monomer units.

3.2.5. Determination of Antimicrobial Activity

Antimicrobial activity of chitosan extracted from *Saccharomyces cerevisiae* was tested against *Staphylococcus aureus*. Zone of inhibition were observed to evaluate chitosan activity against.

3.2.5.1. Preparation of Bacterial Stock Solution

An individual colony of *S. aureus* was selected and picked up from an MSAB culture plate using a sterile inoculum loop. The colony was then aseptically transferred into 5mL of sterile distilled water inside biosafety hood. The solution was mixed well and kept at 37 ºC for 24 h.

3.2.5.2. Preparation of Chitosan Films

4 mg chitosan obtained from *S. cerevisiae* was dissolved into 20 mL of 1% acetic acid to make a chitosan concentration of 2 g/L. The solution was then poured onto a 9 cm diameter plastic petri plate. The solution was allowed to dry at room temperature for 48 h. Produced chitosan film was separated from the petri plate using a sterile knife. The film was cut into small circular pieces to have a diameter of approximately 6 mm to 8 mm. These pieces were kept inside a sterile petri plate until further use.

3.2.5.3. Preparation of Chitosan Solution

Chitosan stock solution was prepared with 40 mg chitosan powder dissolving into 10 mL of 1% acetic acid which makes 4.0 g/L chitosan solution. The stock solution was used to prepare different concentrations of chitosan solution by diluting with 1% acetic acid such as 3.5 g/L, 3.0 g/L, 2.5 g/L, 2.0 g/L, 1.5 g/L, 1.0 g/L, 0.5 g/L and a control of 1% acetic acid solution. Standard solution was prepared using commercially available Tetracycline dissolved in distilled water at a concentration of 1.0 g/L.

3.2.5.4. Spreading Bacteria onto Agar

24 h old bacterial stock solution was used as inoculum. Inside biosafety hood, 100 µL bacterial stock solution was poured onto a previously prepared sterile Mueller Hinton Agar plate. After spreading the bacteria evenly throughout the agar surface, it was allowed to dry for 3-4 min and then kept upside down until next step of the experiment.

3.2.5.5. Placing Chitosan Films onto Agar

Chitosan films were placed onto freshly mounted bacterial agar medium using a sterile stainless steel tweezers. Using the same tweezers, the films were pressed lightly to ensure that they remain attached to the agar surface. The agar plate was then kept upside down at 37ºC for 24 h.

3.2.5.6. Preparing Wells for Chitosan Solution

Gas sterilized 1 mL syringe was cut cross-section wise using a sterile blade. The syringe was pressed into freshly mounted bacterial agar medium to create evenly sized wells. 50 µL of each test solution was poured into each well. The plates were kept in an upright position at 37ºC for 24 h.

CHAPTER 4

RESULTS

RESULTS

In this chapter, experimental results have been presented that include culture and growth of the fungal species and their ability to produce chitosan. The results also include required culture days for the species to obtain maximum yield of chitosan. Characterization results of the chitosan samples have been shown using graphical representations and calculations to determine Degree of Deacetylation (DD%) were included in the Appendix section. Selection of the comparatively better candidate for chitosan production was shown and chitosan extracted from this species was used in the subsequent experiments such as antimicrobial activity testing reporting zone of inhibitions.

All the experiments were done in duplicates at least and average values were reported with respective standard deviation error bars.

4.1. Culture of *Aspergillus niger*

Growth of *Aspergillus niger* was investigated using surface culture method in Potato Dextrose Broth (PDB) medium. The species grew well in the culture medium and underwent clear morphological changes as shown in Figure 4.1. At Day 1 after inoculation, the fungus created a thin and whitish layer on the surface of the medium. Black spores became visible at Day 2 while making the fungal mat comparatively stronger. Within Day 3 and Day 4, black spores completely covered the top of the fungal mat. The fungal mat showed little change in morphology by visual inspection afterwards except slight extension of some parts into the liquid culture media. The bottom of the mat remained yellowish white.

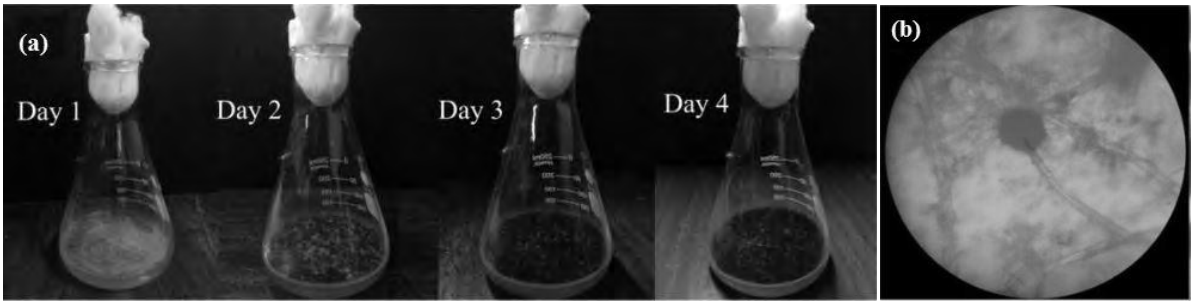


Figure 4.1: (a) Gradual change in morphology of *A. niger*, (b) *A. niger* under light microscope

Biomass growth of *Aspergillus niger* in grams per liter of the culture media at different days of anaerobic surface culture condition have been shown in Figure 4.2. The biomass increase showed consistency up to Day 8 which represents growth phase of the species. The maximum biomass yield was reached at this point to be 7.166 ± 0.47 g/L. Then the biomass underwent a slight decrease to 6.0785 ± 0.13 g/L at Day 10. It increased again at Day 12 and remained consistent through the next couple of days representing the stationary phase.

When the biomass was treated with sodium hydroxide, significant amount of biomass materials were removed. The remaining materials after centrifugation are the alkali insoluble materials (AIM). Dry weights of AIM in gram per liter of the culture media have also been included in Figure 4.2. Highest amount of AIM was found at Day 8 and the value was 1.64 ± 0.28 g/L.

From Figure 4.2, approximately 22.9% of the dry biomass was obtained as alkali insoluble materials (AIM). However, at Day 16, AIM was found to be approximately 24.4% of the dry fungal biomass, which was the highest fraction of AIM obtained by the experiments.

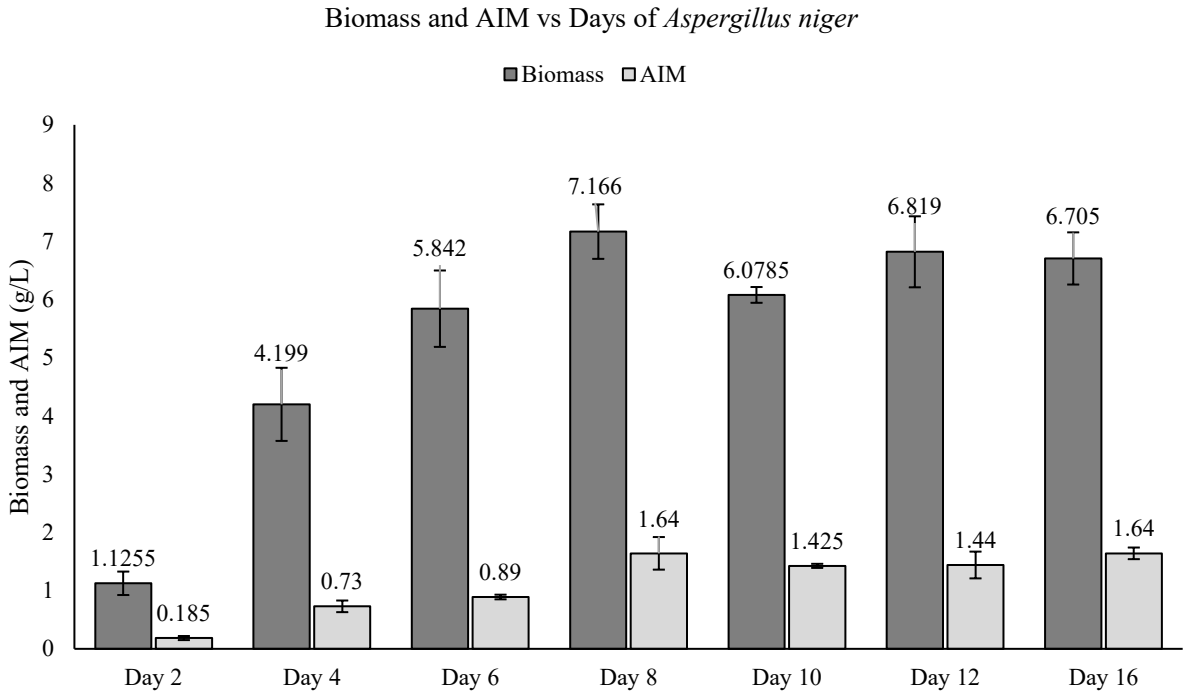


Figure 4.2: Dry biomass and alkali insoluble materials (AIM) of *A. niger* at different days

4.2. Chitosan Yields

The unit of the chitosan yield was taken as dry chitosan weight in milligram per gram of dry biomass weight. The yields were calculated at different days of culture for both *Aspergillus niger* and *Saccharomyces cerevisiae*.

4.2.1. Yields of Chitosan from *Aspergillus niger*

Amount of chitosan extracted at different days of *A. niger* culture is shown in Figure 4.3. Chitosan was absent in the fungal biomass at Day 2. The yield of chitosan started increasing from Day 4 and continued to increase consistently up to Day 12. At this point a clear maximum was found and the value was 16.15 ± 0.95 mg/g. At Day 16, however, the yield decreased slightly.

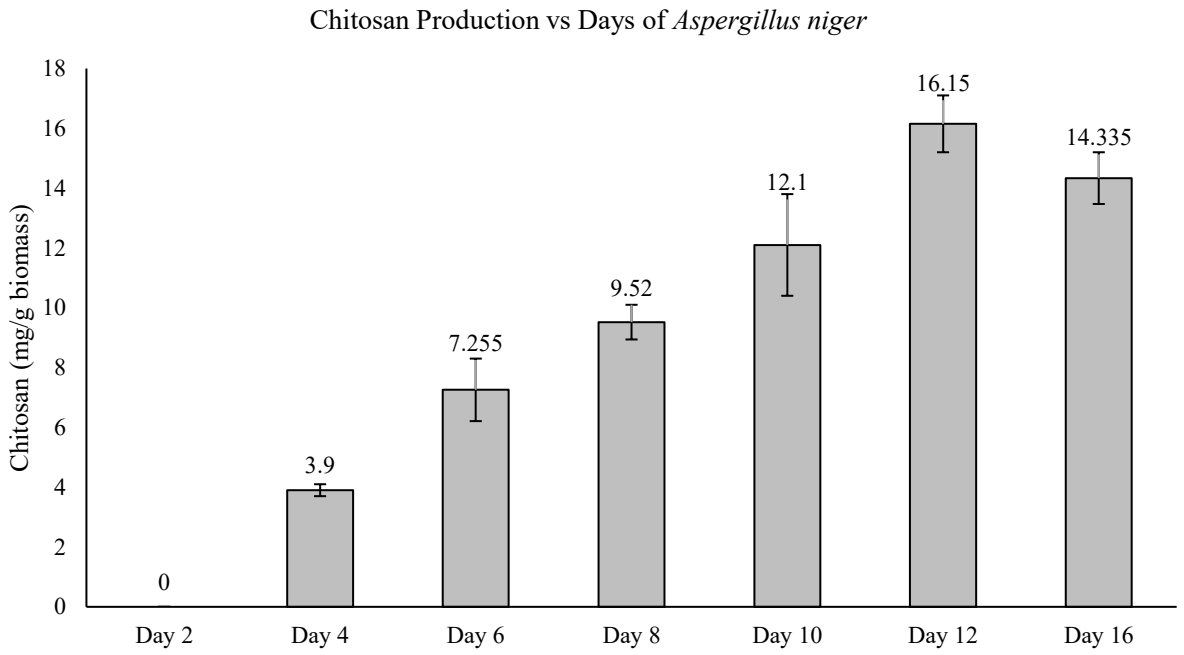


Figure 4.3: Chitosan production from *A. niger* at different days

4.2.2. Yields of Chitosan from *Saccharomyces cerevisiae*

The other fungal species studied for chitosan extraction was *Saccharomyces cerevisiae*. Sporulated yeast cells were identified under light microscope as shown in Figure 4.4. These photos were taken at Day 4 of the nutrition starvation condition in sodium acetate solution. At 100 X magnification, clear budding of *Saccharomyces cerevisiae* cells were visible. Both vegetative and sporulated cells were present in the sample.

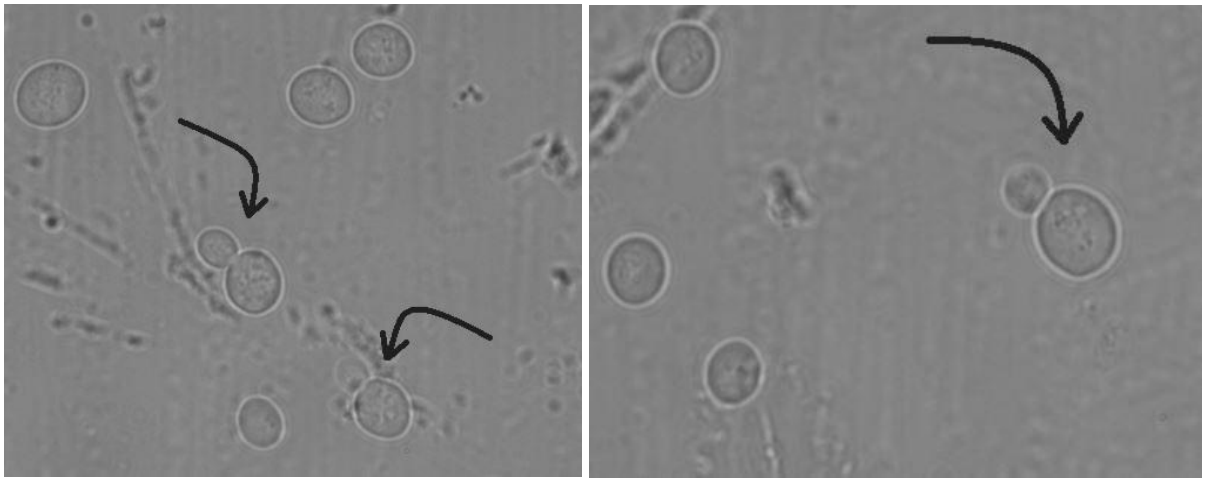


Figure 4.4: Sporulated S. cerevisiae under light microscope (100 X magnification)

In the control experiment, *S. cerevisiae* was found to give a chitosan yield of 10.05 ± 0.25 mg/g. Under sporulation condition (treatment with sodium acetate solution), the fungal species showed consistently increasing yields which continued until Day 4. At Day 5, however, the yield became lower. Figure 4.5 clearly showed a maximum yield obtained at Day 4 and the value was 20.85 ± 0.35 mg/g.

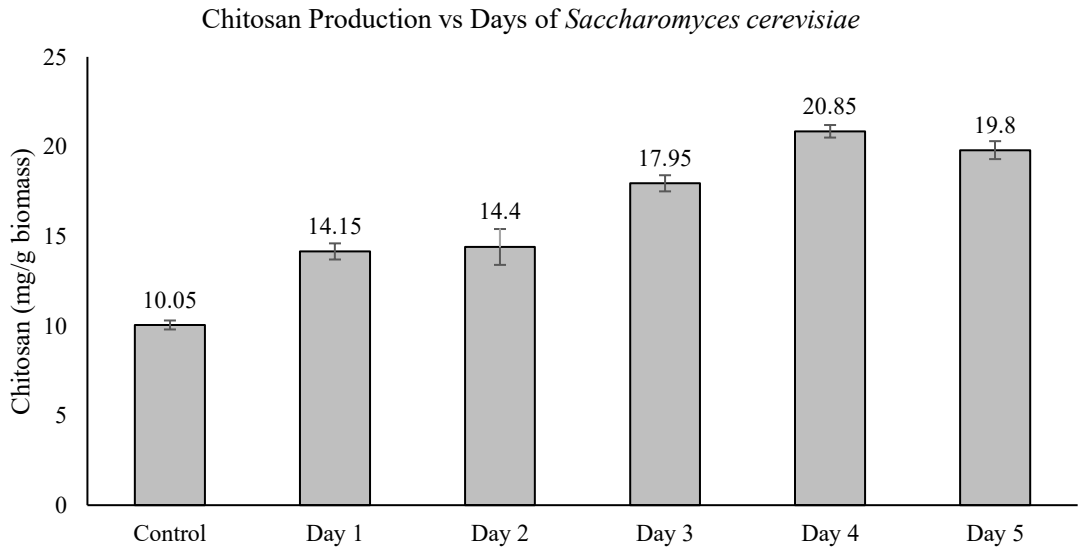


Figure 4.5: Chitosan production from *S. cerevisiae* at different days

4.3. Characterization of the Extracted Chitosan

4.3.1. Degree of Deacetylation using FTIR

FTIR spectra of the extracted chitosan samples from *Aspergillus niger* and *Saccharomyces cerevisiae* are presented in Figure 4.6 and Figure 4.7 respectively. Using the data obtained from FTIR spectroscopy, degree of deacetylation was calculated using Equation 3.1.

$$DD\% = 100 - (A_{1655}/A_{3450}) \times (100/1.33) \quad \text{..... (3.1)}$$

FTIR spectroscopy showed results in Transmittance (%). Absorbance was calculated from the transmittance values using Equation 4.1.

$$A = 2 - \log(T) \quad \text{..... (4.1)}$$

4.3.1.1. Degree of Deacetylation of *A. niger* Chitosan

The FTIR result for the *A. niger* chitosan was presented in Figure 4.6. Intense peaks were visible near 1655 cm^{-1} and 3450 cm^{-1}. Transmittance values were found to be 27.85% and 8.336%.

From these data degree of deacetylation (DD%) was calculated using Equation 3.1 and Equation 4.1. The value was found to be 61.39%. Detailed calculation was shown in the Appendix.

4.3.1.2. Degree of Deacetylation of *Saccharomyces cerevisiae* Chitosan

FTIR result for *S. cerevisiae* chitosan was presented in Figure 4.7. The figure also showed peaks near 1655 cm^{-1} and 3450 cm^{-1}. Transmittance at 1655 cm^{-1} and 3450 cm^{-1} were found to be 41.3% and 16.56% respectively. The degree of deacetylation (DD%) was calculated to be 63.41%.

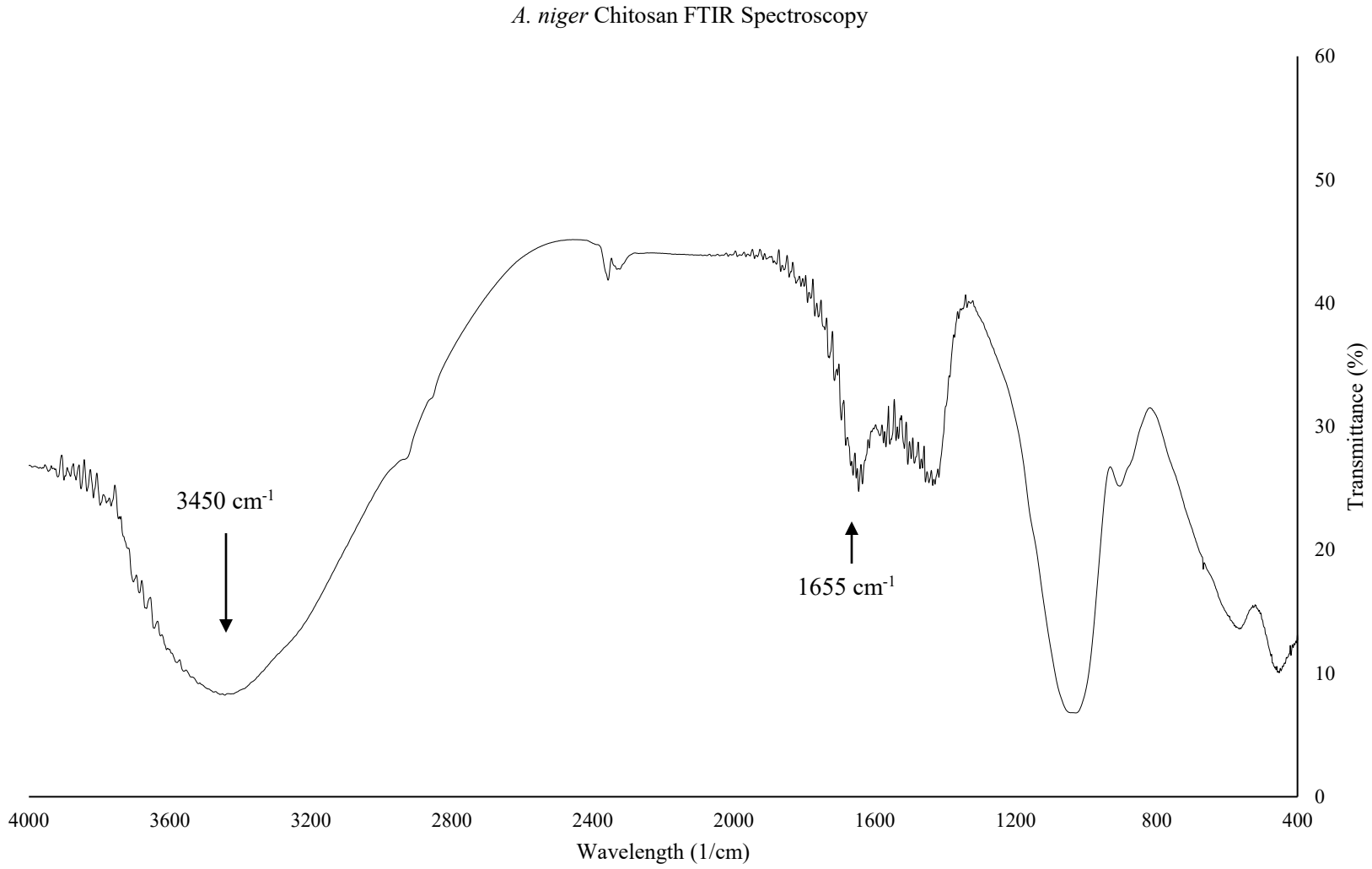


Figure 4.6: FTIR results of *A. niger*

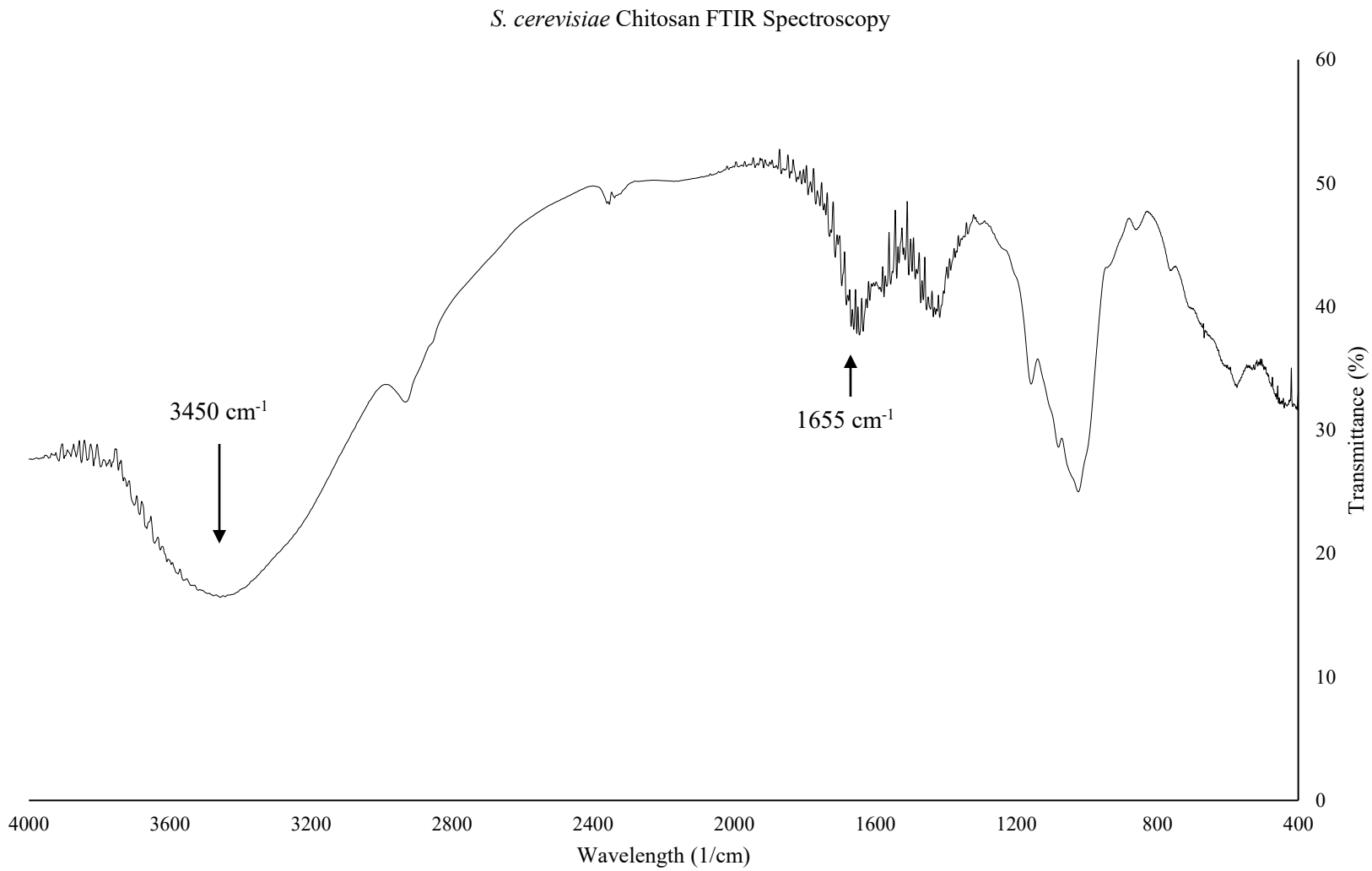


Figure 4.7: FTIR results of *S. cerevisiae*

4.3.2. Degree of Deacetylation using Acid Base Titration Method

Chitosan samples that were obtained from culture of *A. niger* and *S. cerevisiae* were further characterized using acid base titration method. Values of degree of deacetylation were calculated using Equation 3.2.

$$DD\% = 2.03 \times (V_2 - V_1) / [m + 0.0042 \times (V_2 - V_1)] \quad \text{.....(3.2)}$$

4.3.2.1. Degree of Deacetylation from *A. niger* Chitosan

The titration curve for chitosan obtained from cultures of *A. niger* was shown in Figure 4.8.

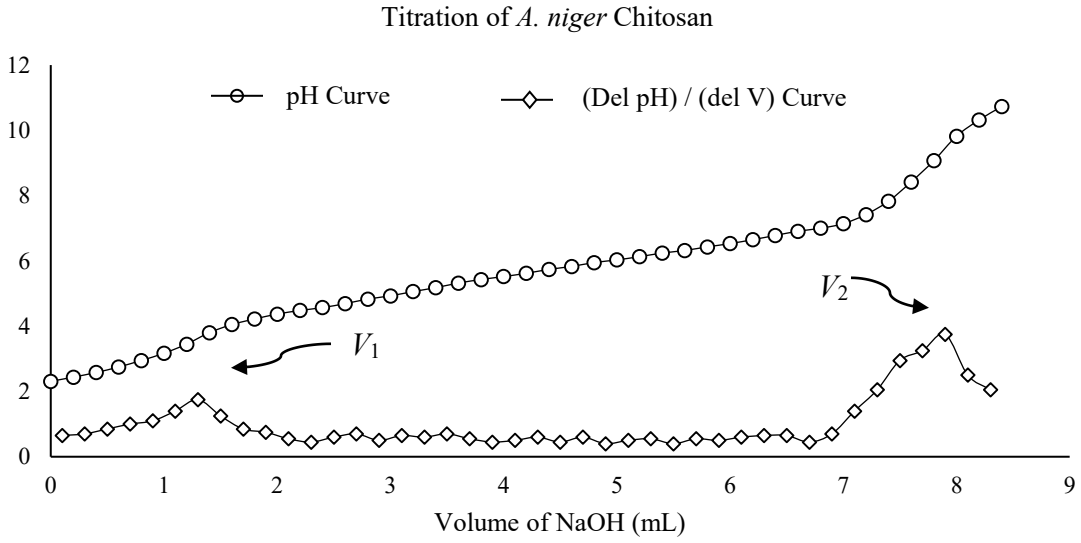


Figure 4.8: Acid base titration curve for *A. niger* chitosan

From the figure, it is observed that adding sodium hydroxide increases the pH consistently. After addition of 1.2 mL NaOH, the pH underwent a sharp rise. In the derivative curve, this point was clearly visible and taken as a value for V_1. The curve showed a second sharp rise after addition of 7.9 mL of NaOH. This point was taken as V_2.

Using these data, the degree of deacetylation (DD%) was determined using Equation 3.2 and the value was found to be 59.61%. Detailed calculations were included in the Appendix.

4.3.2.2. Degree of Deacetylation from *S. cerevisiae* Chitosan

Acid base titration curve for S. cerevisiae was shown in Figure 4.9. From the derivative curve, sharp rises of pH was observed after adding 1.9 mL and 7.8 mL of NaOH. These values were therefore taken as V_2 and V_1 respectively.

From these data, degree of deacetylation (DD%) was calculated to be 53.28%. Detailed calculations were included in the Appendix.

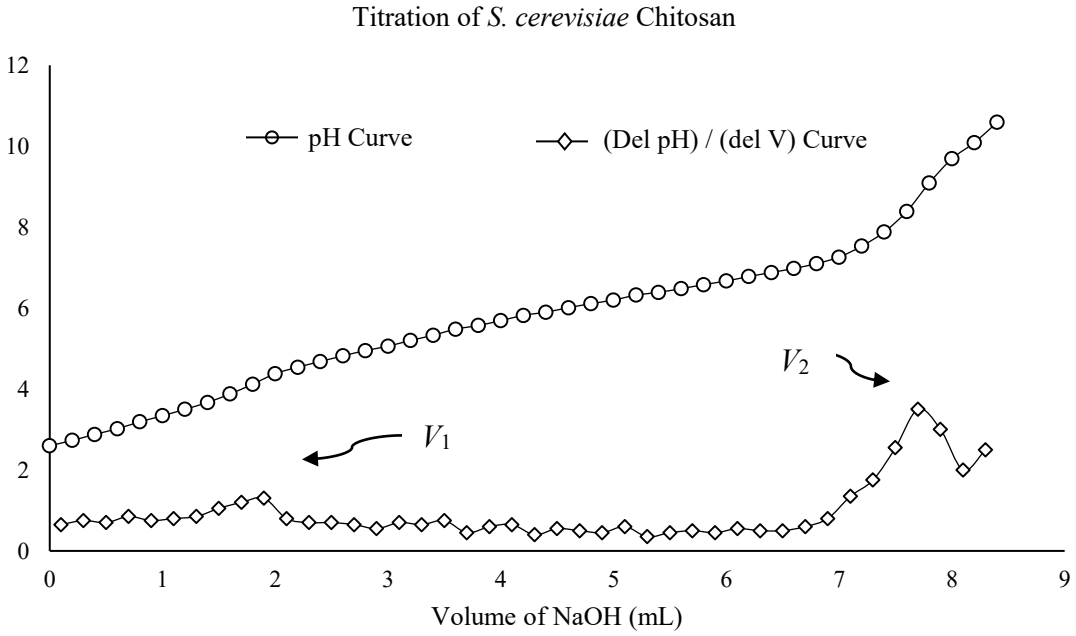


Figure 4.9: Acid base titration curve for

4.4. Antimicrobial Activity of Chitosan determined by Zone of Inhibition

Chitosan obtained from *Saccharomyces cerevisiae* was used for further studies. Chitosan thus obtained was used to determine the zone of inhibition in the antimicrobial activity testing experiments. Agar well diffusion method was applied to obtain the results.

4.4.1. Isolation of *Staphylococcus aureus*

Staphylococcus aureus was identified by visual inspection from 1 day old MSAB (Mannitol Salt Agar Base) agar plates. The agar plate was shown in Figure 4.10. The part of the medium which displayed changed color (the original red color becomes bright yellow) is the part that contains *S. aureus* species.

The yellow color represents production of ammonia and thus a pH lower than 7.0 that distinguishes *S. aureus* from other *Staphylococcus* species.

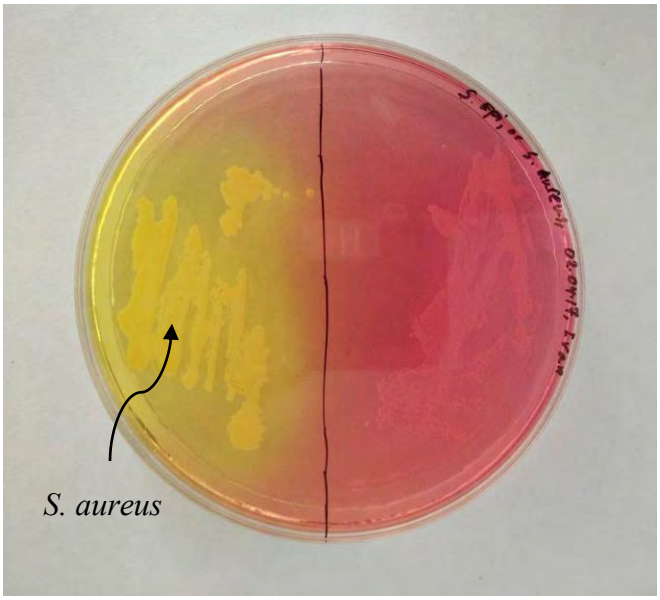


Figure 4.10: Isolation of *S. aureus* in MSAB

4.4.2. Zone of Inhibition Method

Antimicrobial effect of chitosan was tested using zone of inhibition method. Generally, the activity of an antimicrobial agent against a specific bacterial species can be evaluated by placing the agent in the agar plate containing the bacterial culture and observing a circular zone inside which no bacteria can survive. The better the activity of antimicrobial agent, the higher the zone diameter becomes.

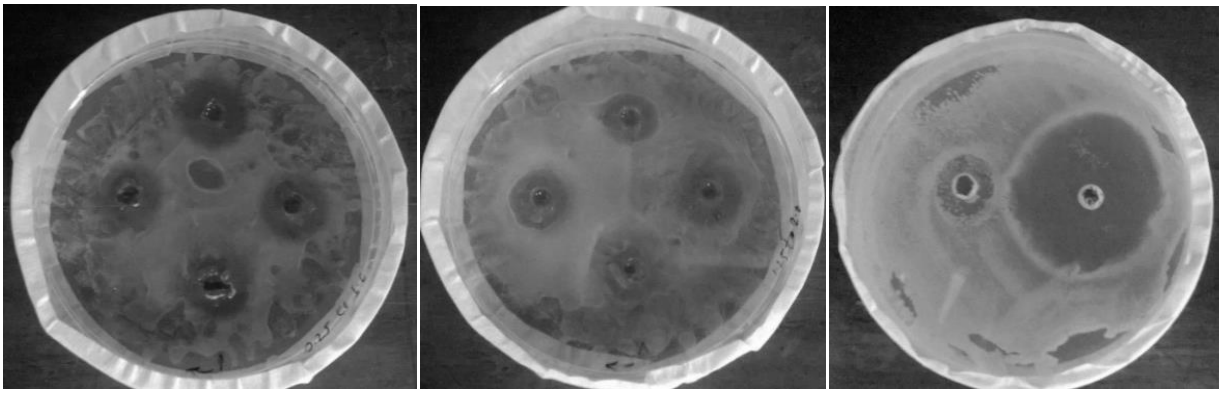
4.4.2.1. Zone of Inhibition using Chitosan Films

Films produced from *Saccharomyces cerevisiae* at concentration of 2 g/L in 1% acetic acid were tested to find zone of inhibitions against *Staphylococcus aureus*. However, chitosan films showed no zone of inhibitions. Therefore the experiment indicated zero antimicrobial activity against the isolated *S. aureus*.

4.4.2.2. Zone of Inhibition using Chitosan Solutions

Antimicrobial activity of the chitosan solution was observed through clearly visible zones of inhibition. The MHA (Mueller Hinton Agar) plates were shown in Figure 4.11.

Figure 4.11 (a) and Figure 4.11 (b) shows zone of inhibition at different chitosan concentrations. Figure 4.11 (c) presents zone of inhibition of 1% acetic acid (control) and 1 g/L Tetracycline (standard).



(a) (b) (c)

Figure 4.11: Zone of inhibition shown by: (a) chitosan concentration 0.5 to 2 g/L; (b) chitosan concentration 2.5 to 4 g/L; (c) 1% acetic acid (control) and 1 g/L Tetracycline (standard)

The diameters of the zones of inhibition were measured. These values were then plotted against the corresponding concentration of chitosan present in the agar wells for antimicrobial action. Zones of inhibition from the experiment have been presented in Figure 4.12.

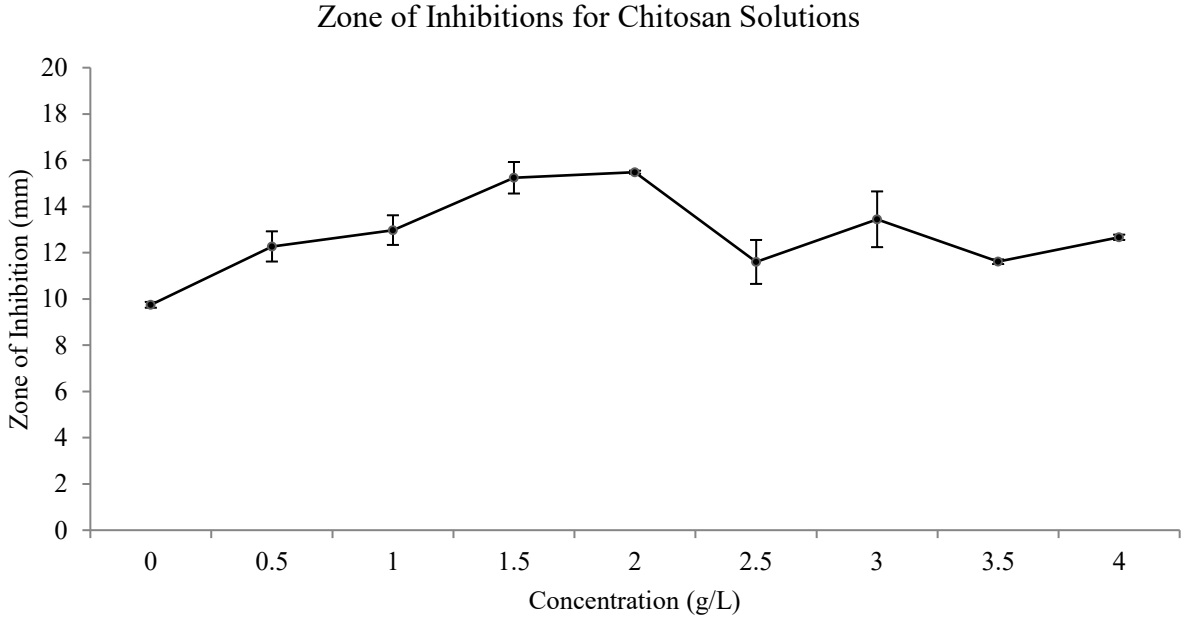


Figure 4.12: Diameter of zone of inhibition shown by chitosan at different concentrations

According to the figure, 1% acetic was antimicrobial itself giving a zone of inhibition of 9.75 ± 0.13 mm. However, inhibition zones with presence of chitosan were consistently higher compared to the negative control of 1% acetic acid. Zone of inhibition reached a maximum with an average diameter of 15.48 ± 0.07 mm for chitosan at 2 g/L concentration. Commercially available Tetracycline, on the other hand, showed 29.46 ± 2.68 mm diameter at a concentration of 1 g/L.

CHAPTER 5

DISCUSSION

DISCUSSION

In this chapter, major findings of this work are discussed according to the results obtained from this study. Limitations and conflicting results are also presented. The experimental results are also compared with those from literature.

5.1. Growth of *Aspergillus niger*

In this work, *Aspergillus niger* was isolated from onions in the laboratory and the species was identified easily from its unique morphology. The study showed flexibility of *A. niger* strain selection for chitosan production.

According to the study, *Aspergillus niger* biomass grew to a maximum at Day 8 and the value was 7.166 ± 0.47 g/L. The biomass decreased at Day 10 and remained lower than the maximum. The reason for this decrease in biomass amount can be accounted for by the deficiency of nutrition in the culture medium [84].

Previous studies reported various amounts of maximum growth for *Aspergillus niger* at different days. The biomass growth depends on several factors. Works of Shehu and Bello [85] showed that, growth of *Aspergillus* species depends upon several environmental factors such as relative humidity and temperature. The amount of biomass also depends strongly on fungal strains and culture media. It had been shown that two different strains of *Aspergillus niger* gave maximum biomass amounts of 19.8 g/L and 17.3 g/L at 5th day of cultivation in Potato Dextrose Broth medium [86]. Maghsoodi *et al.* [84] used Sabouro Dextrose Broth and found 8.57 g/L biomass at 6th day of culture. These results are comparable to the findings of the current study.

5.2. Extraction of Chitosan from Cultures of *A. niger* and *S. cerevisiae*

The maximum amount of chitosan from *A. niger* was found to be 16.15 ± 0.95 mg/g dry biomass at 12th day. On the other hand, *S. cerevisiae* showed a maximum of 20.85 ± 0.35 mg/g chitosan yield. In both cases, the chitosan yield decreased afterwards. Possible explanation for this could be consumption of chitin and chitosan in the cell wall by the microorganism itself due to lack of nutrients [87]. Another reason could be hydrolysis of polymers by diffused hydrolytic enzymes as the biomass became concentrated [88].

The experimental results showed that *S. cerevisiae* was better producer of chitosan compared to that of *A. niger* in terms of yields. Although the control experiment showed presence of chitosan in raw *S. cerevisiae*, rise of chitosan yields indicated increasing amount of ascospore formation on yeast cell wall and significantly validated sporulation of the yeast cells [108]. In our study, *A. niger* showed a more consistent trend which makes it more predictable than *S. cerevisiae* for producing chitosan. Both the fungal species had shown good potential to be used as alternative source of chitosan, though extraction from *S. cerevisiae* was more efficient.

The capability of a fungal species to produce chitosan varies with strains of that species and stage of lifecycle. Yield was also reported to be dependent upon temperature, pH, carbon source, nitrogen source and other culture conditions [92]. Previous studies showed different amounts of fungal chitosan obtained from different fungal species. For example, Amorim *et al.* investigated chitosan yields of two fungal species, *Mucor racemosus* and *Cunninghamella elegans* [89]. They used YPD medium and applied submerged culture technique. The chitosan yield was reported as 35.1 mg/g and 20.5 mg/g respectively. The use of corn steep liquor and honey as agro-industrial

nitrogen and carbon sources gives a chitosan yield of 29.3 mg/g of dry biomass of *Rhizopus arrhizus* [90].

In a previous study, the effect of heavy metals (Cu and Zn) on chitosan production of *Absidia corymbifera* grown in corn steep liquor was tested [74] and the obtained chitosan yield was 67.29 mg/g. In the study of Pochanavanich and Suntornsuk [76], who used Potato Dextrose Broth as culture media, chitosan yield was found to be 107 mg/g dry cell from *Aspergillus niger*. Soybean meal influenced chitosan production as a nitrogen source by *Aspergillus niger* and the chitosan yield was found to be 17.053 mg/g [75], which is quite similar to the present study. *Cunninghamella bertholletiae* was cultured using sugar cane juice and molasses as an alternative medium and the chitosan production was reported as 128 mg/g [77]. A more recent study showed that *Cunninghamella elegans* cultivated on a mixture of corn steep liquor and papaya peel juice gave a yield of 15.63 mg/g [91].

Chitosan extraction from *Saccharomyces cerevisiae*, on the other hand, was a novel extension of the previous work. Haini Zhang *et al.* [93] used yeast spores as chitosan beads. Pochanavanich and Suntornsuk [76] worked with two yeast species, namely *Zygosaccharomyces rouxii* and *Candida albicans* and they reported chitosan yields as 36 mg/g and 44 mg/g respectively.

To the best of our knowledge, there have been no studies for extraction of chitosan from *S. cerevisiae* and there is a potential scope of future work to get high yields of chitosan from this species by optimization of sporulation conditions.

5.3. Characterization of Extracted Chitosan

5.3.1. FTIR Results

FTIR peaks of chitosan samples of the two species were quite similar. It also had similarity with the results obtained in a previous study [84] as shown in Figure 5.1. All these graphs showed peaks at 1655 cm^{-1} and 3450 cm^{-1}.

Usually, amide band I and amide band II are used as characteristic bands for chitosan *N*-acetylation. Chitosan samples obtained from the two fungal species in this experiment showed peaks near 1655 cm^{-1} which represents amide I bond, although sometimes peaks near 1630 cm^{-1} is also taken along with peaks at 1655 cm^{-1} to identify presence of amide I bond [110]. Sharp peaks were also observed near 1480 cm^{-1} which indicates amide II bond. These peaks attributes to C=O stretching [78] and N-H bending vibrations [111].

Intense and broad peaks were observed near 3450 cm^{-1} for both of the chitosan samples. This peak represents OH stretching vibration and it is often used as a reference band to determine degree of deacetylation of chitosan [109]. Broad peaks near this region were also caused by amine N-H symmetrical vibrations [112].

Another characteristic peak was observed near 1070 cm^{-1} or 1030 cm^{-1} that represents the polysaccharide structure caused by skeletal vibrations involving C-O-C stretching bands.

5.3.2. Degree of Deacetylation of Chitosan Samples

Results from FTIR data were used to determine the degree of deacetylation (DD%) of the two fungal species. DD% of *Aspergillus niger* and *Saccharomyces cerevisiae* chitosan were found to be 61.39% and 63.41% respectively. On the other hand, DD% obtained using acid base

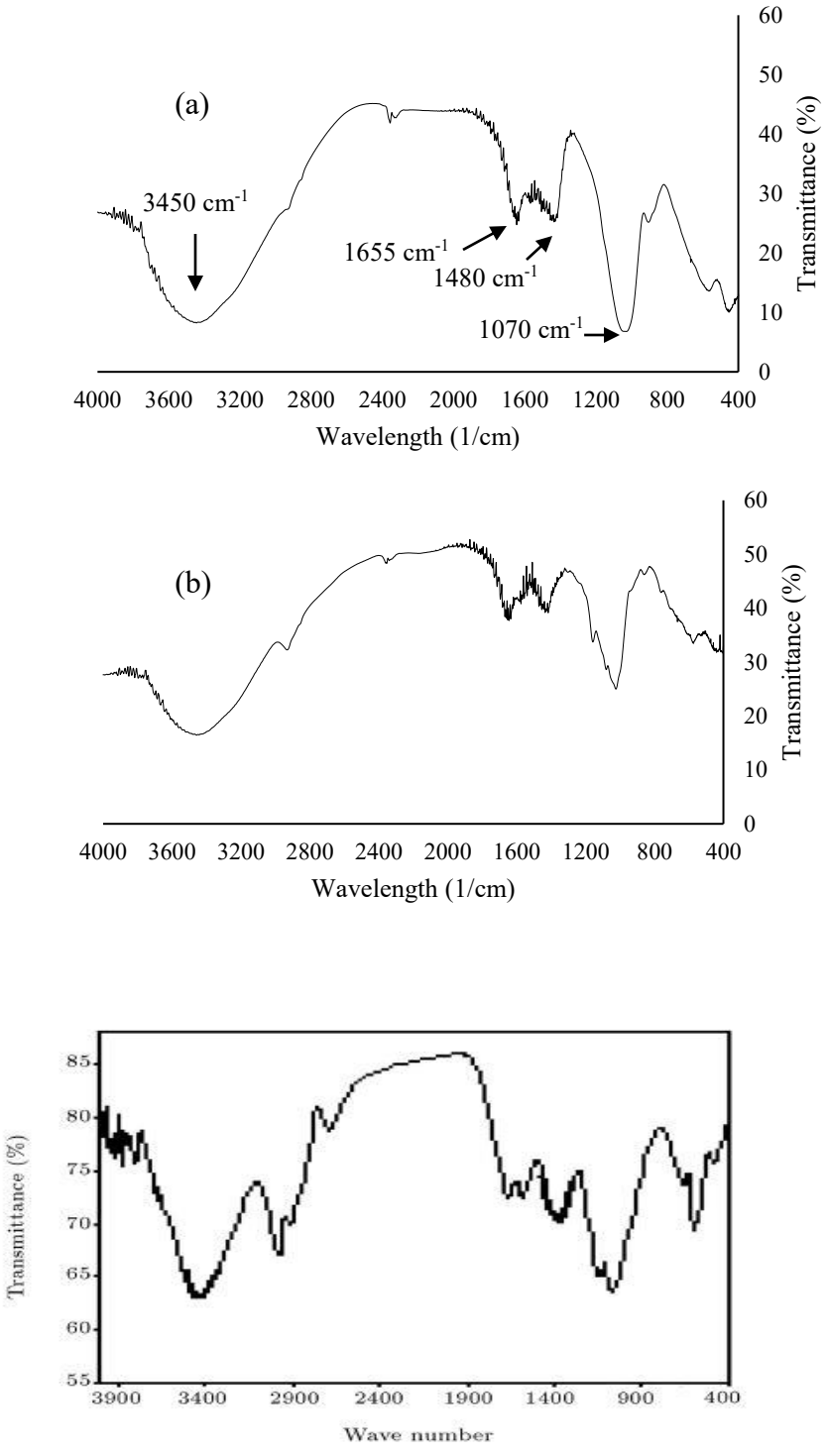


Figure 5.1: FTIR graphs of (a) *A. niger* chitosan; (b) *S. cerevisiae* chitosan and (c) Data from a previous study [84]

titration method was 59.61% and 53.28% respectively. Acid base titration showed lower DD% in both the cases. The possible reason could be poor experimental accuracy, precipitation of chitosan during the experiment and difficulty of mixing for higher pH values [78].

Value of the DD% is a measure that separates chitosan from chitin. Generally, chitin having degree of deacetylation greater than 50% becomes chitosan [94]. In terms of DD%, good quality chitosan was obtained from the present study.

Chitosan deacetylation from *Aspergillus niger* was observed by Danny Javier Balanta Silva *et al.* [95] to be 74.15% which was comparable to the currently obtained results. Chitosan extracted from waste mycelia of *A. niger* from a citric acid production plant reported a chitosan deacetylation of 73.6% [96]. Bhuvaneshwari and Sivasubramanian [97] reported a degree of deacetylation from A. niger chitosan to be 85.9%.

The values of degree of deacetylation depend upon several factors such as fungal species, strain, culture media of fungi etc. For example, S. Chatterjee *et al.* [98] reported that degree of deacetylation ranged from 82.8% to 89.8% for *Mucor rouxii* as different culture media was used. Another study by Yang Lei *et al.* [99] revealed that deacetylation varied between 86.5% and 91.27% with change of culture media for *Rhyzopus oryzae*. Three fungal species namely *Mucor rouxii*, *Cunninghamella elegans* and *Rhizopus sp.* were studied by M. Ghareib *et al.* [100]. The study reported the deacetylation to be 80.3%, 80.3% and 81.5% respectively. Vaingankar and Juvekar [92] found 79.89% deacetylation from *Absidia butleri* chitosan. A medicinal fungus (*Fomitopsis pinicola*) was investigated for chitosan production and the obtained degree of deacetylation was 73.1% [101].

5.4. Antimicrobial Activity of Chitosan

Antimicrobial activity experiments were carried out using chitosan obtained from *S. cerevisiae* because it was comparatively a better producer of chitosan with higher yields and lower time required to reach the maximum production capacity. The degree of deacetylation (DD%) was also good.

5.4.1. Zone of Inhibition using Chitosan Films

Chitosan films were used at a chitosan concentration of 2 g/L to find zones of inhibition against *S. aureus*. The experiments did not show any zone of inhibition indicating that chitosan film did not possess an antimicrobial activity against the selected species. Moreover, the bacterial species grew on the thin chitosan films. A previous study by Foster and Butt [115] reported that films made with chitosan, which was purchased from Sigma Aldrich (>85 DD%), showed no zone of inhibition against *S. aureus* and *E. coli*.

On the other hand, a previous study had reported that chitosan (from a commercial source) films showed inhibitory effect against *Listeria monocytogenes* found in food surfaces [116]. Interestingly, the films produced for that study was more gel-like instead of being dry that was used in the present study. Chitosan exhibits antimicrobial nature due to surface-surface interaction between chitosan polymer chain and microbial cell wall [1].

Thus, the present study suggested that dry chitosan films did not interact with bacterial cell wall.

5.4.2. Zone of Inhibition using Chitosan Solution

The results of the present study showed clear zones of inhibition against gram positive *Staphylococcus aureus* using chitosan solution at different concentrations. The larger the diameter of the zone of inhibition, the more effective was the antimicrobial action against *S. aureus*. Although, the control experiment (1% acetic acid) had shown zone of inhibition against the species, results with chitosan were consistently better, validating the effect of antimicrobial activity of the extracted chitosan. From the experiments, maximum diameter of inhibition zone was obtained at 2 g/L chitosan concentration and the value was 15.48 ± 0.07 mm. A previous work had reported that for chitosan extracted from cuttlebone of *Sepia kobiensis*, a value of inhibition zone between 11 mm to 15 mm was considered as good activity against *S. aureus* [102]. Thus, in terms of inhibition zones, the fungal chitosan showed promising results for use of antimicrobial activity in applications where antibiotic usage is undesirable such as chitosan based films for food preservation [113], chitosan bandages for wound healing [114] etc.

In the present study, when the chitosan concentration exceeded 2 g/L, a decrease in zone of inhibition diameter was observed. This incident has a possible explanation in terms of polymer chain arrangements. With lower concentration of polymer, interaction between the neighboring chains becomes lower which leads to better molecular distribution in the solvent and therefore availability of the charged sites for external coupling can be maximized [103]. Lower number of chain-chain bonds also increases interfacial interactions of active sites [104].

On the other hand, formation of hydrogen and covalent bonds amongst the functional groups of the chitosan chains becomes higher when chitosan concentration rises which reduces dispersion and leads to a coiled conformational structure [105]. This creates a spatial restriction to the

functional groups of chitosan chain and results in a less number of charged sites available for binding with the bacterial cell wall [106].

Annaian Shanmugama *et al.* [102] reported a good antimicrobial activity (inhibition zone diameter 11 mm to 15 mm) of chitosan from cuttlebone of *Sepia kobiensis* against *S. aureus*. Rejane C. Goy *et al.* [106] used commercial chitosan and observed a maximum zone of inhibition at 1.5 g/L concentration of chitosan against the same species and the value of zone diameter was 9.2 mm. Works of Assainar and Nair [107] revealed that different strains of *S. aureus* showed different diameters of inhibition zone while using commercial chitosan and the maximum was found to be 30 mm.

CHAPTER 6

CONCLUSION AND FUTURE WORK

CONCLUSION AND FUTURE WORK

6.1. Conclusion

The present study aimed to extract chitosan from some locally available fungal sources. Between the two fungal species studied, *Saccharomyces cerevisiae* was found to be a better producer of chitosan compared to *Aspergillus niger*. *S. cerevisiae* had shown a maximum chitosan yield of 20.85 ± 0.35 mg/g at 4th day, while *A. niger* had shown a maximum yield of chitosan to be 16.15 ± 0.95 mg/g at 12th day. In terms of yield and days of treatment, *S. cerevisiae* was comparatively more efficient than *A. niger* in chitosan production.

Quality of the chitosan samples were evaluated by determining their degree of deacetylation (DD%). Using the FTIR spectra, DD% of chitosan from *A. niger* was determined to be 61.39%. On the other hand, DD% of chitosan from *S. cerevisiae* was found to be 63.41%. Using acid base titration method, DD% of chitosan from *A. niger* was calculated to be 59.61%, while chitosan obtained from *S. cerevisiae* showed a DD% of 53.28%.

Thin films made from extracted chitosan exhibited no zone of and hence did not show antimicrobial activity against *Staphylococcus aureus*. On the other hand, chitosan solutions in 1% acetic acid showed substantial antimicrobial activity against selected bacterial species by exhibiting clear zones of inhibition. At chitosan concentration of 2.0 g/L, the zone of inhibition was the maximum and the diameter of the inhibition zone was 15.48 ± 0.07 mm.

The availability, ease of maintenance, relatively simple culture methods, good yields of extractable chitosan having good deacetylation and antimicrobial property clearly indicates that fungi can be an excellent alternative source for chitosan production.

6.2. Recommendations

For purposes of increasing yields of chitosan from fungal species, several approaches may be undertaken.

- Growth media composition for *Aspergillus niger* can be optimized for higher chitosan yields. Addition of different nutrients, sucrose and salts can significantly increase chitosan yield. Other factors such as temperature, pH etc. might also be optimized to improve the results.

- *Saccharomyces cerevisiae* can be cultured using yeast culture media so that larger number of fresh cells can afterwards contribute to sporulation under nutrition starvation conditions. Potassium acetate can be applied instead of sodium acetate as sporulation medium [117].

- For better and more accurate characterization of samples of chitosan from fungal species, further experiments can be done such as: NMR (Nuclear Magnetic Resonance) Spectroscopy, X-ray Spectroscopy, UV Spectroscopy, more accurate pH-metric titrations etc. NMR is considered the most accurate and often taken as a standard for other characterization methods, but it is highly expensive [5.34]. X-ray Spectroscopy is the mostly used and versatile means of characterization for all forms of materials including chitin and chitosan and UV spectroscopy can detect *N*-acetyl-glucosamine in chitin and chitosan samples [5.32]. Non-spectroscopic methods include pH-metric titration which can be an inexpensive and quick method to obtain nearly accurate results.

- In the antimicrobial activity testing, the negative control experiments to inhibit *S. aureus* were done using 1% acetic acid, which gave a zone of inhibition of 9.75 ± 0.13 mm. Instead of using 1% acetic acid to make chitosan solutions, 0.1% or 0.2% acetic acid can be used. The lower concentration of acetic acid as a negative control will ensure a clear demonstration of chitosan activity against *S. aureus*.

6.3. Scope of Future Works

Based on the findings of this study, the followings are proposed for extending the present body of work.

- Various types of fungal species such as *Rhizopus oryzae*, *Mucor rouxii*, *Gongronella butleri*, *Absidia* spp. etc. and mushrooms such as *Agaricus bisporus* can be investigated, since literature indicates that they give high chitosan yields. Also, waste biomass of *A. niger* from a citric acid production plant can be utilized. Techno-economic and environmental analysis can be carried out to realize if this is a cost effective, clean method of use of spent biomass from industry.

- Submerged culture technique can be applied to grow *A. niger* which would increase the biomass production and chitosan production at a greater extent.

- As sporulation is necessary to obtain chitosan from yeasts, sporulation media and environmental factors such as temperature and pH can be optimized to get higher chitosan yields from *S. cerevisiae* [56].

- To obtain higher degree of deacetylation, deacetylation reaction might be optimized using various concentrations of sodium hydroxide.

- Disc diffusion method can be applied to test antimicrobial efficacy of chitosan along with well diffusion method. In disc diffusion method, chitosan solution is poured on 6 mm diameter sterile filter papers and allowed to dry at room temperature. Afterwards they are placed onto previously prepared bacteria mounted agar plates and observed to find zones of inhibition. Experiments can be done to get minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC). Minimum concentration of the antimicrobial agent that inhibits bacterial growth is considered as minimum inhibitory concentration (MIC). For example, if the bacterial species is cultured in liquid medium, the lowest level of antibacterial agent concentration at which the liquid medium does not show any turbidity can be taken as MIC of that agent for that particular species. On the other hand, MBC is a measure that involves the lowest concentration of antimicrobial agent that can kill bacteria in a previously cultured broth. MIC and MBC measurement can be utilized to demonstrate antimicrobial efficacy of chitosan solutions in the future studies.

- Different bacterial species such as gram positive *Stahpylococcus* spp., *Streptococcus* spp., *Clostridium*, *Listeria* etc. and gram negative *E. coli*, *Helicobacter pylori*, *Klebsiella pneumoniae* etc. and various human pathogenic fungal species such as *Candida albicans* can be taken in order to test antibacterial and antifungal efficacy of the extracted chitosan.

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APPENDIX

DETERMINATION OF DEGREE OF DEACETYLATION

1. FTIR (Fourier Transform Infrared Spectroscopy) Method

Chitosan from *Aspergillus niger*

Transmittance at 1655 cm^{-1}, T_{1655} = 27.85 %

Therefore, Absorbance at 1655 cm^{-1}, A_{1655} = 2- log (T_{1655}) = 0.555

Transmittance at 3450 cm^{-1}, T_{3450} = 8.336 %

Therefore, Absorbance at 3450 cm^{-1}, A_{3450} = 2- log (T_{3450}) = 1.079

From Equation 3.1, *DD* % = 100 - (A_{1655}/A_{3450}) × (100/1.33)

= 61.39 %

Chitosan from *Saccharomyces cerevisiae*

Transmittance at 1655 cm^{-1}, T_{1655} = 41.3 %

Therefore, Absorbance at 1655 cm^{-1}, A_{1655} = 2- log (T_{1655}) = 0.384

Transmittance at 3450 cm^{-1}, T_{3450} = 16.56 %

Therefore, Absorbance at 3450 cm^{-1}, A_{3450} = 2- log (T_{3450}) = 0.781

From Equation 3.1, *DD* % = 100 - (A_{1655}/A_{3450}) × (100/1.33)

= 63.41 %

2. Acid Base Titration Method

Chitosan from *Aspergillus niger*

V_1 = 1.2 mL, V_2 = 7.9 mL, m = 0.2 g

Using Equation 3.2, DD % = $2.03 \times (V_2 - V_1) / [m + 0.0042 \times (V_2 - V_1)]$

= 59.61 %.

Chitosan from *Saccharomyces cerevisiae*

V_1 = 1.9 mL, V_2 = 7.8 mL, m = 0.2 g

Using Equation 3.2, DD % = $2.03 \times (V_2 - V_1) / [m + 0.0042 \times (V_2 - V_1)]$

= 53.28 %.