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Research Article

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Saccharomyces Cerevisiae as an untapped source of fungal chitosan for antimicrobial action[1]

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ABSTRACT

Despite being widely available, *Saccharomyces cerevisiae* has not been widely explored for direct extraction of chitosan biopolymer for antimicrobial applications. In our study, *S. cerevisiae* from Baker's yeast and *Aspergillus niger* from moldy onion extracts are studied as alternative sources of chitosan; and *S cerevisiae* chitosan tested for antimicrobial efficacy. The properties of *S. cerevisiae* chitosan are compared with moldy onion chitosan and shrimp chitosan extracted from shrimp shells. Chitosan extracted from *S. cerevisiae* is tested for antimicrobial efficacy against *Staphylococcus Aureus.*

The maximum yields of fungal chitosan are 20.85 ± 0.35 mg/g dry *S. cerevisiae* biomass at 4th day using a culture broth containing sodium acetate, and 16.15 ± 0.95 mg/g dry *A. niger* biomass at 12th day. The degree of deacetylation (DD%) of the extracted fungal chitosan samples from *S. cerevisiae* and *A. niger* are found to be 63.4%, and 61.2% respectively, using Fourier Transform Infrared Spectroscopy. At a concentration of 2 g/L, *S. cerevisiae* chitosan shows the maximum inhibition zone diameter of 15.48 ± 0.07 mm.

[1] Part of the results of this manuscript have been presented in the Fifth International Conference for Chemical Engineering, ICChE 2017.

Baker's yeast *S cerevisiae* biomass and *A. niger* from moldy onions has not been previously explored as a source of extractible fungal chitosan. This study gives insight that *S. cerevisiae* and *A. niger* from agricultural or industrial wastes could be a potential biomass source for production of the chitosan biopolymer. The *S. cerevisiae* chitosan displayed effective antimicrobial properties against *S aureus*, indicating the viablitiy of *S cerevisae* as a resource for extraction of high-quality chitosan.

Keywords: Baker's yeast, *Aspergillus niger*, FTIR, degree of deacetylation, zones of inhibition

1. INTRODUCTION

Chitin and chitosan are valuable biopolymers found in biomass resources. Removal of acetyl groups from a percentage of the N-acetyl-D-glucosamine units in chitin chains leads to formation of chitosan, and if the copolymer has higher than 50-60% D-glucosamine it is categorized as chitosan.[1] [2] (Fig 1).

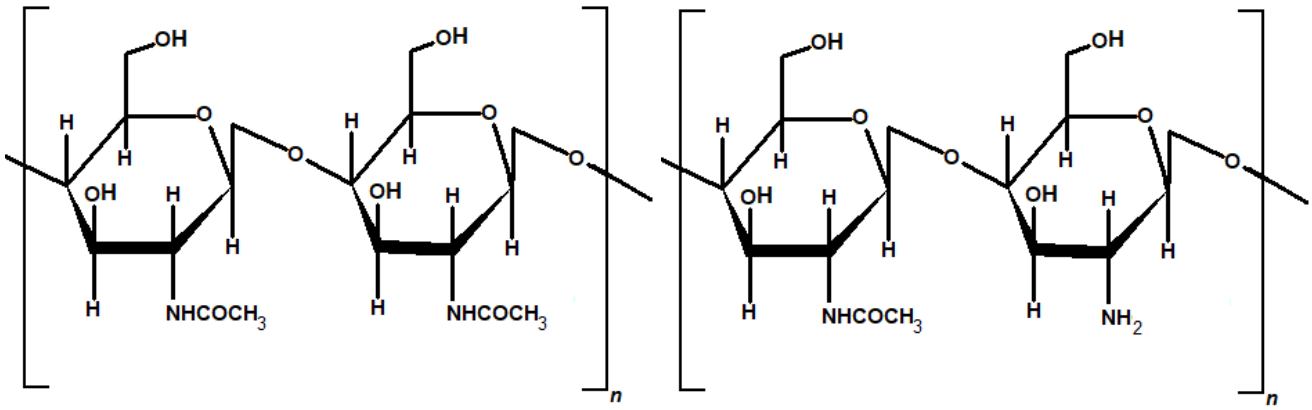

Fig 1 Molecular structure of chitin and chitosan

The polycationic nature of chitosan, high charge density, and presence of free amine groups in the chain enables it to go through chemical and enzymatic modifications as well as crosslinking.[3][4] Consequently, chitosan has its applicability in diverse areas. It is used in biomedical field such as drug delivery, gene transport, wound healing, stem cell technology and tissue engineering due to its exceptional biocompatibility and antimicrobial activity.[5]-[6] Chitosan is also a biodegradable polymer, and used in agricultural sectors as well as pharmaceutical, food and in some fields of biotechnology such as enzyme immobilization,[7] and in environmental applications in water treatment plants as flocculent or to remove metal ions from wastewater. [8]·[9]·[10]

Currently, chitin obtained from crustacean shells are the major source of industrial chitosan production. Conversion procedure of chitin into chitosan poses challenges as the product quality shows a dependence on the source of chitin.[11] Some other drawbacks of the current industrial production using crustacean shells involve the variation of raw material availability, depending on season and location. Also the chitin requires harsh processing steps such as use of strong acids and alkalis. [12] In order to obtain sustainable and inexpensive routes to produce chitosan, studies have been devoted to find alternative sources of chitin and chitosan.[13] Since chitosan is a major structural component of fungal cell wall, research is recently being focused on fungal chitosan production.[14][15] The amount of chitosan present in the cell wall of fungi varies widely depending on the species and strains of fungi.[12] Numerous fungal species from the class Zygomycetes such as *Aspergillus*, *Rhizopus*, *Absidia*, *Gongronella* have been studied for chitosan extraction. [14][16][17] Chitosan is synthesized in the spores of *Saccharomyces cerevisiae* (also known as baker's yeast) which is a type of unicellular fungi.

As global market for chitinaceous products is expected to reach a volume of $4.2 billion by 2021[18], it is worthwhile to investigate local biota, agricultural wastes and industrial wastes of biotech and food industries as potential sources for chitosan production.[19][20] *Saccharomyces cerevisiae* forms the

second most commonly generated residue in breweries and at the end of fermentation, there is an excess of biomass which can be used for chitosan production. Upto 0.9 million tons of spent brewer's yeast are produced yearly and these can be used for various purposes such as production of β-glucans, monooligosaccharides, B vitamins, minerals and yeast extracts[21] [22]. Many reviews have reported studies where the spent *S cerevisiae* biomass is used for purposes of bioremediation, suggesting that the biomass can be used for industrial repurposing. [23] [20] Brady *et al* separated the cell wall components of *S cerevisiae* for heavy metal adsorption, but reported that the chitinaceous material recovered was contaminated [24].

Studies have been done to understand the pathway of chitosan formation in spore walls of budding yeast.[25] Studies have also reported using whole yeast (*S. cerevisiae)* as antimicrobial agents.[26] It is widely understood that the cell wall of this readily available species has approximately 2% chitin, most of it bound to β-1,3-glucan[27]. In spite of this, to the best of our knowledge, no study has been reported focusing on culture of *Saccharomyces cerevisiae* as a potential source for extractible pure chitosan for biomedical applications.

Aspergillus niger has been the main organism used for biotechnological production of citric acid since the early 1900s. [28] By some estimates, upto 300,000 tons of waste *Aspergillus niger* biomass per year is generated from citric acid production[29]. The fungal genus *Aspergillus* constitutes many species, some of which include opportunistic pathogens or toxin producing species, yet industrially important species, such as *Aspergillus niger*. [30] Additionally, fungal biomass is potentially available as microbial growth on agricultural products or wastes[31]. *Aspergillus niger* is the naturally occurring fungal species in moldy onions, grapes and other agricultural harvest. Thus, moldy onions can be used as an inexpensive and readily available source of *A. niger*. [32] To our knowledge, moldy onions have not previously been used as a source for harvesting the *Aspergillus spp*. for chitosan extraction.

Among the many biomedical applications of fungal chitosan, an important application coming into focus recently is the antimicrobial action. Since synthetic antibiotics have led to resistant microorganisms in the recent years, these natural polymers offer a promise of an antibiotic which will not induce resistance, and having benefits of biocompatibility, non-toxic properties and relative low cost of production. [33]

Antimicrobial action of the chitosan from various sources of chitosan can vary. [34] A particular type of chitosan may be suitable for lowering the activity of one type of microbe, but may not work as effectively on others. Factors such as source of chitosan, molecular weight (MW), degree of deacetylation (DD%) and pH of the environment also affects the action against microbes.[5][35][36]To investigate the antimicrobial action of natural biopolymers, *Staphylococcus aureus* is extensively studied as a gram-positive bacterial species as it is widely spread and is commonly associated with skin and wound infections. [33]

In the present study, *Saccharomyces cerevisiae* (baker's yeast) and a locally isolated strain of *Aspergillus spp.* (from moldy onions) were investigated as sources of fungal chitosan production. The degree of deacetylation of the chitosan samples extracted from the fungal sources were determined and compared to that of shrimp shell-based chitosan samples. Antimicrobial property of fungal chitosan from the source *S. cerevisiae* was also evaluated against *Staphylococcus aureus* using zone of inhibition method. This is the first reported study of using local (wild) strains of fungus for chitosan extraction. To the best of our knowledge, this is the first study investigating *S. cerevisiae* as a directly extractable chitosan source, and also the first study reporting the subsequent testing of the *S. cerevisiae* chitosan for antimicrobial action.

2. MATERIALS AND METHODS

2.1 Microorganisms

Microorganisms used in this study were *Saccharomyces cerevisiae, Aspergillus niger*, *Staphylococcus aureus*. Baker's yeast was used as procured from local market as source of *Saccharomyces cerevisiae*. *Aspergillus niger* spp. was isolated from black molded onions which were procured from local market. *S. aureus* was isolated from human nasal specimen.

2.2 Chemicals

Shrimp (*Macrobrachium rosenbergii*), potatoes (*Solanum tuberosum*) and onions (Allium *cepa*) were bought from local market. Dextrose Monohydrate (GlaxoSmithKline, UK) was also bought from local market. Deionized and distilled water (2-3 µS/cm) obtained using laboratory scaled distilled water plant was used. Laboratory grade ethanol (96% v/v), acetic acid, (CH_3COOH, 100% glacial), hydrochloric acid, (HCl, 37%, fuming), sodium chloride (NaCl), sodium hydroxide pellets (NaOH), sodium nitrate ($NaNO_3$), sulfuric acid (H_2SO_4, 95-97%) and acetone (100%) were obtained from Merck, Germany. Sodium acetate was obtained from Qualikems India and ammonium acetate from Indradhanush Chemicals, India. Biological agars and media, mannitol salt agar base (HiMedia Laboratories, India), Mueller Hinton agar (Lab M Limited, UK), and Potato Dextrose Agar (PDA) (Oxoid, UK) were obtained. Glucose (GlaxoSmithKline, UK) was used as obtained. Commercially available Tetracycline (Tetrax 500 capsule, SQUARE Pharmaceuticals Ltd.) and Ciprofloxacin (Ciprocin 500mg tablet, SQUARE Pharmaceutical Ltd) were obtained.

2.3 Isolation of the Microorganisms

2.3.1 Isolation of* Aspergillus spp. *from local source. Onions with black molds were collected from local market. Using a sterile inoculum loop, spores of the black mold were taken aseptically 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. Detailed description of culture media preparation is

provided in Section S1 in the Supporting Information document. After 3 days of incubation at 37ºC, colonies with black conidia became clearly visible. A single colony of *Aspergillus spp.* 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 labelled and disposed after 10 min exposure to UV light.

A sample of the pure culture of the species was collected after 7 days of incubation and sent to Invent Technologies, Bangladesh. Fungal identification using ITS region sequencing was carried out to determine if the fungal species isolated was *Aspergillus Niger.*

***2.3.2 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. A 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, bacterial colonies appeared as yellow clusters. 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. A sample of the pure culture of the species was collected after 24 h of incubation and sent to Invent Technologies, Bangladesh. Bacterial identification using 16s RNA gene sequencing was carried out to determine if the bacterial species isolated was *Staphylococcus aureus*.

2.4 Culture Conditions

***2.4.1 Conditions for* Saccharomyces cerevisiae**. 1 g of dry Baker's yeast (as a source of *Saccharomyces cerevisiae*) was washed with 1% sodium acetate solution, centrifuged in a centrifuge (Uni Gem MR, Hero Lab, Germany) and re-suspended in 200 mL of 1% sodium acetate. For the control experiments, the dry

yeast cells were washed with distilled water, centrifuged and re-suspended in 200 mL distilled water. For further confirmation of the affect of sporulation conditions, the dry yeast cells were washed with 1% ammonium acetate and re-suspended in 200 mL 1% ammonium acetate solution. All the samples were incubated at room temperature on an orbital shaker (Phoenix Instrument RS-OS 20, Germany) at 150 rpm. The fungal biomass at different days were centrifuged and dried at 60ºC. The dried fungal biomass was weighed and used for subsequent chitosan extraction.

***2.4.2 Surface Culture of* Aspergillus niger** 15 mL of sterilized 0.85% NaCl solution was poured on to a seven-day-old agar plate of *Aspergillus niger*. 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 used as inoculum for surface culture. The preparation of Potato Dextrose broth (PDB) medium is described in the Supporting Information document, Section S1. 1 mL of the inoculum was poured into each 250 mL Erlenmeyer flask containing 50 mL of sterilized PDB medium and kept at room temperature. After desired culture periods, fungal mats were taken out from the flasks, washed with distilled water, dried at 60ºC and used for chitosan extraction.

2.5 Fungal Chitosan Extraction

The extraction procedure for chitosan extraction from fungal biomass from various fungal species has some commonalities in principle. The procedure has been modified from the work of Maghsoodi *et al.*[37] and is briefly described below.

2.5.1 Grinding of fungal biomass. The dried fungal biomass from culture of either *S. cerevisiae* or *A. niger* was ground to fine powder using a mortar-pestle. For fine grinding, a commercial waring blender was used after size reduction in the mortar and pestle, Mass of ground fungal matter was then measured to obtain an estimate of dry fungal yield.

2.5.2 Treating with alkali. The dried fungal mass was mixed with 1 M NaOH at 1:50 (w/v) ratio. The mixture was homogenized using a commercial waring blender. It was then heated at 120ºC for 30 min in an electric oven to denature the proteins in the fungus. The heated content was centrifuged at 5000 rpm for 10 min using a centrifuge (Uni Gem MR, Hero Lab, Germany). The solid matter obtained was placed on a filter paper (Whatman no. 40, GE Lifesciences) and washed continuously with distilled water till the filtrate reached a pH of 7. The residue on the filter paper was dried in an oven at 40ºC overnight and collected as the Alkali Insoluble Material (AIM). The purpose of the step is to denature and dissolve most of the fungal matter in alkali, leaving most of the chitosan undissolved as the solid state in the AIM. The weight of AIM is noted and sent for the next step.

2.5.3 Dissolution of Alkali Insoluble Material (AIM) in acetic acid. The AIM was ground and homogenized with 2% acetic acid at 1:50 (w/v) ratio. The mixture in acetic acid was heated at 95 ºC for 6 h in an oven to ensure the dissolution of chitosan in the acetic acid. After cooling down, the mixture was centrifuged at 5000 rpm for 20 min, and the supernatant liquid was collected as the dissolved chitosan solution.

2.5.4 Precipitation of the chitosan. The dissolved chitosan was then made up to an alkaline pH, to decrease the solubility of the chitosan and precipitate it out. 4 M NaOH was added dropwise until the pH of the solution reached around 10 and the chitosan precipitation was visible. The chitosan was centrifuged at 5000 rpm for 10 min and washed four times with distilled water to bring the pH to 7. Finally, the sample was rinsed with ethanol, centrifuged and dried at 40ºC. The powder thus obtained was weighed and sent for characterization or further experiments.

2.6 Shrimp Chitosan Extraction

The procedure followed has been modified from No and Myers[8] and is described briefly in Section S2 in the Supporting Information document.

2.7 Calculation of Yield of Chitosan

The yield of fungal biomass obtained for every liter of PDB culture media and percentage of Alkali Insoluble Mass (AIM) in the fungal biomass, was calculated as well. Chitosan yield (mg/g fungal biomass) from either *Saccharomyces Cerevisiae* or *Aspergillus niger* was also determined by the following expression.

$$\text{Yield} = \frac{\text{dry mass of chitosan (g)}}{\text{dry fungal biomass (g)}} \times 1000 \qquad (1)$$

2.8 Characterization for Degree of Deacetylation

Degree of deacetylation (DD%) corresponds to the percentage of deacetylated glucosamine units over the total number of glucosamine units in the chitosan chain and determines an important characteristic of chitosan.

2.8.1 Degree of deacetylation (DD) using Fourier Transform Infrared Spectroscopy (FTIR). Infrared spectroscopy analysis of the chitosan samples was done using a Shimadzu FTIR spectrophotometer. 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 mounted in the spectrophotometer. Frequency range of the spectra was from 4000 to 400 cm^{-1} with 45 scans. The resolution was 2 cm^{-1}

Using the data obtained from FTIR spectroscopy of chitosan extracted from *Saccharomyces cerevisiae*, *Aspergillus niger* and shrimp, the corresponding degrees of deacetylation were calculated and the details of the calculation are discussed in the Supporting Information document (Section S3).

2.8.2 Degree of Deacetylation using Acid Base Titration Method. Dried chitosan extracted from either *A. niger, S. cerevisiae* and shrimp shells was dissolved in 0.1 M HCl at 10% w/v. The solution was titrated using 0.1 M NaOH. The pH of the system was measured and plotted against volume of NaOH, V, during titration. The dpH/dV values were also calculated and plotted for increasing volumes of NaOH. Degree of deacetylation (DD%) was determined using the calculation procedure described in detail in the Supporting Information document (Section S4).

2.9 Determination of Antibacterial Activity by Zone of Inhibition

Antimicrobial activity of chitosan extracted from *Saccharomyces cerevisiae* was tested against *Staphylococcus aureus*. Chitosan solutions of various concentrations (from 0.0 to 4.0% w/v) were prepared by dissolving it into 1% (v/v) acetic acid. Bacterial stock solution was spread onto previously prepared Mueller Hinton Agar (MHA) plate. A sterile 1 mL syringe was cut cross-section wise using a sterile blade and sterilized with 70% ethanol. This was used to create evenly sized wells in the agar plate. 50 µL of chitosan solution was poured into each well. The plates were kept upside down at 37 °C for 24 h. The larger the diameter of zone of inhibition, the more effective the antimicrobial action against the bacterial species. The diameters of the zones of inhibition were measured in mm.

3. RESULTS AND DISCUSSION

3.1 Biomass Growth of Fungal species

The sporulated yeast cells (*Saccharomyces cerevisiae*) were identified under light microscope as shown in Fig S2 of the Supporting Information document.

The growth characteristics of a sample of *Aspergillus spp.* isolated from moldy onions was investigated using anaerobic 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 Fig S1(b) of the Supporting Information document. Clear appearance of conidial head with some dispersed conidiae and some attached in chains to the head indicated that the species was of the genus *A. fumigatus* or *A. niger*[38]. After 7 days of incubation of the fungal species in the media, a sample was sent for fungal identification using ITS Region Sequencing. The results showed alignment with strains of *Aspergillus niger* (accession number EU440768.1.) in the 'BLAST' sequences. This indicated with certainty that the species isolated from moldy onions belonged to *Aspergillus niger* and these were taken for further processing for isolation of fungal chitosan.

Biomass growth of the isolated *Aspergillus niger* in grams per liter of the culture media at different days of anaerobic surface culture condition is shown in Fig 2. The increase of biomass was consistent up to Day 8 which represents growth phase of the species. The maximum biomass yield was reached at a value of 7.166 ± 0.47 g/L. Then the biomass underwent a slight decrease to 6.0785 ± 0.13 g/L at Day 10. The reason for this decrease in biomass amount can be accounted for by the deficiency of nutrition in the culture medium.[39] It increased again at Day 12 and remained consistent through the next couple of days representing the stationary phase.

Previous studies reported various amounts of maximum growth for *Aspergillus niger* at different days. The biomass growth of *Aspergillus* species depends upon several environmental factors such as relative humidity and temperature.[40] 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.[41] Maghsoodi *et al.* used Sabouro Dextrose Broth and found 8.57 g/L biomass at 6th day of culture.[39] These results are comparable to the findings of the current study.

Significant amount of biomass materials was removed after the sodium hydroxide treatment. Remaining materials after centrifuge are the alkali insoluble materials (AIM) and the dry weights in gram per liter of the culture media have been included in Fig 2. Highest amount of AIM of 1.64 ± 0.28 g/L was found at Day 8. From Fig 2, approximately 22.9 % of the dry biomass was obtained as alkali insoluble materials (AIM) on Day 8. However, at Day 16, AIM was found to be approximately 24.5 % of the dry fungal biomass, which was the highest fraction of AIM obtained by the experiments. Thus, in terms of fungal biomass obtained, as well as corresponding AIM mass and the percentage of AIM obtained, there is an optimum number of days to culture the *Aspergillus* species. The optimum number of days based on biomass and percentage AIM occurred between 6 to 10 days for our study.

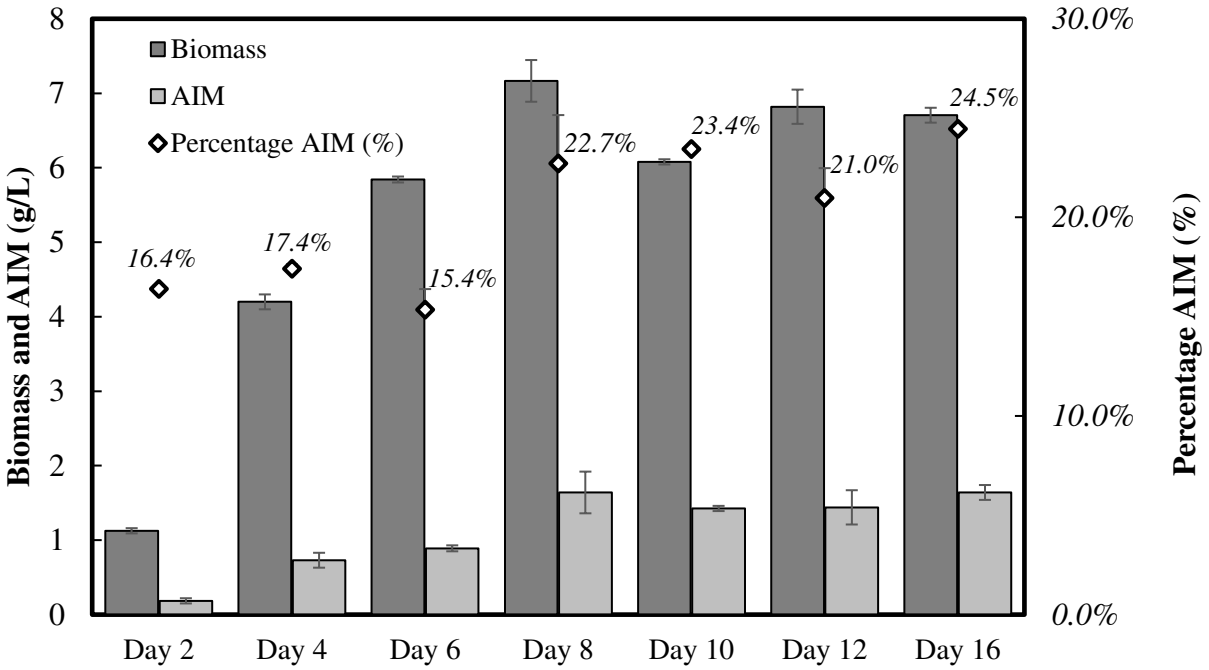

Fig 2 Dry biomass and alkali insoluble materials (AIM) of *A. niger* at different days. The dark grey bars represent the dry biomass (in grams per liter of culture media) obtained at different days of culture and the light grey bars represent the corresponding mass of alkali insoluble material (AIM) from this dry fungal biomass. The AIM as a percentage of dry biomass is marked as diamonds, and the percentages are mentioned in the figure. (Experiments have been done in triplicates).

3.2 Yields of Chitosan from fungal biomass

Under sporulation conditions (treatment with sodium acetate solution), the fungal species *Saccharomyces cerevisiae* showed consistently increasing yields which continued until Day 4. At Day 5, however, the yield became lower. Fig 3 clearly showed a maximum yield obtained at Day 4 and the value was 20.85 ± 0.35 mg/g. In the control experiment, where distilled water replaced sodium acetate in the culture broth, *S. cerevisiae* was found to give a chitosan yield of 10.05 ± 0.25 mg/g at Day 5. 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.[47]

When 1% ammonium acetate was used in the nutrient broth for harvesting the *S cerevisiae* biomass (not shown in Fig 3), it led to 16.3 mg/g chitosan production on Day 3, (almost equal to the 17.95 ± 0.45 mg/g chitosan obtained by using sodium acetate at Day 3). By Day 5, however, the ammonium acetate gave 0 mg/g chitosan production suggesting that it is possible to block meiosis and sporogenesis in yeast by adding ammonium to the sporulation medium and that sporulation inhibition sets in after a few days in the medium. This result indicates that pathways to induce sporogenesis in the yeast which increases the production of chitinaceous products in the cell wall. For instance adjusting carbon-nitrogen nutrient ratio[42], changing the nutrient source[43] or raising the temperatures of culture broths[44] are possible ways to increase chitinaceous cell wall components, leading to increased polymeric chitosan yields from *S cerevisiae*.

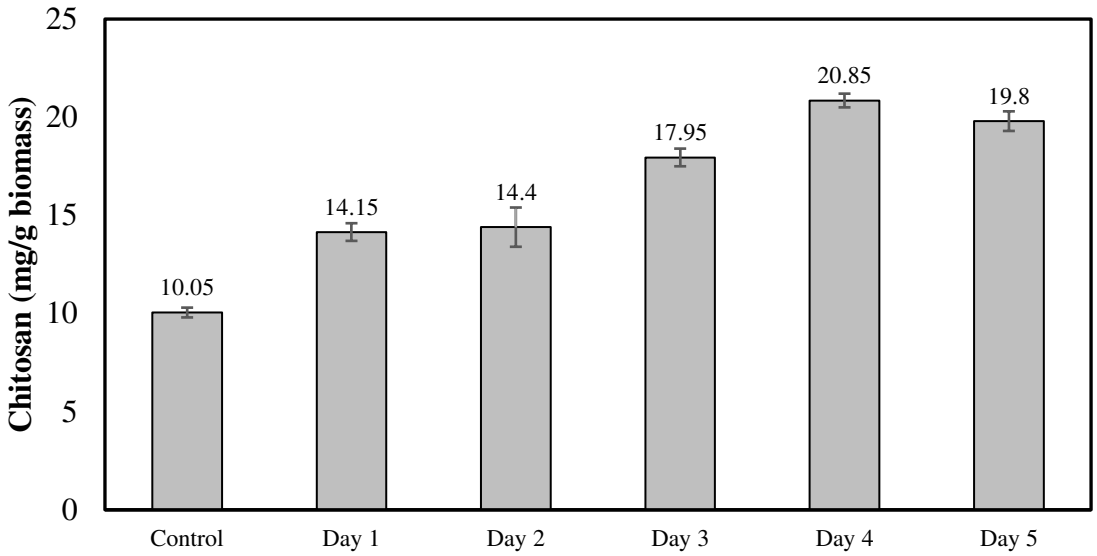

Fig 3 Chitosan production from *S. cerevisiae* at different days

Amount of chitosan extracted at different days of *A. niger* culture is shown in Fig 4. 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.

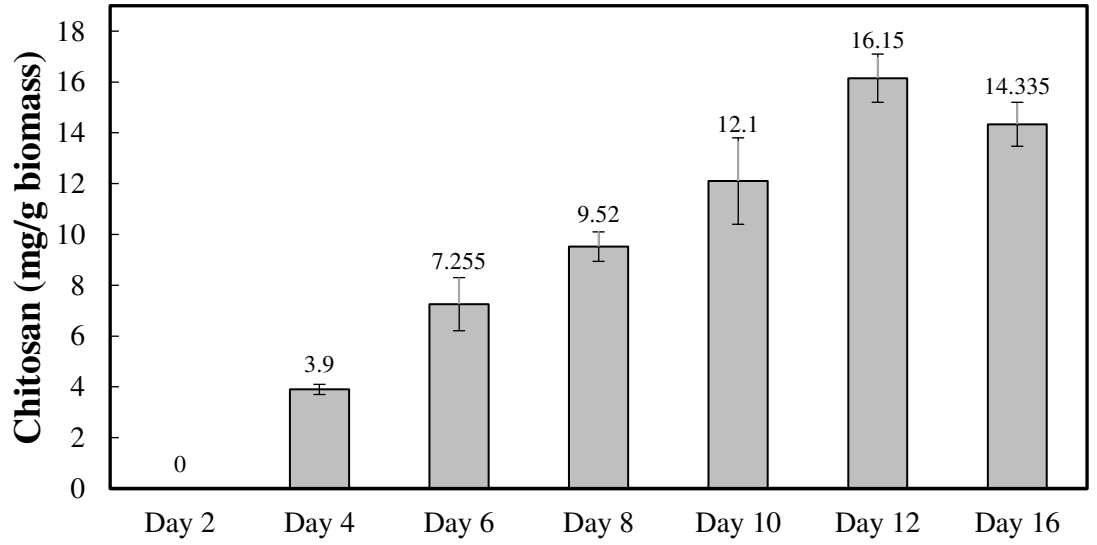

Fig 4 Chitosan production from *A. niger* at different days

For both *A. niger* and *S. cerevisiae*, chitosan yield decreased after reaching a maximum. A possible explanation for this could be the consumption of chitin and chitosan in the cell wall by the microorganism

itself due to lack of nutrients. [45] Another reason could be hydrolysis of polymers by diffused hydrolytic enzymes as the biomass became concentrated. [46] 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. [48] Previous studies showed different amounts of fungal chitosan are obtained from different fungal species. For example, Amorim *et al.* investigated chitosan yields of two fungal species, *Mucor racemosus* and *Cunninghamella elegans*.[49] 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*. [50]

The effect of heavy metals (Cu+ and Zn+) on chitosan production from other fungal species, *Absidia corymbifera* grown in corn steep liquor was tested by other authors and the obtained chitosan yield was 67.29 mg/g. [51] In the study of P. Pochanavanich *et al.*, Potato Dextrose Broth was used as culture media and chitosan yield was found to be 107 mg/g dry cell from *Aspergillus niger*. [16] Soybean meal influenced chitosan production as a nitrogen source by *Aspergillus niger* and the chitosan yield was found to be 17.053 mg/g[52], which is quite similar to the present study. In a more recent studies the yield from *A. niger* mycelium from a local citric acid production plant, was found to be 68.1 mg/g. Previous works have shown that the yield can vary even when using the same species, due to differences in fungi strain, fermentation method, temperature, composition of culture media, and also on the stage of fungal development. [53]

Chitosan extraction from *Saccharomyces cerevisiae* was a novel extension of previous work of using fungal strains as chitosan sources. Haini Zhang *et al.* used yeast spores as chitosan beads.[54] P. Pochanavanich *et al.* 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. [16]

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 further optimization of sporulation conditions.

3.3 Chitosan Yields from Shrimp Shells

The yield of chitosan from shrimp shell was found to be 260 mg/g dry shrimp shell. Several studies for chitosan extraction from local shrimp shells report yields in the range of 152 mg/g [55] to 154 mg/g [56]. Work done by Tharanathan *et al.* shows that crustaceans of different genus and species have varying chitin content, and thus, by extension, varying amounts of chitosan. [57]

3.4 Degree of Deacetylation of the Extracted Chitosan

3.4.1 Degree of Deacetylation determination using FTIR method

The three chitosan samples were extracted from local sources: *S. cerevisiae* from yeast, *A. niger* from moldy onions, and local market shrimp shells. Each of the samples were characterized using KBr disc Fourier Transform Infra-Red (FTIR) method in a Shimadzu FTIR spectrophotometer over a frequency range of 4000-400 cm^{-1} with a resolution of 2 cm^{-1}. According to Fig 5(a), the *S. cerevisiae* chitosan, peaks were found at 1644.37 cm^{-1} and 3456.55 cm^{-1}. Intense peaks were visible at 1645.33 cm^{-1} and 3457.52

cm^{-1} for *A. niger* chitosan as seen in Fig 5(b). In Fig 5 (c), the shrimp chitosan showed peaks at 1641.48 cm^{-1} and 3485.49 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.[58] Sharp peaks were also observed near 1480 cm^{-1} which indicates amide II bond. These peaks attributes to C=O stretching [59] and N-H bending vibrations. [60] Intense and broad peaks were observed near 3450 cm^{-1} for both 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.[61] Broad peaks near this region were also caused by amine N-H symmetrical vibrations.[62] 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.

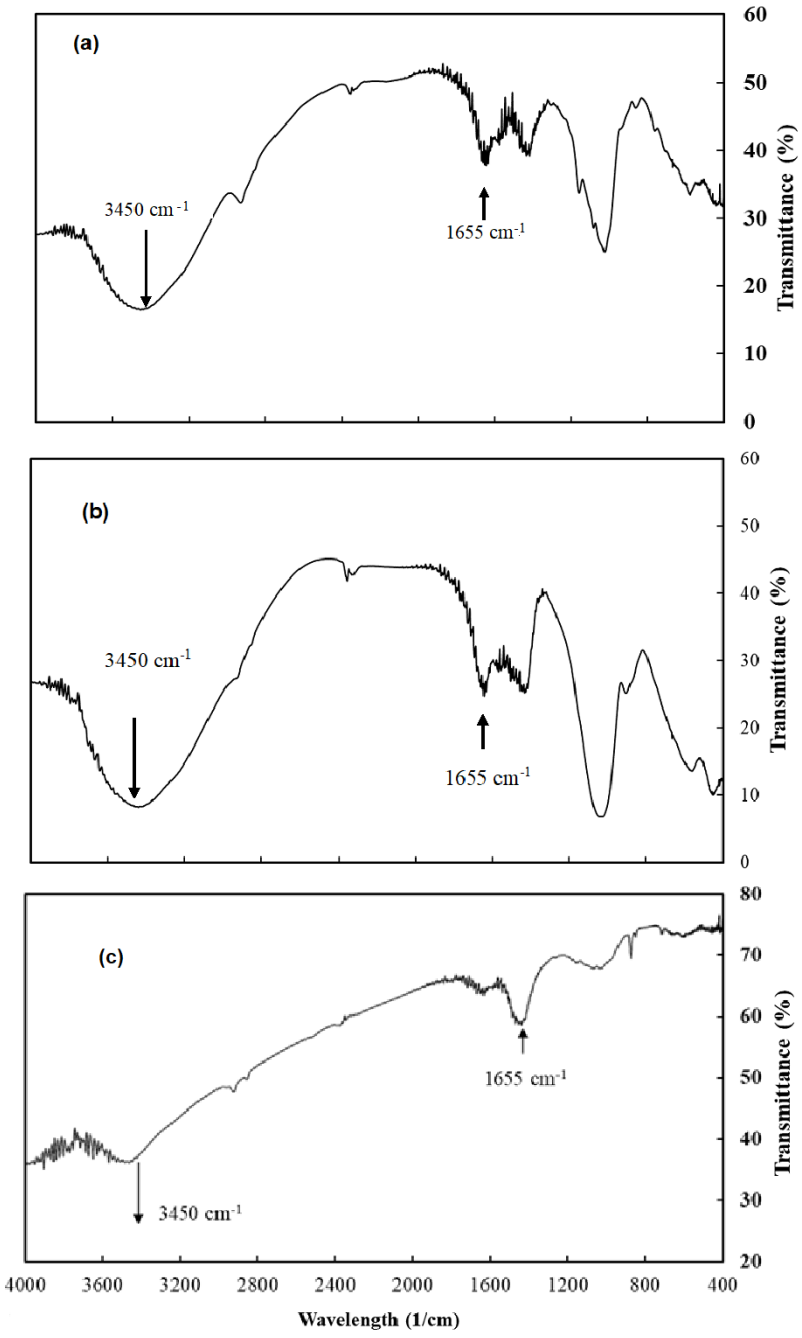

Fig 5 FTIR results of chitosan extracted from (a) *S. cerevisiae* (b) *A. niger* (c) shrimp shells

The degree of deacetylation (DD %) was determined using the Equation S2 of the Supporting Information document. Detailed calculation was shown in the Supporting Information document**.** Using the above-

mentioned formula, the degree of deacetylation was determined to be 63.4%, 61.2% and 67.44%, for chitosan samples obtained from *S. cerevisiae, A. niger* and shrimp, respectively.

3.4.2 Degree of Deacetylation determination using Acid-base titration method

The titration curves for chitosan obtained from cultures of *S. cerevisiae*, *A.niger* and shrimp are shown in Fig 6 (a-c). The curves with the circles represent the pH curve. The curve with the diamonds represents the dpH/dV curve, from which the points of inflexion V_1 and V_2 are determined.

From the figure, it is observed that adding sodium hydroxide increases the pH consistently. Significant increase in pH is visible at two distinct values of NaOH volume. This phenomenon is clearly seen in the derivative curve with the two maxima points. The first maximum appears due to excess HCl neutralization and the second one appears because no more H^+ in the amine group of chitosan is left to react with the added NaOH [59]. DD % obtained using acid base titration method for *S. cerevisiae*, *A. niger* and shrimp was 53.28%, 59.61% and 60.39% respectively.

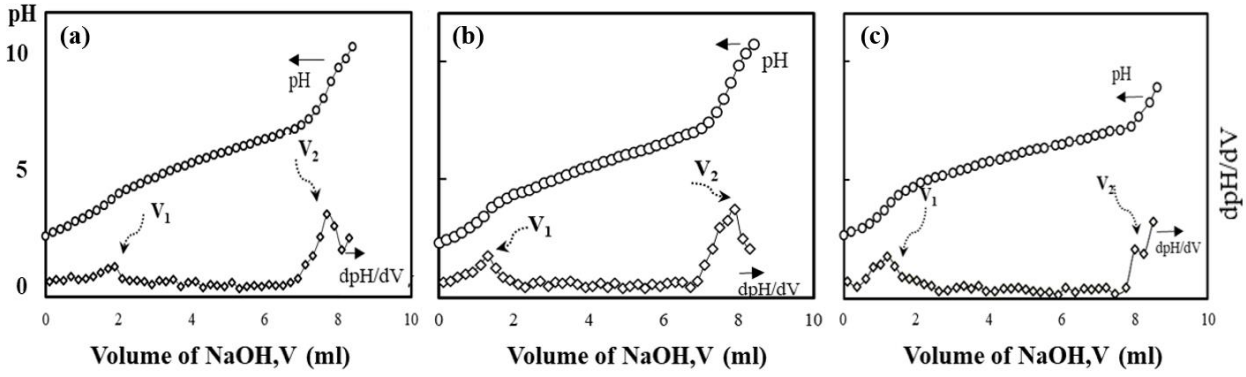

Fig 6 Acid base titration curve for chitosan obtained from (a) *S.cerevisiae* (b) *A. niger* and (c) shrimp shells

Acid base titration showed lower DD% in all the cases. The possible reason could be the precipitation of chitosan during the experiment and difficulty of mixing at higher pH values[59]. Value of the DD% is a

measure that separates chitosan from chitin. Generally, the biopolymer having a degree of deacetylation greater than 60%, found by acid-base titration, FTIR, UV-VIS, NMR or other methods is considered chitosan.[63] However since the acid-base titration method is liable to give lower values of degrees of deacetylation, this method should be used as a less expensive and readily available method, and the results should be complemented using IR, UV-VIS and NMR.

Chitosan deacetylation from *Aspergillus niger* was observed by Danny Javier Balanta Silva *et al.* to be 74.15 % which was comparable to the currently obtained results. [64] Chitosan extracted from waste mycelia of *A. niger* from a citric acid production plant reported a chitosan deacetylation of 73.6 %.[65] S. Bhuvaneshwari *et al.* reported a degree of deacetylation from *A. niger* chitosan to be 85.9 %.[65]

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.* reported that degree of deacetylation ranged from 82.8 % to 89.8 % for *Mucor rouxii* as different culture media was used. [66] Another study by Yang Lei *et al.* revealed that deacetylation varied between 86.5 % and 91.27 % with change of culture media for *Rhyzopus oryzae*. [67]Three fungal species namely *Mucor rouxii*, *Cunninghamella elegans* and *Rhizopus sp.* were studied by M. Ghareib *et al.*.[34] The study reported the deacetylation to be 80.3 %, 80.3 % and 81.5% respectively. Vaingankar *et al.* found 79.89 % deacetylation from *Absidia butleri* chitosan.[48] A medicinal fungus (*Fomitopsis pinicola*) was investigated for chitosan production and the obtained degree of deacetylation was 73.1 %.[68] For the fungal chitosan derived from *A. niger* and *S. cerevisiae*, the degree of deacetylation obtained will depend on fermentation conditions such as nutrient broth and temperature, and further studies can be carried out to correlate these parameters with DD% .

3.5 Antimicrobial Activity of Chitosan against *S. aureus* determined by Zone of Inhibition

Chitosan samples obtained from *Saccharomyces cerevisiae* were used to determine zone of inhibition in antimicrobial activity testing experiments, since it was comparatively a better producer of chitosan with higher yields.

3.5.1 Isolation of *Staphylococcus aureus*

Staphylococcus aureus was identified by visual inspection using MSAB (Mannitol Salt Agar Base) agar plates (Supplementary Information document, Fig S3). After 24 h of incubation of the bacterial species in agar plates, a sample was sent Bacterial identification using 16s RNA gene sequencing. The results showed 99% alignment with strains of *Staphylococcus Aureus* in the 'blast' sequences. One such strain has the accession number KU212139.1.

3.5.2 Zone of Inhibition

Antimicrobial activity of the chitosan solution was observed through zones of inhibition (Fig 7). Fig S2 of the Supporting Information document shows zone of inhibition of 1% acetic acid (control) and 1 g/L Tetracycline (standard). The measurable parameter that is directly linked to the extent of antibacterial activity is given by the diameter of the clear zones observed after a 24-hour incubation period. The larger the diameter of the zone of inhibition, the more potent is the antimicrobial action against the test pathogen, in this case, *Staphylococcus aureus.*

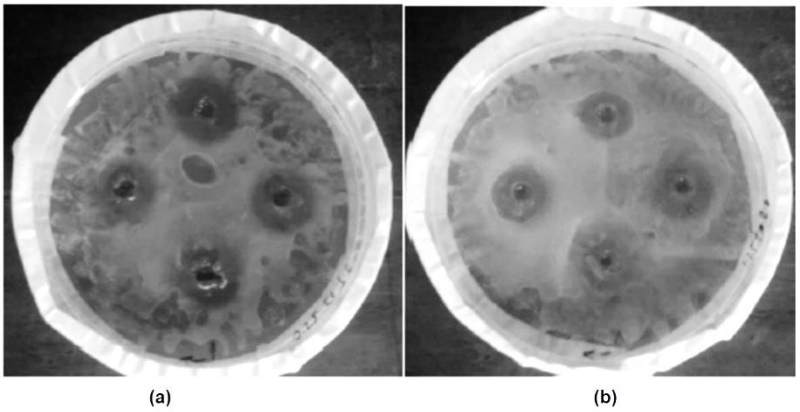

Fig 7 Zones of inhibition shown by chitosan extracted from *S. cerevisiae*: (a) chitosan concentration 0.5 to 2 g/L; (b) chitosan concentration 2.5 to 4 g/L

The diameters of the zones of inhibition were measured and 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 Fig 8.

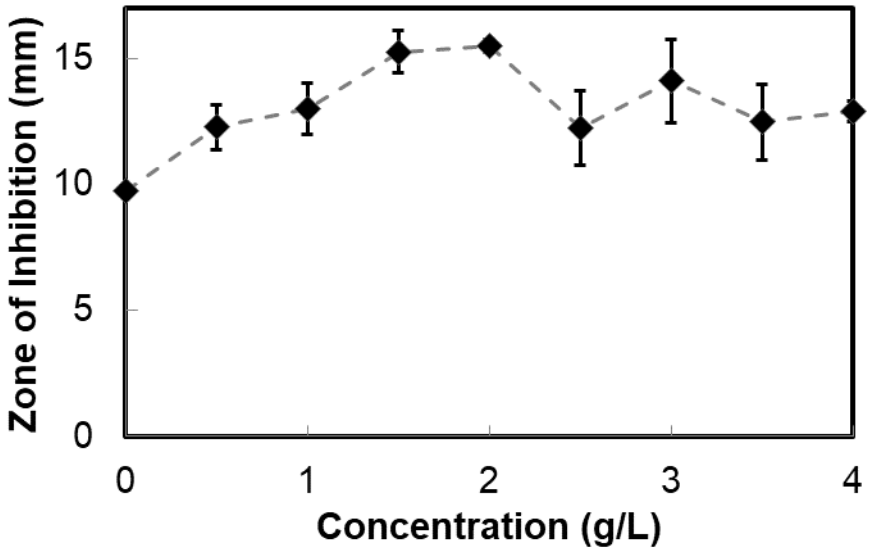

Fig 8 Diameters of zones of inhibition shown by *S. cerevisiae* chitosan at different concentrations

The results of the present study showed clear zones of inhibition against gram positive *Staphylococcus aureus* using chitosan solution at different concentrations. 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 ± 0.15 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*. [69] A study by Ghareib *et. al.* showed zones of inhibition against *S. aureus* of 29 mm, 30 mm and 29 mm using chitosan as antimicrobial agents which were extracted from *Mucor, Rhizopus* and *Cunnighamella* strains respectively. [34] Another study reported zones of inhibition of about 9 or 10 mm when using chitosan extracted from endocholenic fungi including *A. niger.* [70] Rejane C. Goy *et al.* 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. [71] Works of Assainar *et al.* 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.[72] Tayel and his group have carried out extensive studies to use fungal chitosan for antimicrobial applications and the fungal species used as sources include *A.niger, M. rouxii. C. elegans etc* and the microbes used as pathogens were primarily *S. aureus* and *E. coli*. [73] In one such, study the authors have extracted chitosan from *C. elegans* and a 1% w/v solution (or 10g/L) of chitosan in 1% acetic acid yielded a zone of inhibition of 38.2 ± 1.3 mm against *S. aureus* using the well diffusion method. [74] A study of antimicrobial activity of cell lysates of *S cerevisae* by Fakruddin et al gives zone of inhibition of 10.3 mm against *S aureus* bacteria, which is lower than the average zones of inhibition obtained in this study, which ranged from 12.2 to 15.5 mm

Therefore, in terms of inhibition zones, the fungal chitosan showed promising results for use of antimicrobial activity in applications where broad spectrum strong antibiotic usage is undesirable such as chitosan-based films for food preservation and [75]chitosan bandages for wound healing. [76]

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. [77] Lower number of chain-chain bonds also increases interfacial interactions of active sites. [78]

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. [79]This creates a spatial restriction to the functional groups of chitosan chain and results in a smaller number of charged sites available for binding with the bacterial cell wall. [71]

Fungal chitosan extracted from *S. cerevisiae* has excellent potential to act as a mild antimicrobial agent which can be used in prevention of food spoilage, wound healing applications, bioclarification and other such applications. It is known that the parameters, degree of deacetylation and source of chitosan play vital roles when antimicrobial efficacy is considered. Also, the microbe against which the chitosan acts is a factor to be considered in antimicrobial studies.[80] Thus further, studies are warranted to understand the action of *S. cerevisiae* based chitosan with different degrees of deacetylation and against various microorganisms.

4. CONCLUSION

Locally available fungal species were used to extract chitosan from the respective fungal biomasses. *Saccharomyces cerevisiae* was obtained from locally obtained Baker's yeast and used as fungal biomass after 24 hours of culture. *Aspergillus niger* was isolated from onions in the laboratory and the species was identified easily from its unique morphology. *S. cerevisiae* showed an optimum number of days at which yield could be maximized, and 20.85 ± 0.35 mg/g chitosan was obtained on Day 4 of sporulation conditions of the yeast. Yields of chitosan from isolated *A. niger* showed a clear maximum on Day 12 of culture of the fungal biomass, giving 16.15 ± 0.95 mg/g. These results indicate that even though the culture of these organisms have an inherent flexibility, it would be useful to optimize the number of days at which the fungal biomass should be harvested for maximum yield of chitosan

The fungal chitosan sources investigated in this study are potentially available for commercial extraction at lowered costs. The spent biomass from brewing or baking using *S. cerevisiae* could serve as biomass sources. Strains of *S cerevisiae* are found on fruits, in leaves and in trunks of various plant species, particularly on damaged grape berries, [81] harvesting of which could lead to potential chitosan sources. *A. niger* is the primary microbe used for production of citric acid. It can be postulated that the spent *A. niger* biomass of the citric acid production reactor can be considered as a source of extractable chitosan.[82] The thousands of tons of waste biomass produced in industrial processes or as part of agricultural waste every year are a largely untapped resource for production of a non-toxic and biocompatible form of chitosan.

The samples of *S. cerevisiae* chitosan showed antimicrobial potency against a test pathogen, *Staphylococcus aureus.* The chitosan samples showed promise for use in various biological and biotechnological applications such as chitosan-based films for food preservation, infection prevention and

in wound healing applications. Literature on chitosan suggests that a continuation of this study would be beneficial to investigate the antimicrobial action of the modified chitosan obtained from *S. cerevisiae* to modulate properties such as dissolution, pH at which chitosan is active and surface charge, in order to enhance the antimicrobial properties of the fungal chitosan thus obtained. [83][84]

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DECLARATION

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Availability of data: The data that support the findings of this study are available from the corresponding author, NI, upon reasonable request.

AUTHOR CONTRIBUTION

Md. Masifrul Afroz (MA), KM Prottoy Shariar: (PS), Md. Nayeem Hasan Kashem (NH), Nafisa Islam (NI)

• Conceptualization: NI and MA; Methodology: MA, PS and NH; Formal analysis and investigation: MA, PS and NH; Writing - original draft preparation: MA and NI; Writing - review and editing: MA, PS, NH and NI, Funding acquisition: NI; Resources: , NI; Supervision: NI

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