Chitosan from Zygomycetous Fungus, *Benjaminiella poitrasii*: Scale up for Biomass Production and Biophysical Characterization of Biopolymer

by

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Under the supervision of

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Affectionately dedicated to. . . . . .

Aai, Dada

&

Family

CERTIFICATE

This is to certify that the work incorporated in this Ph.D. thesis entitled, "*Chitosan from zygomycetous fungus*, Benjaminiella poitrasii*: Scale up for biomass production and biophysical characterization of biopolymer*", submitted by *Ms. Shamala Rajendra Mane* to the Academy of Scientific and Innovative Research (AcSIR) in fulfillment of the requirements for the award of the Degree of *Doctor of Philosophy in Science* embodies original research work carried-out by the student. We, further certify that this work has not been submitted to any other University or Institution in part or full for the award of any degree or diploma. Research material(s) obtained from other source(s) and used in this research work has been duly acknowledged in the thesis. Image(s), illustration(s), Figure(s), table(s) etc. used in the thesis from other source(s), have also been duly cited and acknowledged.

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DECLARATION BY RESEARCH SCHOLAR

I, Shamala Rajendra Mane hereby declare that the work incorporated in the thesis entitled 'Chitosan from zygomycetous fungus, *Benjaminiella poitrasii*: Scale up for biomass production and biophysical characterization of biopolymer' submitted by me to the Academy of Scientific and Innovative Research (AcSIR), for the degree of Doctor of Philosophy in Biological Sciences has been carried out by myself. All the work has been carried out at CSIR-National Chemical Laboratory, Pune under the guidance of Dr. Bhushan Chaudhari (Research supervisor) and Dr. Mukund Deshpande (Research Co-Supervisor). The reported work is original and has not been submitted to any other university or institution for the award of any degree or diploma. Such material, as has been obtained from other sources, has been duly acknowledged.

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Ms. Shamala Rajendra Mane

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Abbreviations

Abbreviation	Full form
AIF	Alkali insoluble fraction
BpHCNps	*B. poitrasii* hyphal chitosan nanoparticles
BpYCNps	*B. poitrasii* yeast chitosan nanoparticles
CDA	Chitin deacetylase
CLSI	Clinical laboratory standards institute
CSL	Corn steep liquor
D_2O	Deuterium oxide
DDA	Degree of deacetylation
DOE	Design of experiments
DLS	Dynamic light scattering
EGC	Ethylene glycol chitin
FTIR	Fourier transformed infrared
Glc-NAc	N-acetylglucosamine
GPC	Gel permeation chromatography
KBr	Potassium bromide
kDa	Kilo Dalton
lpm	Litre per minute
MCNs	Marine chitosan nanoparticles
MIC	Minimum inhibitory concentration
MW	Molecular weight

NAD(H)	Nicotinamide adenine dinucleotide (reduced)
NADP(H)	Nicotinamide adenine dinucleotide phosphate (reduced)
NMR	Nuclear magnetic resonance
OD	Optical density
RH	Relative humidity
Rpm	Revolutions per minute
TPP	Tripolyphosphate

ABSTRACT

Chapter 1: Introduction

Chitosan is a natural glucosamine polymer [β- (1→4)-linked] synthesized by the deacetylation of chitin [β- (1→4)-linked], *N*-acetylglucosamine polymer. Landings of marine organisms such as crabs, lobsters, and shrimps are the primary commercial source of chitin/chitosan. Fungi, by definition, are organisms that contain chitin as a primary structural component in the cell walls. The fungal cell wall is composed mainly of chitin, chitosan, glucan, and mannan. Therefore, the fungi can be an alternative to marine waste for isolating chitin/chitosan. The possible fungal sources for chitin and chitosan extraction include waste fungal biomass generated from biotech industries and fermentation of zygomycetous fungi. The fungal chitosan is advantageous due to its homogenous, highly deacetylated nature and is available throughout the year. The different classes of fungi vary in their cell wall chitin and chitosan content. The zygomycetes contain higher chitosan as compared to other classes of fungi.

Benjaminiella poitrasii is a well-studied zygomycetous, dimorphic fungus with high chitin/chitosan contents in the mycelial form of the fungus. The present study examines the potential sources for fungal chitosan production and determines its antimicrobial potential against different human and plant pathogenic organisms. Along with the chitosan production and characterization, the synthesis of fungal chitosan nanoparticles to check enhancement in their antifungal potential, if any, against human pathogenic organisms is also carried out.

Crab and shrimp shells are the most popular commercial sources of chitin. Squids, oysters, and cuttlefish are among the other marine sources. Chitin is manufactured and destroyed at a rate of around 10^{12} kg per year.

Chitin from crab shells is converted to chitosan using an alkali treatment in most countries per year for commercial purposes. However, due to the discontinuous supply and seasonal variations of the marine sources, fungi could be a viable alternative. The fungi can be readily grown in the laboratory on cheap nutrients, simple chemical procedures can recover cell wall material, and consistent quality and supply of the raw material are possible. Further, the alkali treatment used for isolation of chitosan is not eco-friendly; Chitin deacetylase (CDA), an enzyme that catalyzes the deacetylation of chitin, is feasible alternative to obtain chitosan.

The zygomycetous fungi are the potential sources for chitosan production. The recent observations suggested that non-zygomycetous plant- and insect- pathogenic fungi also have a high proportion of chitosan in the cell walls. So, to find out the potential source for chitosan production, the objectives of the thesis are designed as follows:

Section I: Optimization for biomass production and improving extractable cell wall chitosan contents from zygomycetous fungus *B. poitrasii*

Section II: Exploring the potential of agriculturally important ascomycetous fungi *M. anisopliae* and *M. verrucaria* for chitosan production: A value addition to low cost-high volume products

Section III: **Comparative analysis of chitosan from different fungi with special emphasis on zygomycetous dimorphic fungus *B. poitrasii*: Evaluation of its chitosan nanoparticles for the inhibition of human pathogenic fungi.**

Chapter 2: Materials and Methods

1. Media Optimization and fermentation Methods: Optimization of growth parameters by one variable at a time approach (OVAT)**,** Optimization of biomass using L18 Taguchi design of experimental (DOE) array, Optimization of different inoculums, Scale-up for biomass production

using solid-state and submerged fermentation, Submerged fermentation of yeasts, Cultivation of mushroom**.**

2. Microbiological methods: Isolation of fungal cell wall, extraction of fungal chitosan, Light microscopy.

3. Biochemical methods: Preparation of crude culture filtrate (CCF), Chitin deacetylase (CDA) enzyme assay, Protein estimation by Lowry and Bradford method, Chemical and enzymatic deacetylation of mycelial biomass using fungal CDAs, Clinical Laboratory Standards Institute's (CLSI) broth microdilution assay, mycelial extension inhibition assay, Red Blood Cell **(**RBC) Hemolysis assay**.**

4. Analytical methods: ^{1} H- Nuclear Magnetic Resonance (^{1}H-NMR) Spectroscopy, Fourier Transform Infrared (FT-IR) spectroscopy, Viscometry studies of molecular weight determination, Gel permeation chromatography (GPC), Dynamic light scattering (DLS), Nanotracking analysis (NTA), High-Resolution Transmission Electron Microscopy (HR-TEM), Field Emission Scanning Electron Microscopy (FE-SEM).

Results

Chapter 3: Optimization for biomass production and improving extractable cell wall chitosan contents from zygomycetous fungus *B. poitrasii*

Chapter 3a: Optimization of biomass from zygomycetous fungus *B. poitrasii* for chitosan production by one variable at a time (OVAT) approach

B. poitrasii, a dimorphic zygomycetous fungus contains more chitosan in the mycelial cell wall than the cell wall of its yeast form. The optimized medium containing yeast extract, peptone, $MgSO_4$, KH_2PO_4, trace metals (Fe^{2+}, Mn^{2+} Zn^{2+}, and Co^{2+}) solution, and 1% starch produced 10-12 g/L (dry wt.) of mycelial biomass in 48 h in a 2 L fermenter. Using 1N NaOH treatment from

1 g of dried biomass 51.00±0.52 mg of chitosan of 42.82 kDa molecular weight and 94.24% degree of deacetylation was extracted. With *M. anisopliae* CDA, chitosan yield was 59.00± 0.84 mg, while upon treatment with *B. poitrasii* CDA, it increased to 78.05± 0.58 mg/g of dry wt. of biomass. The chitosan dissolved in 2% acetic acid showed higher antifungal activity against *Candida albicans* (MIC_{90} 0.025 mg/mL) and *Candida glabrata* (MIC_{90} 0.2 mg/mL) than chitosan extracted from marine source (MIC_{90}>1.6 mg/mL), suggesting the use of fungal chitosan in healthcare.

Chapter 3b: Taguchi design of the experimental approach to increase the biomass and cell wall chitosan contents of zygomycetous dimorphic fungus *B. poitrasii*

A dimorphic fungus *B. poitrasii* contains high chitin/chitosan (35% of the cell wall) in the mycelial (M) form than its yeast (Y) form (20% of the cell wall). However, the relative proportion of chitosan is more in yeast form cells (chitosan: chitin ratio, 6:1) than mycelial cells (chitosan: chitin ratio, 3:1). Using the Taguchi design of experimental (DOE) approach, interactions among eight different parameters showed that carbon source (starch, 10 g/L), incubation time (48 h), inoculum (M and Y mixed 10%), yeast extract (6 g/L) and peptone (10 g/L), were optimum for maximum biomass production. Under these conditions, the chitosan yield from the mycelia was 60.89±2.30 mg/g of dry biomass, while that from the yeast cells was 28.29±2.7 mg/g. The molecular weights of chitosan isolated from M and Y cells were 41.28 kDa and 21.72 kDa, respectively, as measured by gel permeation chromatography. The degree of deacetylation (DDA) of chitosan as measured by FTIR was lower (87.3-88.5%) than measured using ^{1}H-NMR (88.17-90.72%). Furthermore, chitosans from M and Y cells inhibited the growth of plant pathogenic *Fusarium oxysporum* CMI113138 (MIC_{90} 0.1 and 0.4 mg/mL) and *Ustilago*

maydis PRL 1549 (MIC_{90} 0.4 and 0.8 mg/mL) at lower concentrations as compared to chitosan isolated from marine sources (MIC_{90} 0.8 and >1.6 mg/mL).

Chapter 4: Exploring the potential of agriculturally important ascomycetous fungi *M. anisopliae* and *M. verrucaria* for chitosan production: A value addition to low cost-high volume products

One of the major concerns for using entomopathogenic fungi as mycoinsecticide is the production cost, as it is a high volume, low-cost product. The mycelial biomass after removal of conidia can be used as a raw material for the isolation of chitosan, as value addition, and effective cost reduction. In this study, entomopathogenic *M. anisopliae* strains and another ascomycetous fungus, *M. verrucaria*, that produces cuticle degrading and mycolytic enzyme complex extracellularly were used for the isolation of low molecular weight chitosan. From 10 g of dried biomass (i) *M. anisopliae* MTCC5190 124.4±2.98 mg of chitosan of 0.77 kDa molecular weight and 87.80% degree of deacetylation (DDA measured by ^{1}H NMR) was extracted; (ii) of an extracellular CDA producer *M. anisopliae* strain MCC1197, yielded 180.8±4.79 mg of 1.36 kDa molecular weight and 82.05% DDA; (iii) after the separation of crude enzyme complex *M. verrucaria* MTCC5191 the 193.5±2.98 mg of chitosan of 2.38 kDa molecular weight and 86.34% DDA was isolated.

Chapter 5: **Comparative analysis of chitosan from different fungi with special emphasis on zygomycetous dimorphic fungus *B. poitrasii*: Evaluation of its chitosan nanoparticles for the inhibition of human pathogenic fungi.**

The cell wall chitosan was extracted from fungi belonging to different taxonomic classes *viz., B. poitrasii* (Zygomycetes, dimorphic), *Hanseniaspora guilliermondii, Issatchenkia orientalis, Pichia membranifaciens, Saccharomyces cerevisiae* (Ascomycetes, yeasts), *Agaricus*

bisporus, and *Pleurotus sajor-caju* (Basidiomycetes). The maximum chitosan yield was 60.89±2.30 mg/g of dry mycelial biomass of *B. poitrasii*. The degree of deacetylation DDA) of chitosan extracted from different fungi, as observed with ^{1}H-NMR, was in the range of 70-93%. *B. poitrasii* chitosan exhibited the highest DDA (92.78%). The characteristic absorption bands were observed at 3450, 1650, 1420, 1320, and 1035 cm^{-1} by FTIR. Compared to chitosan from marine sources (Molecular weight, MW, 585 kDa), fungal chitosan's showed a lower MW (6.21 to 46.33 kDa). Further, to improve the efficacy of *B. poitrasii* chitosan (Bp), the nanoparticles (Np) were synthesized by using the ionic gelation method and characterized by dynamic light scattering (DLS). For yeast and hyphal chitosan nanoparticles (BpYCNp and BpHCNp), the average particle size was <200 nm with polydispersity index were 0.341±0.03 and 0.388±0.002, respectively, and the zeta potential values were 21.64±0.34 and 24.48±1.58 mV, respectively. The *B. poitrasii* chitosans and their nanoparticles were further evaluated for antifungal activity against human pathogenic *C. albicans* ATCC 10231, *C. glabrata* NCYC 388, *Candida tropicalis* ATCC 750, *Cryptococcus neoformans* ATCC 34664, and *Aspergillus niger* ATCC 10578. BpHCNps showed lower MIC_{90} values (0.025-0.4 mg/mL) than the chitosan polymer against the tested human pathogens. The study suggested that nano-formulation of fungal chitosan, which has low molecular weight and high %DDA, is desirable for antifungal applications against human pathogens. Moreover, chitosan and its nanoparticles were hemocompatible and are therefore safe for healthcare applications.

Chapter 6: Conclusions and Future prospects

This chapter highlights the important findings from this research topic and their possible future prospects.

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Chapter 5: Comparative analysis of chitosan from different fungi with special emphasis on zygomycetous dimorphic fungus *B. poitrasii*: Evaluation of its chitosan nanoparticles for the inhibition of human pathogenic fungi

Chapter 6: Conclusions and Future prospects

Chapter 1
Introduction

1.1 Fungi

The fungi are saprophytic and spore-producing eukaryotic organisms that contain chitin in their cell wall. The group of fungi mainly consists of yeast, molds, and mushrooms (Warnock, 2012). The reproduction in fungi is either asexually by budding, fragmentation, production of spores or sexually through meiosis and fusion (Gow *et al.*, 2017). Marine wastes including crabs, shrimp, and prawns are used in case of large-scale production of these biopolymers. The alternative source for these polymers includes the fungal biomass such as raw material or fermentation of zygomycetous fungi generated from industrial waste. The fungal biomass can also be utilized for wastewater treatment, bioremediation, and as a source of single-cell protein. *Paecilomyces* sp. has been used to remove arsenic (III) from the solution using modified fungal biomass (Acosta Rodriguez *et al*., 2013). For detoxification of hexavalent chromium, dead fungal biomass of *Aspergillus niger, Rhizopus oryzae, Saccharomyces cerevisiae, and Penicillium chrysogenum* has been used (Park *et al*., 2005). In the comparison of marine sources, fungal biomass can provide alternative industrial raw material sources of chitosan. The advantage of using fungi as a source of chitosan is three-fold *viz*., homogenous, highly deacetylated, and available around the year (Ghormade *et al*., 2017).

1.2 Structural organization of fungal cell wall

Fungal cell walls are essential for cell viability, morphogenesis, and pathogenesis. It is composed of glycoproteins and polysaccharides, mainly glucan, mannan, chitin, and chitosan (Gow *et al*., 2017). The composition greatly influences the ecology of the fungus and is highly regulated in response to environmental conditions and imposed stresses (Moreno *et al.*, 2008).

The role of structural and matrix components of the cell walls, such as chitin/ chitosan, α- and β-glucans, proteins, lipids, uronic acids, hydrophobins, sporopollenin, and melanins reviewed

earlier by Feofilova, 2010. The different components of the fungal cell wall has described in details in further sections. The schematic representation of structural organization of the fungal cell wall is depicted as below in Figure 1.1.

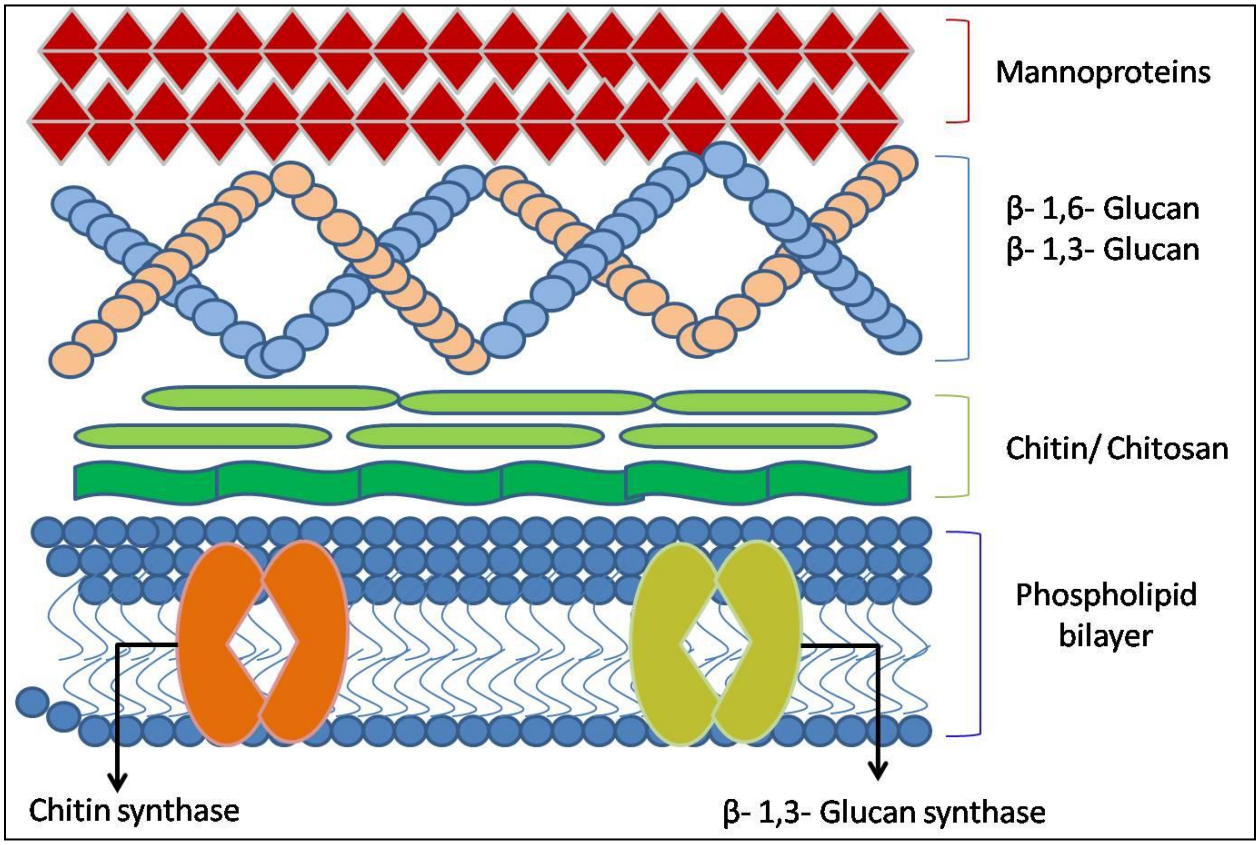


Figure 1.1 Schematic representation of the structural organization of fungal cell wall (Adapted from Mane *et al*., 2022a)

1.2.1 Glucans

Glucans are polysaccharides made up of glucose units. In most of the fungi, a branched β-(1,3) and β-(1,6) glucan linked with chitin via β-(1,4) linkage is a main structural component. There are mainly two types of glucans in fungi, namely, α-glucans and β-glucans. Most cell-bound α-glucans are made of glucosyl units joined by α-(1,3) linkage and are water-insoluble. Some fungi

have α-(1,4) bound glucose units in their cell walls either to interconnect the linear α-(1,3) glucan chains or in an alternated way with α-(1,3) bound glucose units, e.g. in *A. niger* (Ruiz-Herrera, 2012). The most abundant β-glucan in the fungal cell wall is β-(1,3) glucan, which makes up between 65-90% of the whole β-glucan content (Bowman & Free, 2006). A large amount of the β-(1,3) glucan is covalently bound to β-(1,6) glucan in the form of branching polysaccharides (Shahinian & Bussey, 2000). The multibranched β-glucans can be firmly bound to the cell wall or loosely bound, accumulating around the fungus as slime or gelatinous material (Ruiz-Herrera, 2012). The synthesis of β-glucan in filamentous fungi starts at the hyphal tip and sides of cell growth and branching.

1.2.2 Mannan/ Mannan Oligosaccharides

Mannan is a highly branched polysaccharide made up of α-(1→6) linked mannose units with α-(1→2) and α-(1→3) linked side chains. These side chains are also known as mannan-oligosaccharides (MOS). In some cases, additional compounds such as glucose, D-galactose, D-xylose, and phosphate are covalently linked to a protein or peptide moiety. The surface properties of the cell wall are determined by mannoproteins which represent 30-40% (by weight) of the total cell wall (Moreno *et al*., 2008).

1.2.3 Glycoproteins

All fungal cell walls have a protein component tightly interwoven within the chitin and glucan-based structural matrix. These proteins are glycoproteins that have passed through the secretory pathway in transit to the cell wall. Filamentous fungi such as *S. cerevisiae* and *Candida albicans* cell walls contain roughly 30–50% protein by dry weight, representing approximately 20–30% protein of the cell wall by mass. Recent empirical study demonstrated 15% protein content in the *Neurospora crassa* hyphal cell wall by dry weight (Virag & Griffiths, 2004).

1.2.4 Chitin

Chitin is a biopolymer made of N-acetylglucosamine units containing β-(1,4) glycosidic linkages. It is a structural component in many living organisms, including cell walls of fungi, insect cuticles, arthropods, sponges, and beaks of cephalopods (Tharanathan & Kittur, 2003). The percent content of chitin in these organisms is listed in Table 1.1. The chemical structure of chitin is similar to cellulose except for the hydroxyl group in the C-2 position of cellulose is replaced by the acetamido group in chitin. The annual production of chitin accounts for 10^6–10^7 tons. It is used as the raw material for the commercial production of chitosan. Chitin can be enzymatically or chemically deacetylated into chitosan (Dhillon *et al*., 2013).

In nature, three forms of chitin are available, *viz*., α, β and γ. α-chitin is composed of antiparallel chains of β-(1,4) linked *N*-acetyl glucosamine (GlcNAc). The strong intermolecular bondings are present in the shells of crustaceans like shrimp and crabs and fungi having α-chitin. β-chitin has parallel alignment of chains and which is commonly found in squid pens. While γ-chitin present in insects, has two chains parallel in one direction and a third chain goes antiparallel to them (Alvarez, 2014). In nature, pure form of chitin is present only in diatoms. The extracellular β-chitin spines of the centric diatoms such as *Thalassiosira fluviatilis* are completely acetylated and not associated with other substances (Lindsay & Gooday, 1985). However, in most of the organisms it is a polymer of both GlcNAc and glucosamine with varying percentages. Usually, in chitin >70% acetylation is expected while by definition chitosan has degree of acetylation < 30-40% (Ghormade *et al*., 2017).

Table 1.1 Chitin content of different fungal organisms

Organism	Chitin content (%)
Fungi	5-60
Worms	20-38
Squids	3-20
Scorpions	30
Spiders	38
Cockroaches	35
Silkworms	44
Crabs	70

1.2.5 Chitosan

Chitosan, a natural β-(1→4)-linked glucosamine polymer, is the deacetylated form of chitin, commonly found in the exoskeleton or cuticles of many invertebrates and in the cell wall of most of the fungi such as mucoralean fungi (Muzzarelli *et al*., 1994). The chitosan content varies among the different class of fungi as depicted in Table 1.2. It has been extracted mainly from zygomycetous fungi such as *Mucor rouxii* (Synowiecki & Al-Khateeb, 1997), *Mucor racemosus*, *Cunninghamella Elegans* (Amorim *et al*., 2001), *R. oryzae*, *Absidia coerulea* (Davoust & Hansson, 1992), and *B. poitrasii* (Mane *et al*., 2017a). Chitosan extraction has also been reported from other fungi such as *Lentinus edodes*, *Pleurotus sajor-caju*, *Zygosaccharomyces rouxii*, *C. albicans* (Pochanavanich & Suntornsuk, 2002), *M. anisopliae*, and *M. verrucaria* (Mane *et al*., 2017b). Chitosan has a number of applications in areas, *viz*., cosmetics (hand and body creams, shampoos, and toothpastes) (Aranaz *et al*., 2018), agriculture (pest control and seed coats) (Xing *et al*., 2015), pharmaceuticals (drug carrier, contact lenses or eye bandages, adhesives,

antimicrobial compounds), food industry (additives, dyes and nutrients) (Manigandan *et al*., 2018) and wastewater management (flocculating, chelating agents) (Desbrieres & Guibal, 2018).

Table 1.2 Chitosan content and % degree of deacetylation (DDA) of chitosan from different class of fungi

Organism	Extracted chitosan (g/kg of dry biomass)	DDA (%)	Reference
Absidia coerulea	107.3	90	Rane & Hoover, 1993
Absidia glauca	59.4	75.6	Hu *et al*., 1999
Cunninghamella bertholletiae	55	88.20	Amorim *et al*., 2006a
Ganoderma lucidum	83.2	80.29	Ospina *et al*., 2015
Gongronella butleri	40-60	87	Nwe & Stevens, 2002a
Mucor rouxii	60-77	82.8	Chatterjee *et al*., 2005
Penicillium chrysogenum	57	86	Tianqi *et al*., 2007
Rhizopus arrhizus	29.3	86	Cardoso *et al*., 2012
Rhizopus oryzae	58.0	89-90	Tai *et al*., 2010
Aspergillus niger	107	83.8-90	Pohchanavanich &
Candida albicans	44		Suntornsuk, 2002
R. oryzae	138		
Lentinus edodes	33		
Pleurotus sajor-caju	12		
Zygosaccharomyces rouxii	36		
Basidiomycetes			
Agaricus bisporus			

Armillaria mellea	10	80-85	Di Mario *et al*., 2008
Auricularia auriculajudae			
L. edodes			
Pleurotus eryngii			
Pleurotus ostreatus			
Trametes versicolor			

1.3 Sources of chitin/chitosan

The primary commercial sources of chitin are crabs, lobsters, and shrimp. For the production of chitosan, crustacean waste is processed with strong alkalis at high temperatures for long periods. However, the supply of marine waste is seasonal and limited, resulting in variability in the source material, leading to heterogeneity in the physicochemical properties of chitosan. Thus there is limited industrial acceptance for this polymer. (Ghormade *et al*., 2017) Several alternative industrial raw material sources of chitin/chitosan have been suggested, but no significant progress has been made in establishing new technologies for the large-scale controlled production. Recent advances in fermentation technology suggest that large-scale culturing of chitosan containing organisms might be an attractive route for producing this polymer. Moreover, fungal chitosan technology can also be used to overcome many of the above mentioned problems. Ghormade *et al*., 2017 reviewed three different ways of chitin and chitosan production is as below (Figure 1.2).

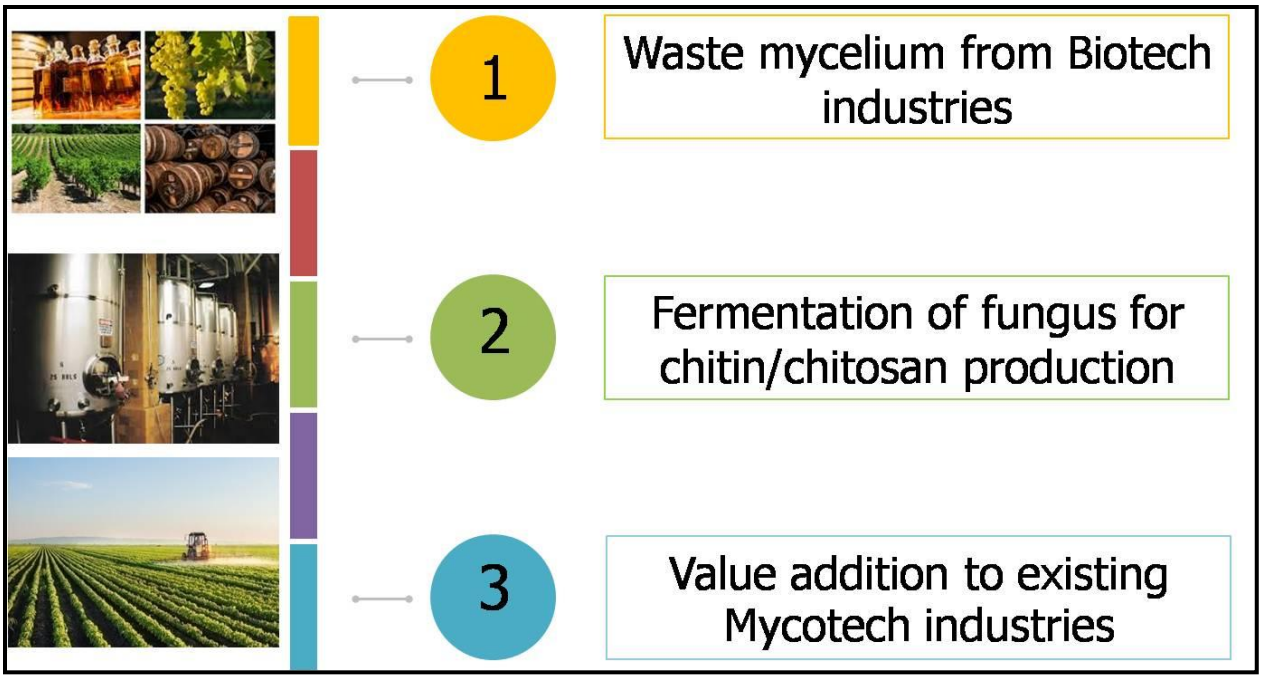


Figure 1.2 Schematic representations of fungal sources for chitosan extraction (Image source: www.google.com)

1.4 Optimization of fungal biomass production using Taguchi design of experimental approach (DOE)

The development of suitable fermentation media is a requisite and important step in efficiently utilizing fermentation technology. The conventional "one-factor-at-a-time" approach involves altering one parameter while keeping all others constant, assuming that the parameters do not interact and that the outcome is a direct function of a single variable. Therefore, optimizing parameters by the conventional approach becomes inaccurate, laborious, and time-consuming Tupe *et al.*, 2007). However, these limitations can be tackled using empirical methods. In empirical methods, there are two approaches, *viz.*, statistical-based and artificial intelligence-based black-box approach. In a statistical-based approach, response surface methodology (RSM) has been extensively used in fermentation media optimization. This technique has also been employed for optimizing enzyme production by microorganisms.

Another tool is the Taguchi design of experiments (DOE) developed by Genichi Taguchi. It involves studying a system by a set of independent variables (factors) over a specific region of interest (levels). Its parameter design concept is related to finding the appropriate design factor levels to make the system insensitive to variations in noise (uncontrollable factors) (Taguchi, 1995). The approach also facilitates the identification of the influence of individual factors, determines the relationship between variables and operational conditions, and finally establishes the performance at the optimum levels obtained with a few well-defined experimental sets. Analysis of the experimental data using the analysis of variance (ANOVA) and factor effects provides an output that is statistically significant in finding the optimum levels. The Taguchi methodology has been used in experimental optimization, such as various biochemical techniques and microbial fermentations (Tupe *et al*., 2007).

Chenthamarakshan *et al*., 2017 optimized laccase production from *Marasmiellus palmivorus* LA1 by the Taguchi method. As laccase production is highly dependent on medium components and cultural conditions, optimizing the same is essential. An orthogonal array (L8) was designed using Qualitek-4 software to study the interactions and relative influence of the seven selected factors by a one-factor-at-a-time approach. The optimum condition formulated was temperature 28 °C, pH 5, galactose 0.8% w/v, cupric sulphate 3 mM, inoculum concentration n=6, and substrate length 0.05 m. Overall yield was increased by 17.6-fold after optimization with a final production of 667.4±13 IU/mL laccase activity.

Kim *et al*., 2005 used this statistical approach to optimize submerged culture conditions to produce mycelial biomass and exopolysaccharides (EPS) from *Agrocybe cylindracea*. Maximum production of mycelial biomass and EPS were obtained at pH 4.0 and 6.0, respectively, at 25 °C. Subsequently, the optimum concentration of each medium component was determined using the

orthogonal matrix method. The optimal combination of the media constituents for mycelial growth was as follows: maltose (80 g/L), Martone A-1 (6 g/L), $MgSO_4$. $7H_2O$ (1.4 g/L), and $CaCl_2$ (1.1 g/L); whereas, for EPS production: maltose (60 g/L), Martone A-1 (6 g/L), $MgSO_4.7H_2O$ (0.9 g/L), and $CaCl_2$ (1.1 g/L). Under the optimal culture condition, the maximum EPS concentration achieved in a 5 L stirred-tank bioreactor was 3.0 g/L, which was about three times higher than that with the basal medium. In another report of exo-biopolymer production, a one-factor-at-a-time and orthogonal matrix method was used to optimize submerged conditions using *Paecilomyces tenuipes* C240 strain (Xu *et al*., 2003). The optimal concentration for enhanced production was determined as glucose (4 g/L), KNO_3 (0.6 g/L), K_2HPO_4 (0.1 g/L), and $MgSO_4.5H_2O$ (0.1 g/L) for mycelial yield, and glucose (3 g/L), KNO_3 (0.4 g/L), K_2HPO_4 (0.1 g/L), and $MgSO_4.5H_2O$ (0.1 g/L) for exo-biopolymer production, respectively. The maximum mycelial yield was 10.18 g/L and exo-biopolymer production of 1.89 g/L. Under optimal culture conditions, the maximum exo-biopolymer concentration in a 5 L stirred-tank bioreactor was 2.36 g/L.

Pundir *et al*., 2018 carried out the optimization of process parameters for the removal of copper and nickel by growing *Aspergillus sp.* using the Taguchi method. The optimized parameters were found to be inoculum concentration (15% v/v), copper/nickel (50 mg/L) at pH 4 and temperature 30 °C. Therefore, the statistical methods of media optimization are essential to study which factors affect the growth of different organisms. Similarly, the Taguchi method of media optimization can be used for the fermentation of zygomycetous fungi for higher production of biomass.

1.5 Bioprospecting of agriculturally important fungi for the extraction of chitosan

In agriculture, many fungi are being used as myco-fertilizers and myco-pesticides. Chemical pesticides and fungicides have harmful effects on crops, and therefore several fungal organisms such as *Beauveria, Metarhizium, Trichoderma* and *Verticillium* are used for the control of insect pests and pathogens (Deshpande, 1999). One of the major concerns is the cost of these bio-agents. As fungi contain chitin and chitosan (5-60%) in their cell wall, the 'waste' biomass can be used as a raw material to isolate these polymers.

Concerning the parameters such as storage stability, the shelf life in and off- the field, and time required to control insect pests and pathogens, chemical pesticides have the edge over mycopesticides adding to their costs. To reduce the cost of production of mycopesticides and value addition, different potential applications such as plant growth-promoting activity, bioremediation of pesticide residues, use of enzymes for biotransformation were reported (Cocaign *et al*., 2013; Garcia *et al*., 2011). Moreover, *M. anisopliae* conidia can be used successfully to control different insect pests in agricultural fields (Tupe, Pathan & Deshpande, 2017). Similarly, the cuticle degrading and mycolytic enzyme (CDE/ME) complex of *M. verrucaria* was reported to effectively control pests and pathogens in grapes (Vidhate *et al*., 2015).

The number of fungi used in mycotechnology was being studied for value addition to their existing products. For example, Shiitake mushroom *L. edode*s produces a polysaccharide, lentinan, an immune stimulant in the body. After its extraction, the fungal biomass was reported to be used to produce chitosan. Using lignocellulosic substrates, *Mucor indicus* can produce ethanol and high amounts of polyunsaturated fatty acids such as γ-linolenic acid. The fungus was reported to produce 62-67% alkali-insoluble material, *i.e.* chitin and chitosan in hyphal form cell

walls (Satari, Karimi & Zamani, 2016). Further, they also reported the use of citrus fruit waste to grow *M. indicus* and *R. oryzae* biomass to produce chitosan. The biomass contained ~0.6-0.7% chitin and chitosan per g of the cell wall. Varelas *et al*., 2016 reported use of yeast lees for β-glucan production as value addition and to reduce the negative costs for wineries. The quantities of mycelial biomass produced are high due to the potential use of agriculturally important fungi in the field. Recently, entomopathogenic fungi such as *Beauveria*, *Metarhizium*, *Nomuraea*, *Paecilomyces*, and *Verticillium* have been studied on a large scale in the field for pest control (Ghormade *et al*., 2010).

For the production of conidia from *M. anisopliae,* solid-state fermentation was carried out by using rice as a substrate. After the separation of conidia from rice, the mycelial biomass was used for the isolation of chitosan. *M. verrucaria* is a saprophytic fungus used to control plant pathogenic fungi and insect pests. *M. verrucaria* secretes high amounts of chitinase along with other mycolytic and cuticle degrading enzymes like β-(1,3) glucanase, protease, and lipase when grown in a medium containing chitin. After the separation of an enzyme, the waste biomass can be used for chitin/chitosan isolation (Mane *et al*., 2017b). It is possible to reduce the cost of agriculture products by using these fungi for chitin and chitosan isolation.

1.6 Effect of different extraction methods on the physical and chemical properties of chitosan

The %DDA and molecular weight are the two basic properties of chitosan which ultimately affect other physical and chemical properties of this biopolymer. The traditional extraction procedures used for extraction of chitosan from crustacean waste are harsh chemical treatments resulting in the production of chitosan with inconsistent physical and chemical properties such as %DDA, MW, solubility and in terms of purity. The extraction procedures are the vital factors in

terms of the solubility and purity of chitosan. Number of factors contributes to the solubility of chitosan like temperature and time of deacetylation and concentration of alkali. Rao *et al*., 2007 carried out the study on intrinsic viscosity, FTIR, and powder X-ray diffraction (XRD) showed that MW and DA were collectively responsible for the solubility in the condition of random deacetylation of acetyl groups, which resulted from intermolecular forces. Hence, it is important to discuss different procedures adapted for isolation of chitosan from fungal sources. The general protocol for the extraction of chitosan includes the acid and alkali treatments in combination with different temperatures. The overview of common extraction methods used for isolation of chitosan from marine and fungal sources is depicted in Figure 1.3

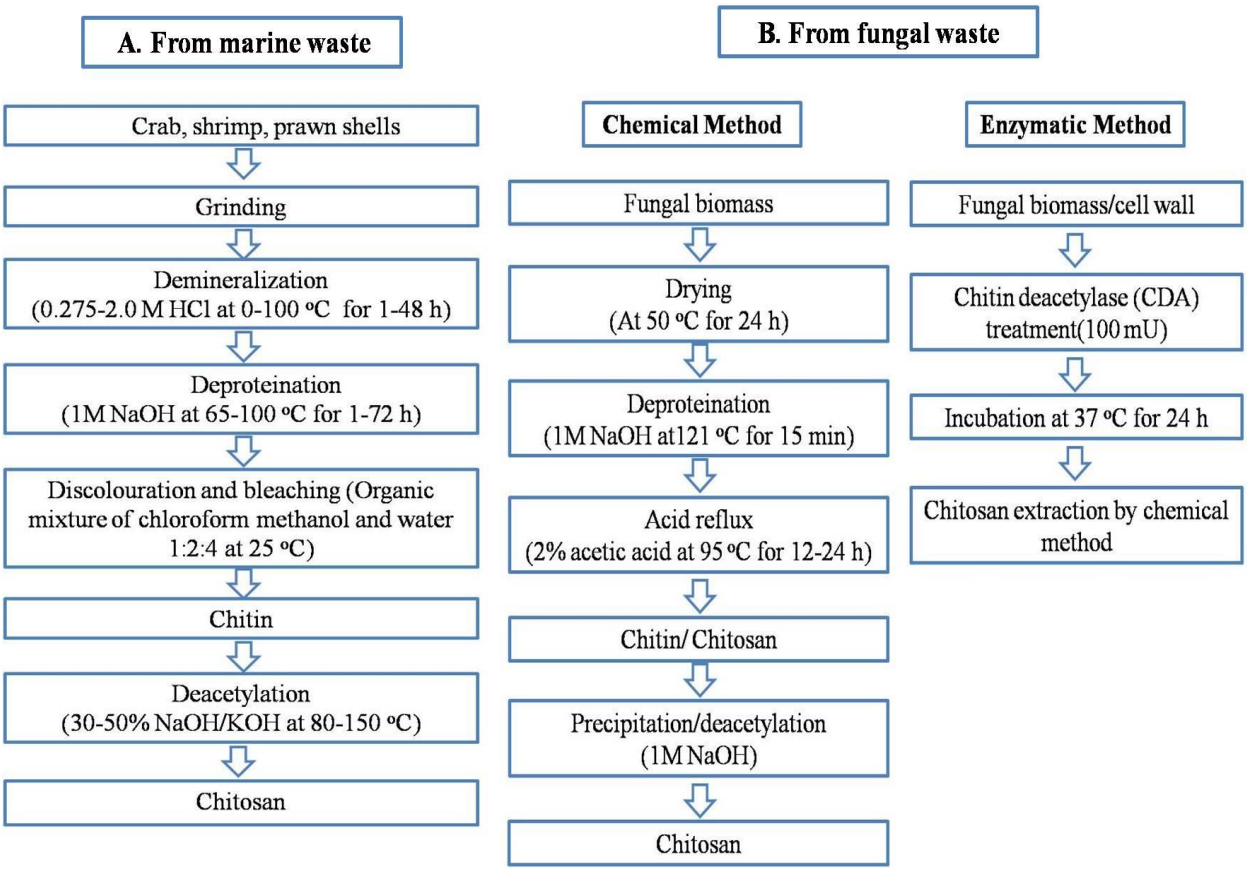


Figure 1.3 Extraction of chitosan from marine (A) and fungal (B) sources (Modified from Sieber *et al*., 2018)

Rane & Hoover, 1993 studied the effect of hot alkali and acid treatments on the extraction of chitosan from the mycelial biomass of *A. coerulea* ATCC 14076. Alkaline treatments were carried out using 1N NaOH with different extraction times at two different temperatures, 95 °C and 121 °C. For the acid treatment, three types of acids *viz*., acetic, formic and hydrochloric acid was used at 95 °C. Maximum yields of chitosan were obtained with alkaline extraction at 121 °C for 30 min and hydrochloric acid extraction at 95 °C for 12 h. The effects on the DA and viscosity of the chitosan of various treatments were also examined.

Hu *et al*., 1998 used caustic soda for extraction of chitosan from *A. glauca*. The study suggested that the higher concentration of caustic soda (50%) in combination with high temperature (121 °C), short contact time (15 min**)**, and the steam environment can avoid severe chain degradation and deacetylation of chitosan. In another study, Niederhofer & Muller, 2004 modified the conventional method of chitosan extraction from fungi for the production of low molecular weight chitosan. The low molecular weight chitosan was extracted directly from the raw material besides the thermal and chemical depolymerization. In this method, the reprecipitation of the alkali insoluble fraction was performed using ethanol. As the low molecular chitosan soluble up to alkaline pH ranges, reprecipitation and washing with ethanol was a crucial step. The use of water for washing between the preparation steps would cause solving and discarding of the low molecular chitosan.

A new method was developed based on the temperature-dependent solubility of chitosan in dilute sulfuric acid to extract chitosan from the zygomycetous cell wall (Zamani *et al*., 2007). The procedures were determined by measuring phosphate, protein, ash, glucuronic acid, and degree of acetylation (DA). The results showed that 8% of the biomass as chitosan. After treatment with 0.5 M NaOH, the AIM contained 45.3% chitosan. Treatment of AIM with 10%

acetic acid resulted in 16.5% acetic-acid-soluble material (AcSM) and 79.0% alkali- and acid-insoluble material (AAIM).

Nwe *et al.*, 2008 investigated chitosan extraction from the fungus *G. butleri* by using Termamyl enzyme which is a starch degradation enzyme. Chitosan in the fungal cell wall exists in two different forms, free chitosan and bounded to β-glucan. According to the results, it was suggested that the linkage of chitosan and β-glucan in the chitosan-glucan complex (CGC) was successively cleaved with the heat stable amylase. According to the results obtained from the specific enzymatic digestion, IR and NMR spectroscopy and nitrogen content analysis, it has been concluded that chitosan and glucan in the fungal cell wall are linked by α-(1, 4) glycosidic bond.

In a study carried out by Kannan *et al.*, 2010 the chitosan was extracted from 15 days old biomass of *Agaricus Sp*, *Pleurotus Sp.* and *Ganoderma Sp.* with different concentrations of alkaline (1-4 M NaOH) and acid treatment (2-10% acetic and hydrochloric acid). According to the report, the production as well as the quality of chitosan significantly affected by the concentrations of acid, alkali, temperature and incubation time. The type of acid used for the extraction also showed a significant difference in the production of chitosan. As compared to the acetic acid, %DDA was higher in case of extraction of chitosan with hydrochloric acid. Hence the concentration and types of acid and alkali should be carefully optimized for the extraction of chitosan with consistent physical and chemical properties.

1.7 Chitin deacetylase (CDA) for enzymatic deacetylation of chitin to chitosan

Chitosan with different %DDA has applications in agriculture, human health care, and other industries. Chemical deacetylation is done using 30–50% NaOH at 60–140 °C. The >95% DDA is achieved at 140 °C (60 and 90 min). However, the chemical treatment causes random

deacetylation patterns. On the other hand, deacetylation with CDA can create non-random patterns of acetylation. However, the crystallinity and insolubility of chitin are the two major barriers that hinder the development of an enzymatic deacetylation process. Therefore, a mild chemical treatment along with enzymatic deacetylation can be a better option (Mane *et al*., 2017a). The conversion of chitin into chitosan is represented as below in Figure 1.4.

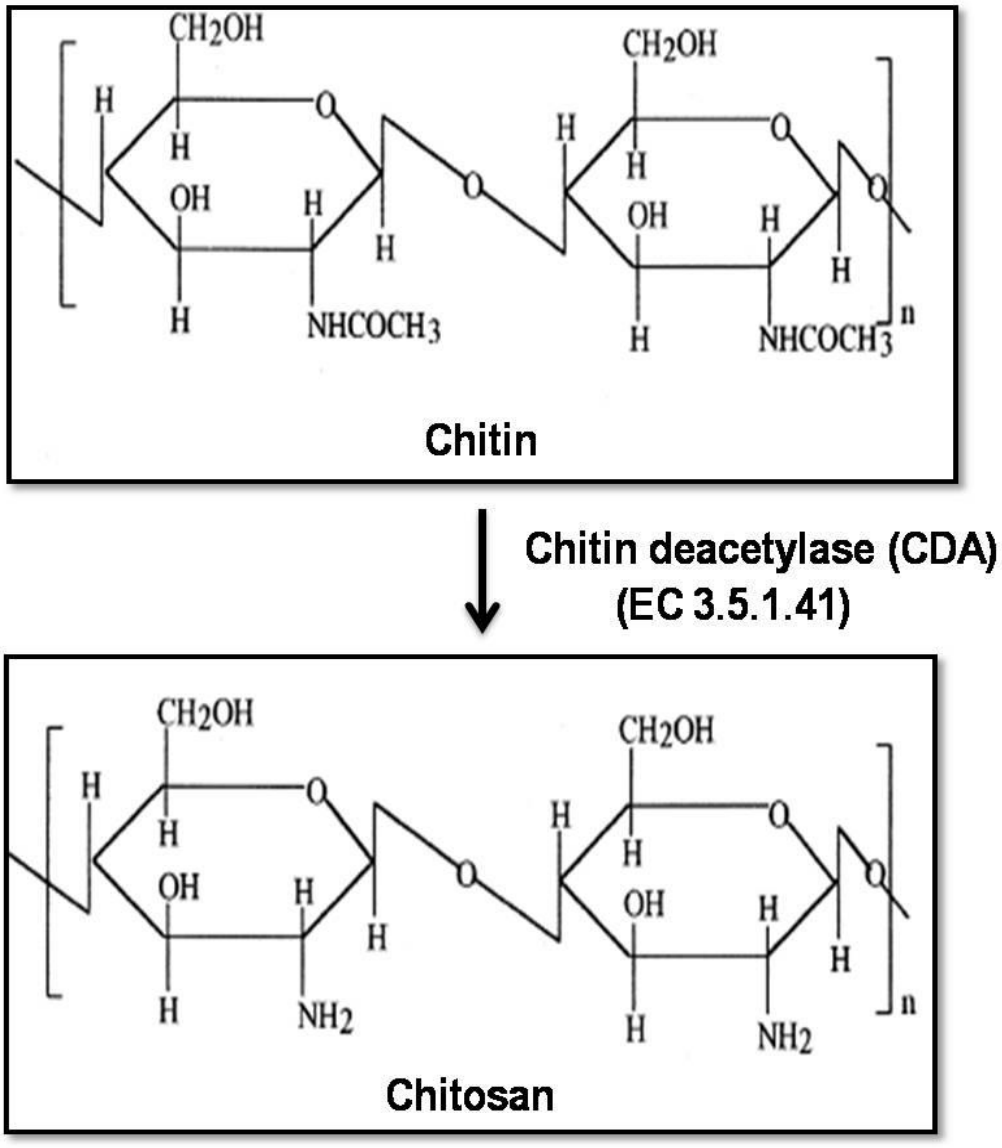


Figure 1.4 Schematic representation of the conversion of chitin into chitosan by chitin deacetylase (Modified from Tsigos *et al*., 2000).

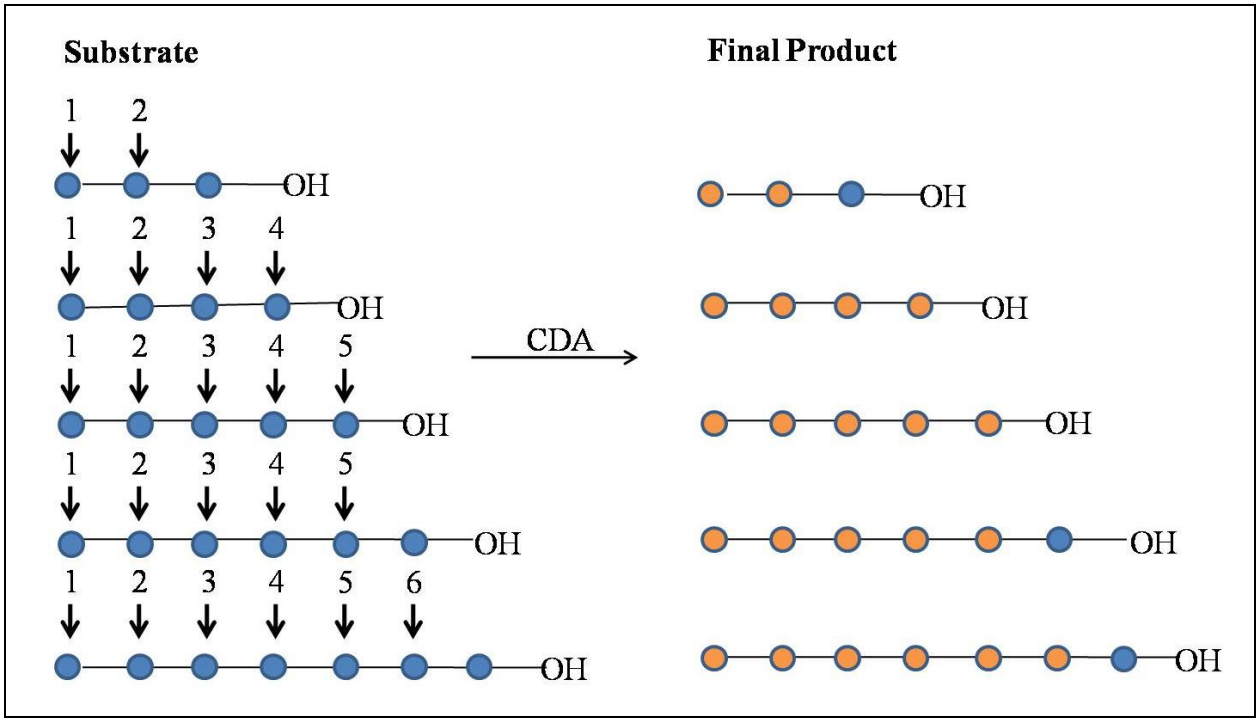
Fungal CDAs have been studied more extensively than those from insects and marine bacteria. Even though several CDA genes have been identified in insects, especially in the peritrophic midgut matrix, their biochemical properties have not been determined yet (Cai *et al*., 2006). CDA from several fungi have been reported (Alfonso *et al*., 1995; Blair *et al*., 2006, Gao *et al*., 2011; Jeraj *et al*., 2006; Kafetzopoulos *et al*., 1993; Suresh, Sachindra & Bhaskar, 2011).

Based on their diverse locations in fungi, CDA have been divided into two subgroups. *M. rouxii* and *A. coerulea* CDA are secreted into the periplasm and are thus called intracellular chitin deacetylases (Araki & Ito, 1975), while *C. lindemuthianum* and *A. nidulans* secrete CDA into the culture medium, thus being called extracellular chitin deacetylases (Alfonso *et al*., 1995). CDA enzymes operate by metal-assisted acid/base catalysis. The general mechanism of CDA was first proposed for *Streptococcus pneumonia* peptidoglycan GlcNAC deacetylase (*Sp*PgdA) when solving its structure by X-ray crystallography (Blair *et al*., 2005). The mode of action of CDA has been studied in both chitosan polymers and chitin oligomers (Tsigos *et al*., 2000).

The mode of action of CDA from *M. rouxii* has been investigated on an ~32% randomly deacetylated water-soluble chitosan substrate, with an average degree of polymerization (DP) of three. Using ^{1}H- and ^{13}C-NMR spectroscopy, it was found that the enzyme hydrolyzed the acetyl groups of the substrate, according to a multiple-attack mechanism with a degree of multiple attacks of three. This is the maximum number of successive deacetylations that the enzyme could achieve because the maximum number of consecutive *N*-acetyl-D-glucosamine residues found in this substrate polymer was three (Srinivasan & Baton, 1997). In zygomycetous fungi, CDA has an important role in fungal growth and is involved in cell-wall chitosan biosynthesis in tandem action with chitin synthase (CS; E.C. 2.4.1.16). It could also be involved in the deacetylation of

chitin oligosaccharides during autolysis after the action of endo-chitinase on cell walls. The CDA is also reported to be involved in ascospore formation in *S. cerevisiae*. Commercially available chitosan is generally produced in a heterogeneous process using concentrated NaOH or KOH (40-50%) at high temperatures taking advantage of the speed and low cost of the production process.

The distribution of GlcNAc and GlcN moieties in this heterogeneously generated chitosan shows a random pattern, highly depending on the conditions applied during the deacetylation (Ng *et al.*, 2007). Therefore, although many studies have been done, variation in biological activities of chitosan is unavoidable and in-depth knowledge on their mode-of-action is scarce. The major reason is that most studies are done with poorly characterized heterogeneous mixtures of chitosan, especially in its PA. Alternatively, chitosan can also be prepared enzymatically by harnessing CDA. The mode of action of chitin deacetylase on chitosan oligomers is as illustrated in Figure 1.5.



N-acetyl D glucosamine residues D-glucosamine

- Numbered arrows indicate the order and site of deacetylation

Figure 1.5 Mode of action of chitin deacetylase on chitin oligomer (Adapted from Tsigos *et al*., 2000)

1.7.1 Current limitations for the use of CDA

Three major challenges need to be addressed while studying CDA. Firstly, the scarcity of well-characterized CDAs, result in diverse chitosan or chitooligosaccharides (COS) products that cannot be ignored. In an interesting article, all the fungal CDAs reported till now, including their biochemical properties, were summarized (Tsigos *et al*., 2000). It was found that there are only a very small number of fungal CDAs that have been fully or partially characterized (Zhao *et al*., 2010). Secondly, there is a lack of easy and accurate approaches to establish the structure of enzymatically deacetylated chitosan or COS products. At present, only a small amount of data has been published to explain the action mode and catalytic mechanism of CDAs on substrates of

chitin (Tsigos *et al*., 2000) or COS (Hekmat *et al*., 2003; Tokuyasu *et al*., 2000) which may be partially due to the technological limitations. Thirdly, the scarcity of an efficient method to break the crystalline structure of chitin, which is highly recalcitrant that cannot be easily tackled by CDA, is also a key issue to overcome. With these situations, two strategies may be considered; either physical or chemical pre-treatment of chitin substrate or integration of a chitin-binding protein (CBP) along with CDA (Zhao *et al*., 2011).

1.8 Applications of chitosan

In recent years, interest in chitosan and COS has increased due to their unique biodegradability, bio-renewability, biocompatibility, physiological inertness, and hydrophilicity. Based on these properties, chitosan and COS have been widely and continuously used in various fields, such as agriculture, cosmetics, water treatment, food industry, pharmaceuticals, and biomedicine. Most biological activities of chitosan are strongly dependent on its DP, which defines the molecular mass of the polymers, DA, which defines its charge density, and pattern of acetylation (PA), which defines the distribution of GlcNAc and GlcN moieties in the chitosan chain (Olicon-Hernandez *et al*., 2017).

1.8.1 Agriculture sector

Chitosan is known to enhance the plant innate defense mechanisms. Chitin and chitosan have been used to control disease or reduce the spread of different pests and pathogens. It can act as a chelating agent for nutrients and minerals. Moreover, chitosan was reported to induce host defense responses such as chitinase and glucanase activation, depolarization of the membrane protein, phosphorylation, lignification, ion flux variations, cytoplasmic acidification, biosynthesis of phytoalexine, synthesis of reactive oxygen species, biosynthesis of jasmonic acid,

callose formation, proteinase inhibitors and the expression of unique early responsive and defense-related genes (Hadrami *et al*., 2010).

To control the growth and spread of many diseases caused due to viruses, bacteria, fungi and pests, chitosan has been used as a foliar application. The chitosan foliar spray reduced local and systemic infection by powdery mildew pathogen, *Blumeria graminis* f. sp. *hordei* in barley as described earlier by Faoro *et al.*, 2008. Along with these applications, chitosan can be applied as a soil amendment to control pathogens like *Fusarium*. Chitosan can also be used as seed coating agent (Orzali *et al*., 2017).

1.8.2 Antimicrobial agent

The main factors affecting the antibacterial activity of chitosan are molecular weight (MW) and concentration. According to some reports, chitosan is more effective in inhibiting the growth of bacteria than chitosan oligomers, and the molecular weight of chitooligosaccharides is critical for microorganism inhibition (<10,000 Da) (No *et al*., 2002). Chitosan can be used as a potential food preservative of natural origin due to its antimicrobial activity against a wide range of foodborne filamentous fungi, yeast, and bacteria (Raafat & Sahl, 2009). The mechanism of the antimicrobial activity of chitosan has not yet been fully elucidated, but several hypotheses have been proposed (Lopez-Moya *et al*., 2019). The most feasible hypothesis is a change in cell permeability due to interactions between the positively charged chitosan molecules and the negatively charged microbial cell membranes (Park *et al*., 2008).

1.8.3 Biomedical applications

Till now, different chitosan-based products are available for biomedical applications. These products mainly include finely divided powder, nonwoven fabrics, porous beads, lyophilized soft fleeces or gels, gauges, laminated sheets, and transparent films (Shariatinia, 2019). Patients

suffering from diseases like diabetes require rapid healing of the wound, and chitosan has been known for its wound healing property for a long time. Chitosan-based hydrogels have also been demonstrated to promote wound healing at different healing stages, and also can alleviate the factors against wound healing such as excessive inflammatory and chronic wound infection. Chitosan can enhance drug absorption due to its mucoadhesive nature. Modification of chitosan through derivatization of the amino and hydroxyl groups by quaternizing with carboxyalkyl, hydroxyalkyl, and acyl derivatives could increase water solubility at higher pH (Wang *et al.*, 2020). Wongpanit *et al.*, 2005 successfully crosslinked the microwave-treated chitin and chitosan films for the wound care application. To overcome the limitations of oral drug delivery, nanotechnology-based formulations are increasingly exploited. Chitosan can be formulated as polymeric nanoparticles for various applications in oral drug delivery. The intestinal absorption of catechin and epigallocatechin gallate can be improved by encapsulating them in chitosan nanoparticles (Dube, *et al.*, 2010). Tamoxifen, an anti-cancer drug, is slightly water-soluble and therefore, permeation of tamoxifen across the intestinal epithelium was increased by formulating tamoxifen into lecithin-chitosan nanoparticles (Barbieri *et al.*, 2015).

1.8.4 Cosmetic treatments

Unlike most other polymers, chitosan, a naturally occurring cationic polymer, can be utilized in the cosmetic industry. Chitosan can behave like a hydrocolloid which facilitates interaction with skin and hair. As chitosan and hair are complementary due to their opposite charge, they can be used as one of the ingredients in shampoo, styling lotions, hair sprays, etc. Different derivatives of chitosan are available for hair and cosmetic solution applications. Most cosmetic products like creams, lotions, nail enamel, lipstick contain different forms of chitin and chitosan (Arnaz *et al.*, 2018). Libio *et al.*, 2016 studied the release of hyaluronic acid in a skin model using chitosan

films prepared in citrate buffers. Skins treated with those films, with or without hyaluronic acid, showed stratum corneum desquamation and hydration within 10 min. The results suggested that the neutralized chitosan citrate film prepared without glycerol promoted a cosmetic effect for skin exfoliation in the presence or absence of hyaluronic acid.

1.8.5 Food industry

Chitosan is of particular interest over synthetic polymers, because it is considered as GRAS (Generally Recognized as Safe) by Food and Drug Administration (FDA). To prevent the growth and accumulation of harmful bacteria, the use of films and blends of natural materials can be a good alternative. Natural biopolymer like chitosan having intrinsic antibacterial activity along with its strong physicochemical properties can be a good choice for packaging materials (Aider, 2010). The major challenge faced by food industry is microbial contamination. Naamani *et al*., 2016 suggested the use of PE coating with chitosan-ZnO nanocomposite to enhance antimicrobial properties of the films. Chitosan-ZnO nanocomposite coatings improved antibacterial properties of PE by inactivating about 99.9% of viable pathogenic bacteria. Hence, it can be used as nanocomposite coating in the development of active food packaging in order to prolong the shelf life of food products (Naamani *et al*., 2016).

1.8.6 Textile materials

Due to the structural characteristics of chitosan, it can be easily blended with conventional textile fibers such as wool, cotton, silk, and polyester with good compatibility and strong interactions. Functional textiles with antibacterial, anti-wrinkle, dyeing, and antistatic properties can be produced using chitosan as a finishing agent or fiber modifier (Morin-Crini *et al*., 2019). For the development of functional fabrics in textile industry, chitosan has been used as an eco-friendly finishing agent. It was originally used as a dye deepening agent in the textile industry. Since it is

a cationic polymer, chitosan is an ideal fixing agent for anionic dyes. Moreover, salt-free dyeing is possible by using chitosan with the help of some other additives (Lim & Hudson, 2003). Table 1.3 depicted the applications of chitosan from different industrial sectors in details.

Table 1.3 Applications of chitosan in various industrial sectors

Sector	Topic	Applications
Agriculture	Plant protection	Protection of plants
	Antimicrobial agent	Coating material: seeds, fruits, vegetables
	Antioxidant	Stimulation of plant growth and plant production
	Horticulture	Increase of crop yields
	Agrochemistry	Reduce the growth of phytopathogenic fungi
Cosmetics	Toiletry	Functional additives
	Hygiene	Moisturizers: maintain skin moisture, tone skin
	Personal care	Thickening agent
	Skin care	Hydrating and film-forming agent
	Oral care	Role in surfactant stability; stabilize emulsion
	Dental care	Antistatic effect
	Hair care	Bacteriostatic
	Cosmeceuticals	Encapsulating agent
	Fragrances	Delivery systems
	Essential oils	Products: shampoos, creams, skin creams, creams for acne treatment, lotions, bath lotions, nail polish
Food industry	Food technology	Additives: fining agent, texture controlling agent,
	Nutrition	natural flavor extender, emulsifying agent, gelling
	Functional foods	agent, color stabilizer, dye-binding properties,
	Food additives	thickener and stabilizer for sauces, flavor extender
	Food mimetic	Improve nutritional quality
		Bioactivities: antibacterial, antifungal, antioxidants
		Preservation of foods from microbial deterioration: protective for fruits

Textiles	Textiles	Dye binder for textiles
	Functional textiles	Impregnated textile materials
	Cosmetotextiles	Binding agent for nonwoven
	Medical textiles	Surface modification of textiles
		Textiles with antibacterial properties
		Textile printing and antimicrobial finishing
		Textile preservative and deodorant agent
Medicine and	Drug delivery	Drug delivery: delivery of antibiotics, peptides,
Biomedical	Biomedicine	proteins, vaccines
	Biomedical engineering	Growth factor delivery
	Biofabrication	Biological response modifier
	Tissue engineering	Gene delivery, targeted delivery, deoxyribonucleic
	Regenerative medicine	acids therapy, gene therapeutics,
	Wound dressing	small interfering ribonucleic acid delivery
		Antifungal, antimicrobial, anti-infectious Hemostatic
		effects

(Adapted from Crini *et al*., 2019).

1.9 Hypothesis and Objectives

So, this study is mainly focused on the important fungi as an alternative source to obtain chitosan with specific properties useful for healthcare applications. As zygomycetous fungi are relatively rich in chitosan than other fungi, *B. poitrasii*, a zygomycetous dimorphic fungus exploited for the chitosan commercial production of chitosan in large quantities for using different optimization and scale up techniques.

Alternately, fungal waste harvested from different industries such as mycotech has good potential for chitosan production as a cheap and reliable source and for value addition too. Increasing use of chitosan, especially low molecular weight in healthcare supports the efforts of mycotech industry for chitosan production. Improvement of chitosan yield using fungal enzyme

chitin deacetylase will reduce the use of harsh alkali and thus can make process eco-friendly. Hence from above discussion we have hypothesized to extract the chitosan from different classes of fungi for commercial production and to explore the fungal chitosan in healthcare applications. Following objectives were designed based on the above hypothesis-

Section I: Optimization for biomass production and improving extractable cell wall chitosan contents from zygomycetous fungus *B. poitrasii*

a) **Optimization of biomass from zygomycetous fungus *B. poitrasii* for chitosan production by one variable at a time (OVAT) approach**
b) **Taguchi design of the experimental approach to increase the biomass and cell wall chitosan contents of zygomycetous dimorphic fungus *B. poitrasii***

Section II: Exploring the potential of agriculturally important ascomycetous fungi *M. anisopliae* and *M. verrucaria* for chitosan production: A value addition to low cost-high volume products

Section III: **Comparative analysis of chitosan from different fungi with special emphasis on zygomycetous dimorphic fungus *B. poitrasii*: Evaluation of its chitosan nanoparticles for the inhibition of human pathogenic fungi**

Chapter 2

Materials and Methods

2.1 Materials

Table 2.1 List of chemicals and source

Name	Source
Agar powder, corn steep liquor (CSL), Luria-Bertani broth (LB), maltodextrins, glucose, mycological peptone, nutrient agar, potato dextrose agar (PDA) powder, sodium chloride, sodium acetate, sodium hydroxide, soluble starch, yeast extract	Hi-media, India
Cobalt chloride, potassium dihydrogen phosphate, magnesium sulphate, ferrous sulphate, manganese sulphate, zinc sulphate	SRL, India
sodium tetraborate (BORAX)	SRL, India
Deuteriated water	Merck, India
Acetic acid glacial, acetic anhydride, Hydrochloric acid, Tween-80	SD Fine Chemicals, India
Chitin, chitosan, ethylene glycol chitin, fluconazole	Sigma-Aldrich, USA
Acetone, ethanol	MP-Biomedicals, USA
RPMI 1640 medium	Hi-media, India
Bergmeyer's Kit	Megazyme, Ireland

2.2 Fungal strains and growth conditions

The fungal cultures used for chitosan production, *B. poitrasii* (NCIM 1240), *H. guilliermondii* (FJ231450), *I. orientalis* (FJ231418), *P. membranifaciens* (FJ231459) and *S. cerevisiae* (FJ231433) were maintained on 1% YPG agar slants (0.3% yeast extract, 0.5% mycological peptone, and 1% D-glucose, and pH 6.5). The yeast cultures were identified by Chavan *et al*., 2009. The mushroom, *P. sajor-caju* DMRP112 was procured from ICAR-Directorate of Mushroom Research, Himachal Pradesh, Solan and maintained on 2% potato dextrose agar (2% PDA). *A. bisporus* (1 kg) was directly procured from local market.

M. anisopliae MCC1197 and *M. anisopliae* MTCC5190 were maintained on 1% YPG (0.3% yeast extract, 0.5% mycological peptone, and 1% D-glucose, and pH 6.5) agar slants. *M. verrucaria* MTCC 5191 was maintained on natural potato dextrose (20% potato and 2% D-glucose, pH 6.5) agar (PDA) slants. All the slants were stored at 4-8 °C.

The human pathogens *C. albicans* ATCC 10231, *C. glabrata* NCYC 388, *C. tropicalis* ATCC 750 and *C. neoformans* ATCC 34664 were maintained on 1% YPG. While the filamentous fungus, *A. niger* ATCC 10578 was maintained on 2% PDA. All the slants were stored at 4 °C.

The plant pathogens, *Xanthomonas campestris* NCIM 5028, *F. oxysporum* CMI113138 and *U. maydis* PRL 1549 were maintained on nutrient agar and on PDA, respectively.

2.3 Optimization of fungal biomass production by one variable at time approach (OVAT)

Yeast cell inoculum of *B. poitrasii* was developed by inoculating 1.6 x 10^7 spores in 100 mL of 1% YPG broth from 3 d old slant and incubating at 37 ºC under shaking condition for 24 h. Before inoculation the yeast cells were washed with sterile distilled water to remove traces of glucose. The following media were used to obtain mycelial biomass: (A) YP (g/L), yeast extract

3, peptone 5; (B) YP modified: YP with (g/L): KH_2PO_4, 5.0; $MgSO_4$, 2.0 and 1.0 mL trace metal solution (mg/mL): $FeSO_4.7H_2O$, 5; $MnSO_4$, 1.5; $ZnSO_4$, 3.34; $CoCl_2$, 2.0; (C) YP modified with 2% corn steep liquor (CSL); (D) YP modified with 0.5% soluble starch; (E) YP modified with 0.5% maltodextrins; (F) YP modified with 1% starch; (G) Optimized medium containing (g/L): yeast extract, 6.0; peptone, 10.0; soluble starch, 10.0 and inorganic salts and trace metal solution as mentioned above. The YP medium along with 20% sweet potato extract was also used for biomass production. The medium was inoculated with 1.6×10^8 yeast cells/L and incubated at 28 ºC for 48 h. The mycelial biomass was separated by filtration using Whatman filter paper no. 1, washed, dried and stored in refrigerator until use.

The optimized medium in shake flask was further used in 2 L fermenter (New Brunswick BioFlo®/CelliGen® 115, Germany) for scale up. The development of an inoculum in optimized medium for fermentation studies is described above. For fermentation agitation was programmed from 250-400 rpm, aeration 2.5 litre per minute (lpm), initial pH 6.5 and was carried out 28 ºC for 48 h.

2.4 Optimization of fungal biomass using Taguchi design of the experimental approach

The optimization of media components and other incubation conditions for *B. poitrasii* biomass was done using the Taguchi DOE. Eight different control factors were identified to evaluate their role in the objective function, *i.e.* optimizing medium for maximum biomass production. The factors selected were starch, yeast extract (YE), peptone, pH, temperature, the ratio $MgSO_4$:KH_2PO_4, inoculum size, and incubation time. The temperature factor has been assigned with only two levels, namely 25 °C and 28 °C, whereas three were selected for the remaining seven factors. Based on the number of factors and levels, L18 ($2^1 \times 3^7$) orthogonal array was used

for the design. In the present study, all 8 columns were assigned with different factors (Table 2.2 and 2.3 depicts the details).

Table 2.2 Factors and their levels used for L18 orthogonal array design

	Level		
Control factor	**1**	**2**	**3**
A. Temperature (°C)	25	28	-
B. Carbon source (Starch) (%)	0.5	1	1.5
C. pH	5.5	6.5	7.5
D. Yeast extract (%)	0.3	0.6	0.9
E. Peptone (%)	0.5	1.0	1.5
F. Inoculum Size (%)	8	10	12
G. Incubation Time (h)	24	36	48
H. $MgSO_4$/ KH_2PO_4 Ratio	1:2	1:2.5	1:3

Table 2.3 Design of experiment to optimize fermentation conditions to increase *B. poitrasii* biomass and chitosan contents using L18 orthogonal array

	Control factors							
Expt. No.	Temp (°C)	Starch (%)	pH	YE (%)	Peptone (%)	Inoculum Size (%)	Incubation Time (h)	$MgSO_4$: KH_2PO_4
1	25	0.5	5.5	0.3	0.5	8	24	1:2
2	25	0.5	6.5	0.6	1	10	36	1:2.5
3	25	0.5	7.5	0.9	1.5	12	48	1:3
4	25	1	5.5	0.3	1	10	48	1:3

5	25	1	6.5	0.6	1.5	12	24	1:2
6	25	1	7.5	0.9	0.5	8	36	1:2.5
7	25	1.5	5.5	0.6	0.5	12	36	1:3
8	25	1.5	6.5	0.9	1	8	48	1:2
9	25	1.5	7.5	0.3	1.5	10	24	1:2.5
10	28	0.5	5.5	0.9	1.5	10	36	1:2
11	28	0.5	6.5	0.3	0.5	12	48	1:2.5
12	28	0.5	7.5	0.6	1	8	24	1:3
13	28	1	5.5	0.6	1.5	8	48	1:2.5
14	28	1	6.5	0.9	0.5	10	24	1:3
15	28	1	7.5	0.3	1	12	36	1:2
16	28	1.5	5.5	0.9	1	12	24	1:2.5
17	28	1.5	6.5	0.3	1.5	8	36	1:3
18	28	1.5	7.5	0.6	0.5	10	48	1:2

The growth experiments were carried out in a 1000 mL Erlenmeyer flask containing 200 mL of optimized medium. The different inoculums were prepared in the respective medium using sporangiospores (1.6 x 10^7/100 mL), incubated for 24 h under shaking conditions and used for inoculations. All flasks were incubated at 28 °C for 48 h on a rotary shaker (180 rpm). The mycelial biomass was harvested by filtration through Whatman filter paper No.1 and washed

with distilled water (3-4 times). The above filtrate solution was centrifuged at 7100 rpm for 20 min to harvest the yeast cells and washed with distilled water several times to remove the starch. The removal of starch was confirmed with the iodine test. For instance, 1 g of wet biomass (yeast or mycelia) was taken into a test tube, and a few drops of iodine solution were added. No change in color indicated the absence of starch in biomass. The dried mycelial and yeast biomass was further used for the isolation of chitosan.

2.5 Effect of different inoculum types on the growth of fungal biomass

B. poitrasii, *viz*., sporangiospores, mycelia, yeast cells, alone and in combination, were used as an inoculum to obtain biomass. *B. poitrasii* growth from slant was gently scraped to obtain sporangiospores, free from yeast, mycelium, and zygospores, as determined by light microscopy. The yeast inoculum was obtained by inoculating 1.6 x 10^7 sporangiospores in 100 mL YPG medium under shaking conditions (180 rpm) at 37 °C for 24 h. To prepare mycelial inoculum, sporangiospores were inoculated in 100 mL YP medium and incubated at 28 °C, 180 rpm for 24 h. The mix inoculum was prepared by growing the sporangiospores in the optimized medium at 28 °C, 180 rpm for 24 h. All the experiments were carried out in a 1000 mL Erlenmeyer flask containing 200 mL of optimized medium, inoculated with different inoculum types and incubated at 28 °C, 180 rpm for 48 h. The biomass was harvested and dried until constant weight.

2.6 Solid state fermentation for conidia production by *M. anisopliae*

The mycelial inoculums of *M. anisopliae* MTCC5190 was developed in YPG medium for 48 h. Further the production of conidia by solid state fermentation using rice as a substrate in Unicorn bags (autoclavable, type/14 with single microvented filter of 0.2 µm, 2 kg capacity, 64 x 36 cm, Unicorn Imp & Mfg Corp, USA) at 28 ºC and 70-80% RH for 14 d was carried out as described

earlier (Kulkarni *et al*., 2008). After harvesting of conidia using liquid extraction (0.1% Tween 80) 14 d, the solid substrate along with mycelial biomass was suspended in distilled water and centrifuged at 10000 x g for 10 min. The supernatant containing mycelial fragments were separated by decantation, washed 3 times with distilled water and centrifuged 1000 x g for 10 min.

2.7 Submerged fermentation for the production of extracellular enzymes by *M. anisopliae* and *M. verrucaria*

The submerged fermentation of *M. anisopliae* MCC1197 was carried out in 1% YPG medium for extracellular, constitutive, chitin deacetylase(s) (CDA) for 72 h (Nahar *et al*., 2004). *M. anisopliae* MCC1197 biomass was separated from the crude CDA by centrifugation at 10000 x g for 10 min and washed repeatedly before its use.

The production of cuticle degrading and mycolytic enzyme (CDE and ME) mixture by *M. verrucaria* MTCC5191 was carried out in submerged fermentation using chitin as a sole carbon source for 7 d as described earlier (Vidhate *et al*., 2015). After separation of CDE/ME enzyme mixture from *M. verrucaria* biomass and unutilized chitin by centrifugation at 10000 x g for 10 min. The biomass was separated from the unutilized chitin at 1000 x g for 10 min, after repeated washing with distilled water.

2.8 Submerged fermentation of yeasts

The yeast cultures were grown in 1000 mL Erlenmeyer flasks containing 200 mL of 1% YPG medium and the flasks were incubated at 28 ºC at 180 rpm, for 48 h. Inoculum (24 h old) of *H. guilliermondii, I. orientalis, P. membranifaciens* and *S. cerevisiae,* were prepared in 1% YPG medium and final concentration of 10^7 cells/100 mL was adjusted using hemocytometer under

light microscope. The yeast biomass was obtained by centrifugation at 8000 x g for 20 min and dried at 50 °C until further use.

2.9 Cultivation of *P. sajor-caju*

The cultivation of *P. sajor-caju* was carried out by using wheat straw as a substrate. The spawn culture was developed by using wheat. The overnight soaking of wheat was carried out in water. After which, it was boiled for about 30 min, drained it on plain surface to remove excess water to retain 50% moisture. Calcium carbonate (20 g/kg of wheat straw) was added to the bags and autoclaved at 121 °C for 30 min. The bags were inoculated with the spawn culture and incubated at 28 °C. Further the cultivation was carried out as described by Tupatkar & Jadhao, 2006.

2.10 Isolation of cell walls of different fungal organisms

All preparations were carried out in the 0-4 °C range. The cells were washed and suspended in cold distilled water and disrupted in a 90's Braun homogenizer by using the glass beads (0.45-0.5 mm) (4-5 cycles of 30s each) through mechanical shaking. The cell wall preparations were observed by light microscopy to confirm the absence of cytoplasmic content (Khale & Deshpande, 1992). The cell wall pellet after centrifugation was washed 5-6 times with cold distilled water then twice with decreasing concentration of cold NaCl solution (5.0%, 2.0% and 1.0%, w/v) followed (10-12 times) by cold distilled water. The cell wall preparations were clean and free from cytoplasmic material as determined by light microscopy. The purified cell wall samples were lyophilized and stored at -20 °C till further use.

2.11 Extraction of chitosan from fungal biomass

Chitin and chitosan were extracted from the isolated cell wall as described earlier (Doiphode, 2007; Khale & Deshpande, 1992). The cell wall pellet was re-suspended in 1N NaOH (1:40 w/v) followed by autoclaving at 121 °C for 15 min. Alkali insoluble mass (AIM) was washed

thoroughly with distilled water, further with ethanol followed by centrifugation at 12000 x g for 10 min. The residue was refluxed in 100 volumes of 2% acetic acid at 95 ℃ for 24 h. The slurry was centrifuged at 12000 x g at 4 ℃ for 45 min. Chitosan was precipitated out from the supernatant by adjusting the pH to 8.5-10 with 1N NaOH. The precipitate was washed several times with chilled distilled water. It was further washed with acetone and dried in oven at 50 ℃, the sample were stored at -20 ℃ till further use. In another experiment, instead of cell wall isolation, the washed *B. poitrasii* mycelial biomass was directly used for the extraction of chitosan.

The commercial chitosan from shrimp shells procured from Sigma-Aldrich, USA (Product No. C3646) was used as reference in the present study, unless and otherwise mentioned.

2.12 Production of extracellular chitin deacetylase by *M. anisopliae* and intracellular chitin deacetylase by *B. poitrasii*

The extracellular constitutive production of chitin metabolizing enzymes particularly CDA by *M. anisopliae* strain was carried out in 1% YPG medium at 28 ℃ for 72 h (Nahar *et al*., 2004). The extracellular supernatant was further ten times concentrated by ultra-filtration (UM 30 Amicon membrane) and used for deacetylation of *B. poitrasii* cell wall.

To obtain intracellular crude CDA from *B. poitrasii,* the fresh mycelial biomass obtained in optimized medium, was homogenized with glass beads in 50 mM sodium tetraborate buffer (pH 8.5), in Braun's homogenizer for 5 cycles of 15 sec each and then centrifuged at 12,000 g for 15 min. The supernatant was transferred to new microfuge tube. This supernatant was used as intracellular crude CDA.

2.13 Chitin deacetylase assay

Chitin deacetylase (CDA) activity was estimated using ethylene glycol chitin (EGC) as a substrate prepared as described earlier with slight modification (Nahar *et al*., 2004). The reaction mixture contained: 100 µL 50 mM sodium tetraborate buffer, pH 8.5, 100 µL EGC (1 mg/mL), and 50 µL enzyme, incubated at 37 °C for 30 min. The reaction was terminated by heating at 100 °C for 15 min. The acetate molecules released were measured by Bergmeyer's method using acetic acid (ACS manual format) assay kit according to manufacturer's instruction. The acetate released was determined based on NADH formation that was measured by increase in the absorbance at 340 nm. One unit of enzyme released 1 µmol of acetate from ethylene glycol chitin per min (Nahar *et al*., 2004).

2.14 Enzymatic deacetylation of *B. poitrasii* cell wall

The cell wall pellet isolated as mentioned under section 2.10 was treated with 10 mL of *M. anisopliae* extracellular and *B. poitrasii* intracellular CDA (100 mU each), separately at 37 °C for 24 h with constant shaking. After 24 h, the pellet was collected by centrifugation and further processed for chitosan extraction. In the control sample, cells were homogenized and chitosan was extracted by 1N NaOH treatment as described in section 2.11. The supernatant obtained after CDA and chemical treatment was used to quantify the released acetate by Bergmeyer's method using acetic acid (ACS manual format) assay kit (Megazyme, Ireland) according to manufacturer's instructions. Based on the concentration of acetate released due to each treatment, the chitosan contents were calculated.

2.15 Biophysical characterization of chitosan

2.15.1 Fourier transform infrared (FT-IR) spectroscopy

The Fourier transform infrared (FT-IR) spectra were taken on a Bruker Optics ALPHA-E spectrometer with a universal Zn-Se ATR (attenuated total reflection) accessory in the 400-4000 cm^{-1} region or using a Diamond ATR (Golden Gate). The chitosan sample was dried at 105 °C for 1 h and consequently grinded for 5 min. It was mixed with KBr and was kept at 105 °C for 1 h and after grinding for overnight. The FT-IR was taken using KBr pellet as a reference as described by Van de Velde & Kiekens, 2004. The DDA was determined by means of the absorbance ratio A_{1320}/A_{1420}. The following formula was used for the % Degree of acetylation (DA) calculation = 31.198 (A_{1320}/A_{1420})-12.20. The % DDA was calculated by using formula: % DDA= 100-% DA.

2.15.2 ^{1}H- nuclear magnetic resonance (NMR) spectroscopy

The samples were prepared by dissolving 10 mg of chitosan in a solution containing 1.96 mL of D_2O and 0.04 mL of concentrated HCl with continuous stirring for 30 min. Samples were lyophilized and dissolved in 1 mL of D_2O. The 600 µL of sample was used for the NMR spectral analysis (Lavertu *et al*., 2003). All the NMR spectra were taken on Bruker AV400 NMR spectrometer. The experiments were carried out at 70 °C at which the solvent (D_2O) peak does not interfere with any of chitosan's peaks. DDA was calculated using formula 1 and 2 integrals of proton ^{1}H of deacetylated monomer (H1-D) and the peak of the three protons of acetyl group (H-Ac) (Hirai *et al*., 1991)

% DDA = H1D/H1D+H1A × 100….. (1)

% DDA = H1D/H1D+HAc/3 × 100… (2)

The signal from protons H2, H3, H4, H5, H6, H6'of both monomers and the peak of acetyl group (H-Ac) was also used for DDA calculation.

% DDA = [1- (1/3 HAc /1/6 H 2-6)] x **100….. (3)**

2.15.3 Determination of viscosity of chitosan

Viscosity measurements were carried out at 25 °C using Ubbelohde-type viscometer (Fisher, Germany). Viscometer was connected to a visco-clock (Schott Visco clock), which automatically recorded the time of flow of solution through the two marks of the viscometer. Every value recorded was an average of 3 measurements. Chitosan samples (0.1%) were prepared in 0.5 M acetic acid and 0.5 M sodium acetate (Kasaai, 2000). The flow time was determined in sec. The average molecular weight was then calculated using the Mark- Houwink equation:

$[\eta] = KMv^{\alpha}$, Where K and α for HAc/NaAc is $119\times10^{5}$and 0.5, respectively.

2.15.4 Molecular weight determination of fungal chitosan by gel permeation chromatography (GPC)

A chromatography system with OHpak SB-800 HQ series columns (Agilent Technologies, USA) was applied to determine the molecular weight of fungal chitosan using gel permeation. The fungal chitosan was dissolved in 2% acetic acid at a final concentration of 2 mg/mL. Further the solution was filtered through 0.22 µ filters to remove debris. The mobile phase used in this study was 0.2 M acetic acid/0.1 M sodium acetate. The filtered chitosan solution (50 µL) was run at 0.8 mL/min flow rate and 40 °C column temperature. Pullulan 20201 Shodex standards were used for the calibration of column. The molecular weight was determination was carried out as described earlier by Zielinska *et al*., 2014 with a refractive index detector.

2.16 Fungal chitosan nanoparticles synthesis and characterization

2.16.1 *B. poitrasii* hyphal and yeast chitosan nanoparticles synthesis

B. poitrasii hyphal as well as yeast and marine chitosan solutions at concentration of 0.5 mg/mL were prepared by dissolving in 10 mL of 2% acetic acid. The synthesis of nanoparticles was carried out by ionic gelation method through the interaction with sodium tripolyphosphate (TPP). A stock of 1.6 mg/mL of TPP was prepared in sterile distilled water. Under continuous magnetic stirring at 1000 rpm, 1.25 mL of TPP solution was added in drop wise manner to 10 mL of chitosan solution (1:5 molar ratios) and nanoparticles were produced by ionic gelation (Kolge *et al*., 2021). The particles were then kept at room temperature for 30 min. The nanoparticles were then centrifuged at 25000 x g for 30 min. The nanoparticles were redispersed in distilled water and used for characterization (Sreekumar *et al*., 2018).

2.16.2 Characterization of synthesized fungal chitosan nanoparticles

Mean particle size (Z-average), polydispersity index (PDI) and zeta potential of the *B. poitrasii* hyphal chitosan nanoparticles (BpHCNps), *B. poitrasii* yeast chitosan nanoparticles (BpYCNps) and marine chitosan nanoparticles (MCNps) were measured by using Malvern Zetasizer Pro. The measurements were carried out in triplicate at 25 °C.

2.16.3 Field emission scanning electron microscopy (FE-SEM) of chitosan nanoparticles

For FE-SEM analysis, chitosan nanoparticle samples were fixed on the metal stub with double adhesive tape and coated with gold under vacuum. The samples were then observed under FEI–NOVA NANOSEM 450 system at 2, 40,000 X magnification.

2.16.4 High resolution transmission electron microscopy (HR-TEM) of chitosan nanoparticles

Particles were visualized using high resolution transmission electron microscopy. To this end, 10 µL of sample was diluted in water (1:10 v/v). Then, 10 µL of the sample was placed onto a copper grid covered for 30 s. Excess liquid was removed using a filter paper and the grids were dried in a desiccator for at least 24 h. Imaging was performed using JEOL-JEM-F200 system.

2.17 Antifungal assay of fungal chitosan

Chitosan extracted from different fungi was dissolved in 2% acetic acid solution at a final concentration of 10 mg/mL. The solution was kept for stirring overnight for dissolution of chitosan. The NaOH (1N) was used to adjust the final pH of the solution to 5. For the determination of antifungal activity of chitosan nanoparticles, the particles dispersed in distilled water were used directly. The antifungal activity was checked against human pathogenic *C. albicans* ATCC 10231, *C. glabrata* NCYC 388, *C. tropicalis* ATCC 750, *C. neoformans* ATCC 34664 and *A. niger* ATCC 10578 by Clinical Laboratory Standards Institute's (CLSI) broth micro-dilution assay as reported earlier by Mane *et al*., 2017a. The stock was prepared in Roswell Park Memorial Institute (RPMI) 1640 medium and added in the first row of a 96-well microtitre plate. The chitosan samples were serially diluted using RPMI 1640 medium in successive wells to get the final concentration in the range of 0.025-5.0 mg/mL. The yeast cells (~2 x 10^3cfu/mL) were grown for overnight and diluted in RPMI 1640 medium for inoculation (100 µL) in the wells of the microtitre plate. For filamentous fungi, 2 x 10^4 spores/mL were used as an inoculum. The plates were further incubated for 24-72 h. Acetic acid (2%) was used as control. After incubation, the absorbance was measured at 600 nm via a microtitre plate reader

(Epoch, Biotech Instruments) to check out the growth. The lowest concentration exhibiting >90% inhibition of growth as compared to the control was described as the MIC_{90}.

2.18 Disk diffusion assay of fungal chitosan against plant pathogenic fungi

To study the antifungal potential of fungal chitosan, hyphal extension inhibition assay was performed as described earlier. Briefly, radial mycelial growth of plant pathogenic fungi namely *F. oxysporum* CMI113138 and *U. maydis* PRL 1549 was developed at the center of 90 × 15 mm petridish containing 15 mL of potato dextrose agar. When colony diameter reached up to 2 to 3 cm, sterile filter paper disc containing 1.0 mg, 2.0 mg and 3.0 mg of chitosan in 2% acetic acid was placed 0.5 cm away from the colony rim. 2% acetic acid served as a control. Further, plates were incubated at 28 °C for 72 h and observed for the inhibition zone around filter paper disk (Berger *et al.*, 2018).

2.19 Antimicrobial assay of fungal chitosan against plant pathogenic organisms

Antibacterial activity of the fungal chitosan was checked against *X. campestris* NCIM 5028 and antifungal activity was checked against *F. oxysporum* CMI113138 by CLSI broth micro-dilution assay as described in Section 2.17. *X. campestris* was grown for 24 h in Luria Bertani (LB) broth. The OD of the culture was adjusted to 0.12-0.14 using RPMI and inoculated (100 µL) in the wells of the microtitre plate. The microtitre plates were incubated for 24 h. *F. oxysporum* spores (2 x 10^4/mL) were suspended in the medium and inoculated (100 µL) in the wells of the microtitre plate. The microtitre plates were incubated for 48 h.

2.20 RBC hemolysis assay

To study the hemocompatibility of chitosan and chitosan nanoparticles, the red blood cell (RBC) hemolysis assay was performed as described by Pathan *et al*., 2020. The chitosan as well as chitosan nanoparticles were tested in the concentration range of 0.039 to 5 mg/mL. Triton X-100 (0.1% (v/v) in PBS will be used as a positive control whereas PBS will be used as a negative control. The experiment was performed in triplicates with two biological replicates and the mean values were considered for the calculation of percent hemolytic activity of chitosan and chitosan nanoparticles.

Chapter 3

Optimization for biomass production and improving extractable cell wall chitosan contents from zygomycetous fungus *Benjaminiella poitrasii*

Chapter 3a

Optimization of biomass from zygomycetous fungus *Benjaminiella poitrasii for* chitosan production by one variable at a time (OVAT) approach

Contents of chapter-3a have been published in a research article……

(Mane *et al*., 2017, Journal of polymer materials, 34, 145-156)

3.1a Introduction

Chitosan, a β-(1-4), 2-amino-2-deoxy-D-glucose (or D-glucosamine) polymer formed by deacetylation of chitin by the action of enzyme CDA (EC 3.5.1.41). It has a wide range of applications from agriculture to human health care products (Kannan *et al*., 2010; Kim *et al*., 2007; Muzzarelli *et al*., 1994). Currently, chitosan is commercially produced from shellfish, shrimp waste, crab and lobster processing using strong alkalis at high temperatures for long periods of time (Knorr, 1994). However, the supply of marine waste is seasonal and limited, resulting in variability in source material. Furthermore, physico-chemical properties of chitosan derived from such processes are heterogeneous (Crestini *et al*., 1996; Kim *et al*., 2007). Since chitosan is required on large scale for variety of applications, it was proposed that it would be difficult to replace the marine sources with other organism in near future (Allan *et al*., 1978; Roberts, 2008).

Ghormade *et al*., 2017 reviewed the potential of fungal waste from mycotech industries as an alternate source for chitosan. Furthermore, other than waste biomass utilization for value addition, number of zygomycetous fungi can be employed for chitosan production, as they contain relatively more chitosan in their cell walls than fungi from other classes (Ghormade *et al*., 2017). In addition, the quality of fungal chitosan in terms of homogenous polymer length, DDA and solubility offers advantages over the crustacean sources (Pochanavanich & Suntornsuk, 2002; Ghormade *et al*., 2017).

Extraction of chitosan was reported mainly from zygomycetous fungi like *A. coerulea, C. elegans, G. butleri, M. rouxii, M. racemosus, M. indicus* and *R. oryzae* (Amorim *et al*., 2001; Davoust & Hansson, 1992; Nwe *et al*., 2002; Satari *et al*., 2015; Synowiecki & Al-Khateeb, 1997). In case of *G. butleri*, Nwe *et al*., 2002 used both solid state fermentation (SSF) as well as

submerged fermentation (SMF) for biomass production. Suntornsuk *et al*., 2002, reported use of soyabean, mung bean, molasses, corn-steep liquor, or sweet potato extract as cheaper alternate substrates to grow *R. oryze* and others for biomass development.

B. poitrasii is a well studied zygomycetous, dimorphic fungus with high chitin/chitosan contents (35% of the cell wall) in the mycelial form of the fungus (Khale & Deshpande, 1992). In the present chapter optimization of *B. poitrasii* biomass production for the extraction of chitosan by using yeast inoculum and the effect of chitin deacetylase treatment to increase chitosan contents were reported.

3.2a Results

3.2.1a Production of *B. poitrasii* mycelial biomass in shake flasks using one variable at time approach (OVAT)

Various combinations of media were used to produce the maximum mycelial biomass of *B. poitrasii*. Within 48 h, the biomass yield in shake flask increased 3.34 times when grown in medium G (optimized medium, 44.50±0.80 g/L of wet biomass and 8.72±0.45 g/L of dry biomass) compared to medium B (YP modified medium, 13.30±0.60 g/L of wet biomass and 2.66±0.25 of dry biomass). In YP modified medium, adding 2% CSL (medium C), 0.5% soluble starch (medium D), 0.5% malto-dextrins (medium E), or 1% soluble starch (medium F) did not significantly increase biomass.

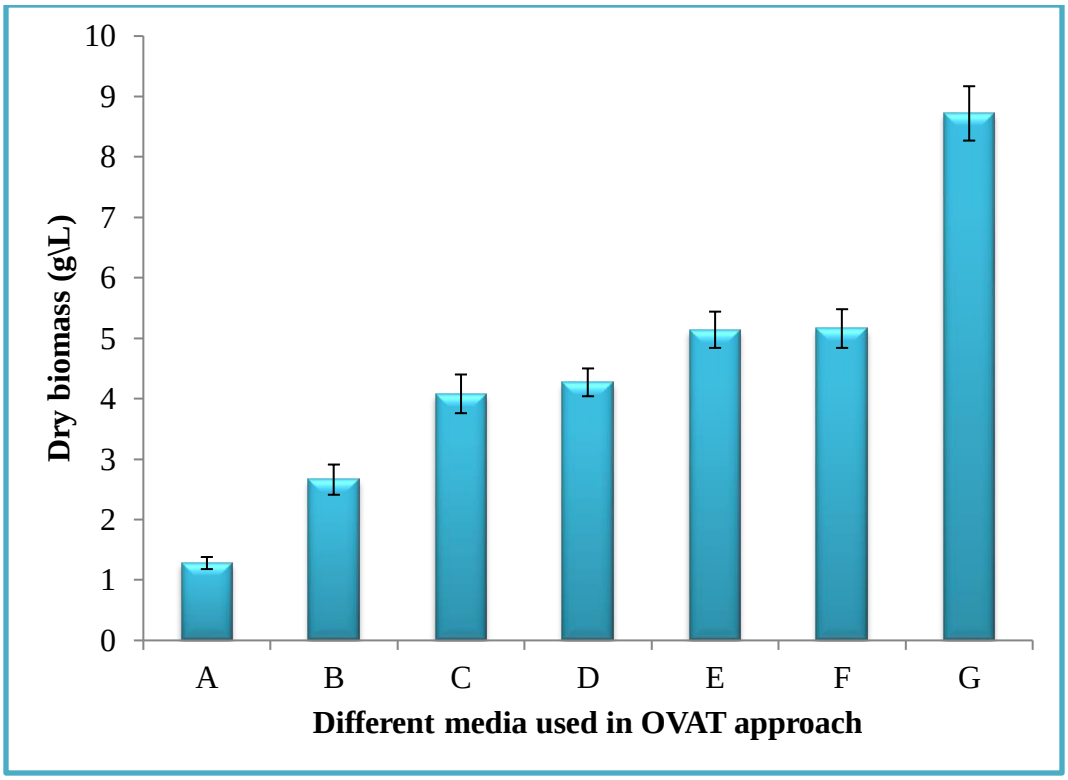


Figure 3.1 *B. poitrasii* biomass production in different nutritional media under shaking condition after 48 h. The following media were used: A, YP; B, YP modified *i.e.* with inorganic salts; C, YP modified with 2% CSL; D, YP modified with 0.5% starch; E, YP modified with 0.5% maltodextrins; F, YP modified with 1% starch and G, optimized medium.

The addition of inorganic salts and trace metals to YP medium increased biomass by 2-fold, from 6.44±0.90 g/L (1.28±0.10 g/L of dry biomass) to 13.30±0.60 g/L (2.66±0.25 g/L of dry biomass) in the YP modified medium (Figure 3.1). Furthermore, with 20% sweet potato extract, biomass yield increased 2.69-fold (17.33±0.80 g/L of wet biomass and 3.46±0.30 of dry biomass) (Table 3.1).

Table 3.1 The yield of biomass and chitosan of *B. poitrasii* grown in different media in shake flasks

Medium	**Wet Biomass wt. (g/L)**	**Dry Biomass wt. (g/L)**	**Chitosan mg/L**
YP (control)	6.44±0.90	1.28±0.10	83.52±5.00
YP modified	13.30±0.60	2.66±0.25	151.67±3.10
YP+SP extract	17.33±0.80	3.46±0.30	226. 00±4.00
Optimized medium	44.50±0.80	8.72±0.45	443.24±4.10
Optimized medium*	46.20±1.00	9.25±0.50	460.47±5.00
Optimized medium** (Fermenter batch)	51.20±1.00	10.00±0.50	510.00±5.20

The yeast cells (1.6 x 10^8/L) were inoculated and flasks were incubated under shaking conditions at 28 ºC for 48 h.

*The 10% (v/v) inoculum developed in optimised medium as described under materials and methods was used in shake flask.

** The inoculum (10% v/v) developed in the optimized medium was used for 2L fermenter.

For biomass production in optimized medium, an inoculum was made by inoculating 1.6 x10^7 spores from a 3 day old slant and incubating at 28 °C under shaking conditions for 24 h. The biomass produced in the shake flask was slightly higher with a 10% inoculum containing both yeast and hyphal cells in equal proportion (Table 3.1).

The mycelial morphologies developed in various media were comparable by microscopically (Figure 3.2). However, when compared to other media, the mycelia on sweet potato medium were thicker.

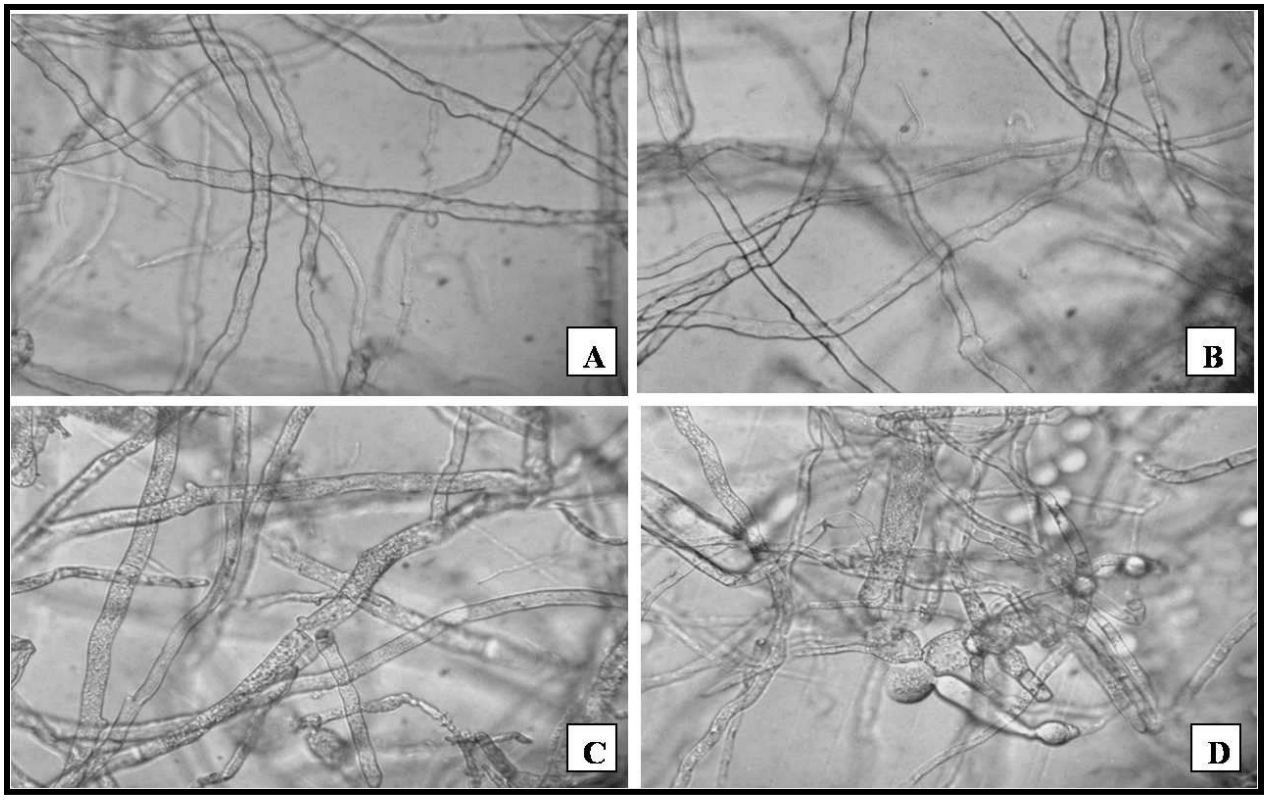


Figure 3.2 Mycelial morphology of *B. poitrasii* in different production media. A, YP medium; B, modified YP; C, modified YP with 20 % sweet potato extract.; D, optimized medium. All images were captured at 400X magnification.

3.2.2a Production of *B. poitrasii* mycelial biomass in fermenter

The production of mycelial biomass optimized in shake flask was successfully up scaled to 2L fermenter. The inoculum (10% v/v) developed in optimized medium was used. The biomass was produced in the optimized medium in 2 L fermenter maintaining pH 6.5, air flow 2.5 lpm and agitation at 250-400 rpm. After 48 h the biomass yield was 51.20±1.00 g (wet wt.)/L (Table 3.1).

After media optimization studies, the amount of biomass obtained was increased by 7.95-fold with simultaneous increase in the yield of extractable chitosan by 6.1-fold *viz*., from 83.52±5.00 to 510.00±5.20 mg/L (Figure 3.3; Table 3.1).

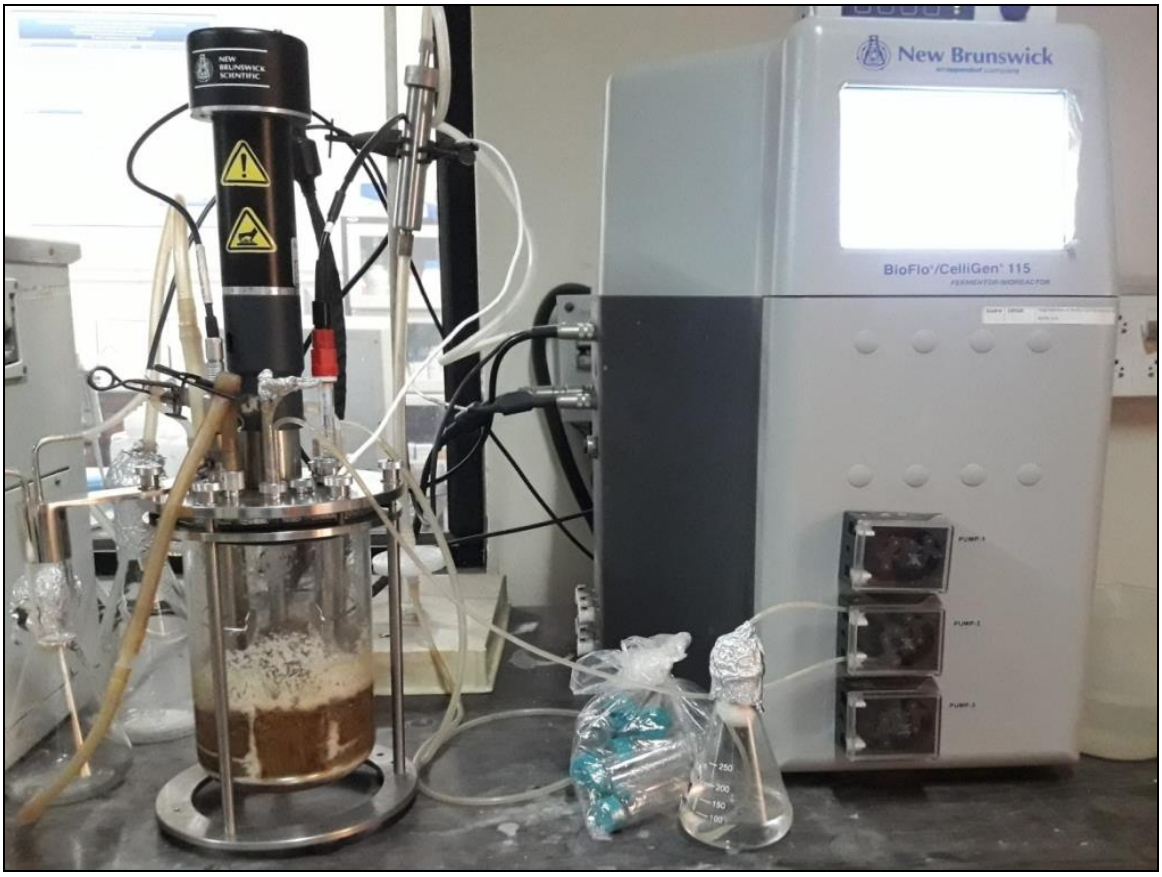


Figure 3.3 Batch Fermentation for *B. poitrasii* biomass production (New Brunswick BioFlo®/CelliGen® 115, Germany) (Fermenter was programmed at agitation from 250-400 rpm, aeration 2.5 lpm, initial pH 6.5, temperature, 28 ºC for 48 h).

3.2.3a Qualitative analysis of fungal chitosan by fourier transform infrared (FT-IR) spectroscopy

IR spectra were used to identify the presence of chitosan extracted from mycelial biomass of *B. poitrasii*. The typical peaks at 1452 and 1556 cm^{-1} for purified chitosan (Sigma Chem. Co. USA) and also for *B. poitrasii* samples were used to confirm the presence of chitosan. The degree of deacetylation calculated from FT-IR was 92.89% for *B. poitrasii* chitosan while for commercial chitosan (Sigma), it was 80.22% (Figure 3.4; Table 3.2).

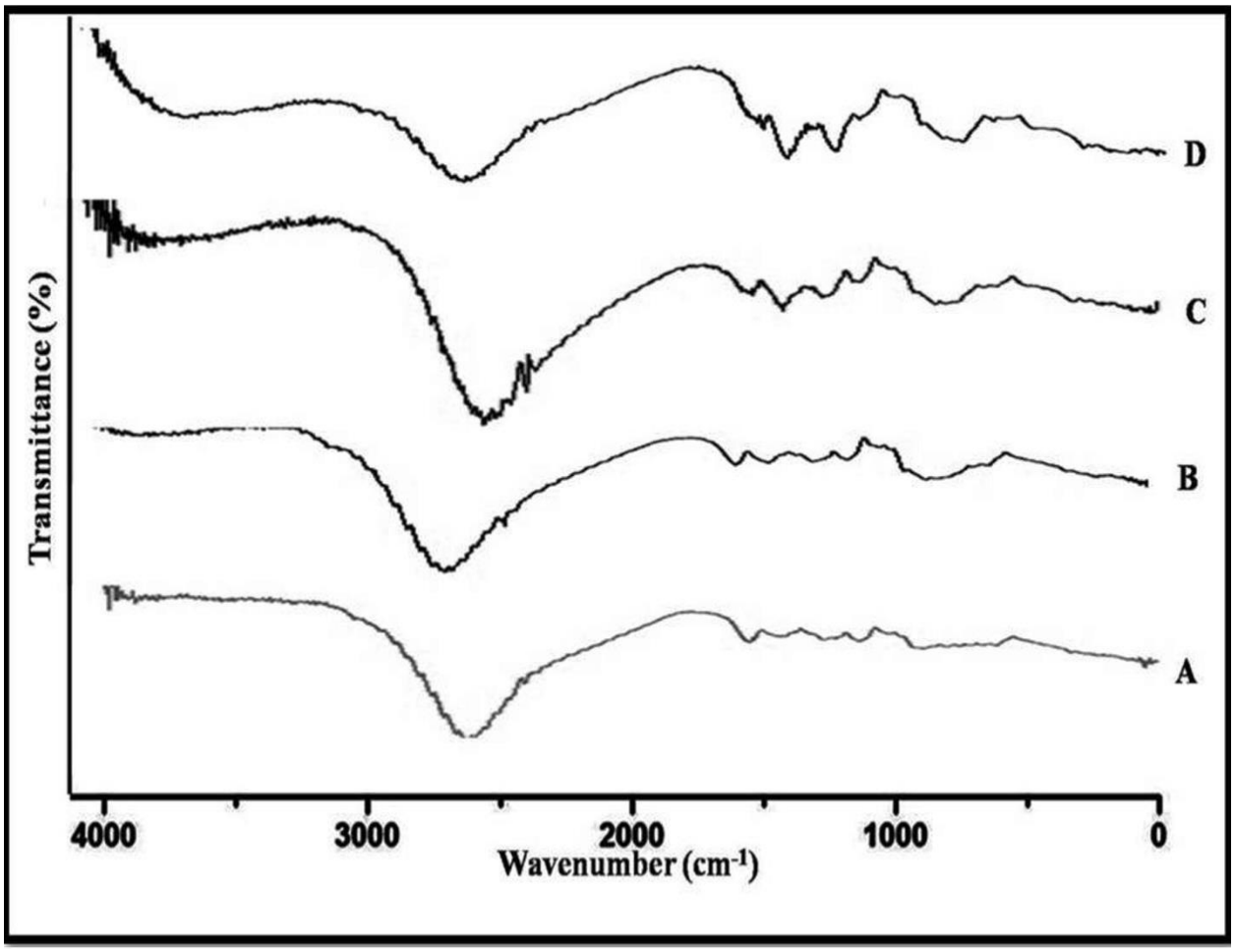


Figure 3.4 FT-IR profiles of chitosan from different sources. A, purified chitosan from Sigma Chem. Co. USA; B, chitosan isolated from *B. poitrasii* grown in YP medium; C, chitosan of isolated from *B. poitrasii* grown in YP modified medium; D, chitosan isolated from *B. poitrasii* grown in optimized medium.

Table 3.2 Degree of deacetylation and molecular weight of chitosan isolated from *B. poitrasii* mycelia grown in different media

Isolated chitosan from *B. poitrasii* grown in media	**DDA (^{1}H-NMR) (%)**	**DDA (FT-IR) (%)**	**Molecular weight (kDa)**
YP	95.15	93.87	42.95
YP modified	94.76	92.77	43.57
Optimized medium	94.24	92.89	42.82
YP modified with 20% Sweet potato extract	ND	ND	37.31
Purified chitosan (Sigma Chem. Co. USA)	85.66	80.22	464.83

ND, Not determined

3.2.4a Quantitative determination of %DDA of chitosan using ^{1}H- nuclear magnetic resonance spectroscopy

^{1}H- NMR was used to calculate the degree of deacetylation. *B. poitrasii* chitosan isolated from biomass grown in different media had 94.24% DDA while the chitosan procured from Sigma showed 85.66% DDA (Figure 3.5; Table 3.2). The %DDA values calculated using FT-IR were marginally lesser than values obtained by ^{1}H- NMR.

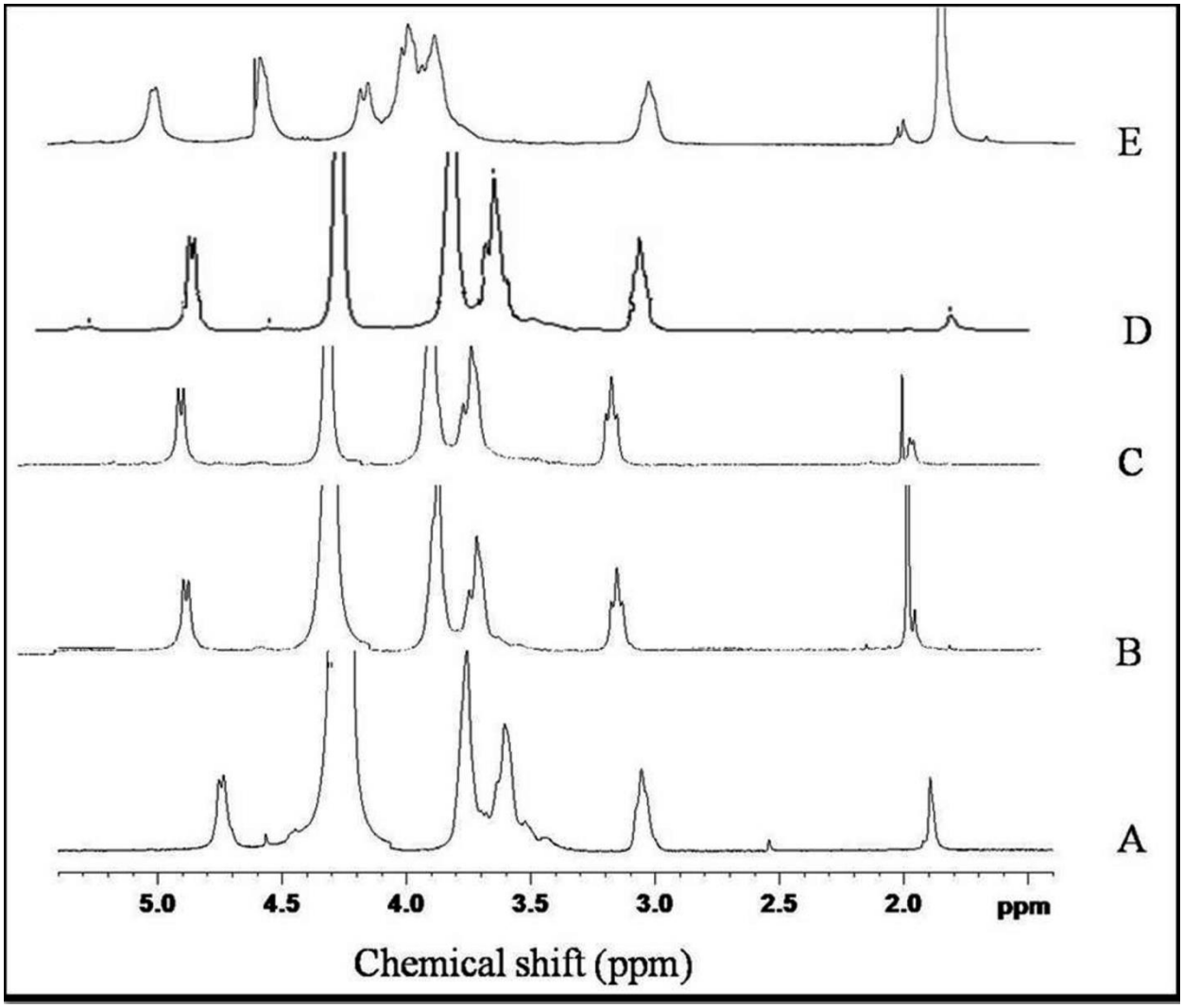


Figure 3.5 ^{1}H-NMR profiles of chitosan from different sources. A, purified chitosan from Sigma Chem. Co. USA; B, chitosan isolated from *B. poitrasii* grown in YP medium; C, chitosan of isolated from *B. poitrasii* grown in YP modified medium; D, chitosan isolated from *B. poitrasii* grown in optimized medium; E, chitosan isolated from *B. poitrasii* grown in sweet potato medium.

3.2.5a Viscometry analysis for molecular weight determination of *B. poitrasii* chitosan

The average molecular weight of *B. poitrasii* chitosan was 42.82 kDa while of commercial chitosan (Sigma) from marine source was almost ten times *viz.*, 464.83 kDa (Table 3.2).

3.2.6a Enzymatic deacetylation of *B. poitrasii* biomass with *M. anisopliae* chitin deacetylase

As compared to the NaOH treatment, chitosan content was increased by 1.16-fold (59.00±0.84 mg/g of biomass) with *M. anisopliae* CDA treatment. When biomass was treated with 1N NaOH followed by *M. anisopliae* crude CDA, chitosan content was increased to 70.70±0.72 mg/g of biomass *i.e.* 1.39-fold as compared to the chemical treatment. However, the treatment with *B. poitrasii* mycelial intracellular crude CDA yielded 78.05±0.58 mg chitosan/g of biomass *i.e.* 1.53-fold more as compared to the NaOH treatment (Table 3.3).

Table 3.3 Chemical and enzymatic deacetylation of *B. poitrasii* mycelia biomass

Sr. No.	Treatment	Chitosan (mg/g dry wt.)	Fold increase
1.	1N NaOH (Control)	51.00±0.52	-
2.	Extracellular CDA of *M. anisopliae*	59.00±0.84	1.16
3.	1N NaOH followed by *M. anisopliae* CDA	70.70±0.72	1.39
4.	Intracellular CDA of *B. poitrasii*	78.05±0.58	1.53

B. poitrasii mycelial biomass (1 g, dry weight) was treated as described under material and methods (Section 2.11).

3.2.7a Antifungal activity of fungal chitosan against human pathogenic *Candida* strains.

B. poitrasii chitosan showed better antifungal activity against *C. albicans* (MIC_{90} 0.025 mg/mL) and *C. glabrata* (MIC_{90} 0.2 mg/mL) than against *C. neoformans, C. tropicalis* and *A. niger* though not comparable with fluconazole. Commercial chitosan from marine source showed MIC_{90} > 1.6 mg/mL against all the fungi tested (Table 3.4).

Table 3.4 Antifungal activity of *B. poitrasii* chitosan against different human pathogens

	Minimum inhibitory concentration (MIC_{90}) mg/mL				
	C. albicans ATCC 10231	*C. neoformans* ATCC 34664	*C. glabrata* NCYC 388	*C. tropicalis* ATCC 750	*A. niger* ATCC 10578
B. poitrasii chitosan	0.025	0.8	0.2	>0.8	0.8
Commercial Chitosan	>1.6	>1.6	>1.6	>1.6	>1.6
Fluconazole	0.008	0.032	0.128	>0.128	>0.128

*Antifungal assay was carried out in 96 well plates as described under materials and methods (Section 2.17).

3.3a Discussion

The chitosan with high degree of deacetylation and low molecular weight can be isolated from cell walls of zygomycetous fungi (Vaingankar & Juvekar, 2014; Wu *et al*., 2005). One of the main challenges was the production of biomass sufficient enough to make the process cost-effective.

The simple yeast extract-peptone and glucose medium was used for the biomass production of *A. coerulea* (Muzzarelli *et al*., 1994). The yield reported was 6.2 g/L (dry wt.) while Nwe *et al*., 2002 obtained 5.63 g/L dry wt. of biomass of *G. butleri* with 10% sweet potato extract medium containing minerals and urea as a nitrogen source. In case of *B. poitrasii* in optimized medium, the yield was 10.00±0.50 g/L (dry wt.), which was more than other zygomycetous sources reported so far (Table 3.1).

Among different zygomycetes screened, Tan *et al*., 1996 reported that *Cunnighamella echinulata* showed high chitosan contents (7.14%) of dry wt. of biomass as compared to other zygomycetes such as *Rhizopus* species, *Mucor hiemalis, A. glauca, G. butleri* and others (2- 6%). While Nwe *et al*., 2002 obtained 11% chitosan from *G. butleri* biomass. In case of *B. poitrasii* the extractable chitosan was 5.1-6.5% (Table 3.1). With *B. poitrasii* CDA treatment it was increased to 7.8% (Table 3.3). On equal activity basis, the *M. anisopliae* CDA treatment increased to only 5.9% (Table 3.3). Nahar *et al*., 2004 reported that the *M. anisopliae* produces different isozymes of CDA extracellularly. They might have different roles during growth, penetration through insect cuticle (conversion of chitin to chitosan to soften cuticle for penetration) and in self-defence from insect chitinase by modifying cell wall chitin to chitosan. The isozyme which is involved in self defense, *i.e.* modification of cell wall from chitin to chitosan, might be important to increase chitosan contents after CDA treatment.

Different extraction procedures were designed to obtain more chitosan in different organisms. In the present investigations the treatment of *B. poitrasii* biomass with 1N NaOH at 121 °C for 15 min and further with 2% acetic acid (0.3 M) at 95 °C for 24 h was found to be effective. The chitosan yield was increased in 2 stage processes, *i.e.* NaOH treatment followed by *M. anisopliae* CDA treatment by 1.39-fold (Table 3.3).

Rane & Hoover, 1993 reported higher chitosan contents with high deacetylation in *A. coerulea* when grown in a medium containing high glucose and protein contents supplemented with minerals as compared to minimal medium, in absence of minerals. Rice and corn as carbon sources supported growth of *R. oryzae* with 601 mg/L chitosan yield (Hang, 1990). While Amorim *et al*., 2006 isolated 580 mg/L chitosan from *C. bertholletiae* using sugar cane juice. Yokoi *et al*., 1998 observed that sweet potato medium was better for *G. butleri* than buckwheat

(*Fagopyrum esculentum*) medium. The chitosan yield was 730 mg/L. While Maw *et al*., 2002 obtained 470 mg/L chitosan with another strain of *G. butleri*. From Table 3.1 and 3.3 it can be suggested that in case of *B. poitrasii* maximum possible yield of chitosan could be 780 mg/L.

Most of zygomycetous fungi are reported to have chitosan with 80-90% DDA. The % DDA of *B. poitrasii* chitosan extracted from mycelial cells grown in different media was in the range of 94-95% (Table 3.2).

Generally low molecular weight chitosans were observed to have high antifungal activity (Alburquenque *et al*., 2010). Kulikov *et al*., 2014 demonstrated that chito-oligomers with molecular weights ranging from 2.09-19.99 kDa had high antifungal activity against several *Candida* strains. *B. poitrasii* chitosan exhibited low molecular weight (42.82 kDa). It showed MIC_{90} in the range 0.025- 0.8 mg/mL against human pathogenic yeasts such as *C. albicans, C. neoformans, C. glabrata* and filamentous *A. niger* (Table 3.4).

Kim *et al*., 2008 reported that *Mortierella* species produced highly active chitin deacetylase which could deacetylate chito-oligomers too. The metabolic engineering in the fermenter for the production of mycelia with more % of chitosan in the cell wall and also an enhanced intracellular CDA activity in *B. poitrasii* could be the next step for the production of highly deacetylated chitosan.

3.4a Summary

In summary present study has focused on the production of mycelial biomass for the extraction of chitosan from the zygomycetous fungus *B. poitrasii*. The media parameters were designed according to the one variable time at time approach. The concentration of yeast extract, mycological peptone and starch plays important role to increase the mycelial biomass. The method for extraction of chitosan was also established. The biophysical properties of fungal

chitosan such as degree of deacetylation and molecular weight were better as compared to the chitosan from marine source. The higher %DDA and low molecular weight of fungal chitosan makes it a good antifungal agent against human pathogenic *Candida* strains.

Chapter 3b

Taguchi design of the experimental approach to increase the biomass and cell wall chitosan contents of zygomycetous dimorphic fungus *Benjaminiella poitrasii*

Contents of chapter-3b have been published in a research article……

(Mane *et al*., 2022, Journal of polymer materials, Article in press)

3.1b Introduction

Chitosan is a natural polymer made up of glucosamine units by deacetylation of chitin. The main commercial source of chitin/chitosan is landings of marine organisms such as, crabs, lobsters, and shrimps. However, the supply of marine waste is seasonal and limited, resulting in variability in the source material. The fungi containing chitin/chitosan in their cell walls can be an alternative commercially viable source. Ghormade *et al*., 2017 extensively reviewed the possible fungal sources for chitin and chitosan production. The fermentation of zygomycetous fungi *viz*., *A. coerulea, B. poitrasii, C. elegans, G. butleri, M. rouxii* and *R. oryzae*, was preferred for chitosan production due to a higher percentage of chitosan in their cell wall (Ghormade *et al*., 2017). The attempts were also made to optimize the fermentation conditions to increase biomass production, ultimately increasing the chitosan yield. Previously, we have optimized the mycelial biomass production in *B. poitrasii* by using a one variable at time (OVAT) approach (Mane *et al*., 2017a).

Xu *et al*., 2003 also used a OVAT and orthogonal matrix method to optimize submerged conditions for *Paecilomyces tenuipes* C240 strain. However, the optimization of fermentation parameters by OVAT approach becomes tedious and time-consuming. Alternatively, statistical methods can be used to study the interaction of variables in generating the process response. Taguchi DOE developed by Genichi Taguchi is one such tool. It involves studying the system by a set of independent variables (factors) over a specific region of interest (levels). Taguchi's parameter design concept is related to finding the appropriate design factor levels to make the system insensitive to variations in noise (uncontrollable factors).

Kim *et al*., 2005 used this statistical approach to optimize submerged culture conditions to produce mycelial biomass and exopolysaccharides (EPS) by *A. cylindracea*. Under the optimal

culture condition, the maximum EPS concentration achieved bout three times higher than the basal medium.

In the present chapter, fermentation conditions were optimized to increase the biomass and extractable cell wall chitosan contents of *B. poitrasii* using the Taguchi DOE approach. The effect of interactions between different fermentation parameters on growth and chitosan contents of *B. poitrasii* were also studied. Further, biophysical properties of chitosan isolated from *B. poitrasii* yeast and mycelia grown under optimized fermentation conditions were studied. Furthermore, the antifungal potential of *B. poitrasii* yeast and hyphal chitosan was also evaluated against plant pathogenic fungi.

3.2b Results

3.2.1b Multiple variable approach for optimization of biomass production by *B. poitrasii*

The experimental data were processed using excel sheets prepared by Dr. P. R. Apte, TIFR, Mumbai, India (http://www.tifr.res.in/~apte) for DOE using the Taguchi approach. The individual influence of the factors at the assigned levels, severity indices for different interactions between factors and ANOVA were calculated. The biomass (dry weight, unless otherwise mentioned) after 48 h of incubation was in the range of 2-6.6 g/L and 0.1-2.83 g/L for mycelium and yeast, respectively (Table 3.5).

Table 3.5 Effect of interactions between different fermentation parameters on growth and chitosan contents of *B. poitrasii* studied using L18 orthogonal array

Expt. No.	Final pH	Mycelia		Yeast cells	
		Biomass (g/L)	Chitosan* (mg/g)	Biomass (g/L)	Chitosan* (mg/g)
1	7.07	2.16 ± 0.33	29.33 ± 0.48	0.63±0.30	8.88±0.32
2	8.25	4.93 ± 0.43	30.28 ± 0.40	0.9±0.10	10.48±0.36
3	8.31	4.76 ± 0.15	31.46 ± 0.45	0.26±0.05	9.15±0.29
4	7.37	5.94 ± 0.48	33.98 ± 0.37	1.7±0.10	12.79±0.21
5	7.90	3.80 ± 0.55	30.71 ± 0.51	1.46±0.15	11.43±0.34
6	7.90	5.32 ± 0.44	33.71 ± 0.53	0.97±0.15	8.28±0.21
7	7.46	4.81 ± 0.21	30.44 ± 0.39	2.53±0.87	14.48±0.36
8	8.20	6.62 ± 0.44	38.49 ± 0.35	0.96±0.15	8.42±0.10
9	8.21	4.11 ± 0.21	28.95 ± 0.44	1.3±0.20	12.44±0.25
10	7.77	4.74 ± 0.39	30.16 ± 0.38	1.56±0.25	13.87±0.83
11	7.03	2.58 ± 0.50	28.88 ± 0.31	2.83±0.12	15.33±0.26
12	7.56	2.50 ± 0.52	31.18 ± 0.38	0.14±0.04	6.41±0.19
13	7.39	5.57 ± 0.15	35.30 ± 0.42	0.5±0.17	7.56±0.37
14	6.81	3.44 ± 0.37	32.65 ± 0.47	0.7±0.26	9.36±0.12
15	7.47	3.86 ± 0.27	33.21 ± 0.25	0.16±0.05	4.49±0.32
16	6.67	3.03 ± 0.94	35.85 ± 0.32	0.26±0.25	6.58±0.23
17	7.90	4.23 ± 0.15	30.34 ± 0.27	0.11±0.01	5.52±0.13
18	7.09	5.08 ± 0.27	29.83 ± 0.28	0.12±0.005	5.79±0.18

*The mycelia and yeast cells were homogenized in liquid nitrogen and chitosan was isolated as mentioned under Materials and Methods (Section 2.11).

The maximum biomass of *B. poitrasii* hyphae (6.50-7.00 g/L) was obtained in experiment no. 8 (Table 3.5). The factors *viz*., incubation temperature of 25 °C, starch (1.5 %), yeast extract (0.9%), peptone (1%), $MgSO_4$ and KH_2PO_4 at 1:2 proportion in the growth medium of pH 8, and incubation time of 48 h, were found to be optimum for production of maximum mycelial biomass (6.62±0.44 g/L) and chitosan (38.49±0.35 mg/g of dry biomass) (Table 3.5). The optimum conditions for yeast biomass production were found to be as of experiment no. 7 (Table 3.5).

3.2.2b Effect of different types of inoculums on the growth of *B. poitrasii* biomass

The yeast inoculum produced higher proportions of yeast cells (3.15±0.05 g/L) and fewer mycelia ((1.37±0.21 g/L). The least biomass was obtained with mycelial inoculum (2.60±0.22 g/L of M and 1.88±0.10 g/L of Y) (Table 3.6).

Table 3.6 Effect of different types of inoculums on the growth and chitosan contents of *B. poitrasii*

Inoculum Type	**Biomass (g/L)**		**Chitosan dry wt. (mg/g of dry biomass)**	
	M	Y	M	Y
Spores	4.27±0.16	1.80±0.18	57.20±1.1	26.23±2.7
Mycelia	2.60±0.22	1.88±0.10	38.10±0.5	27.41±2.4
Yeast	1.37±0.21	3.15±0.05	36.30±0.8	25.63±1.6
Mix (M+Y)	4.71±0.19	2.70±0.10	58.72±1.0	23.64±1.2

M, mycelia; Y,yeast cells.

The inoculum (Y+M) produced the highest biomass (4.71±0.19 g/L of M and 2.70±0.10 g/L of Y) followed by spore inoculum (4.27±0.16 g/L of M and 1.80±0.18 g/L of Y) in the optimized media. Therefore, the mixed inoculum containing both the yeast and mycelium cells was used in further experiments (Figure 3.6).

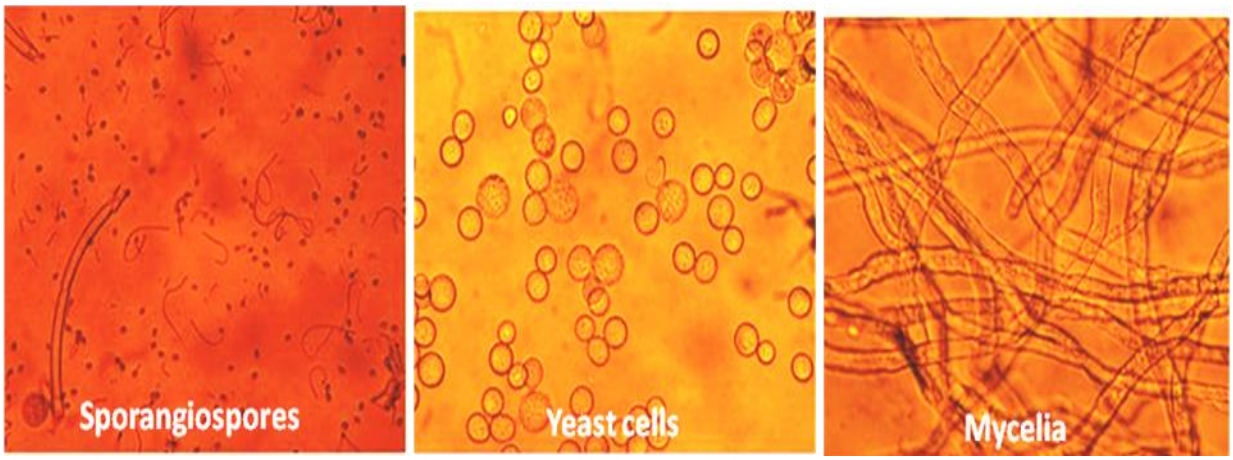


Figure 3.6 Different morphological forms of *B. poitrasii* used for optimization of inoculum. (Adapted from Pathan *et al*., 2017)

3.2.3b Validation of optimized conditions to produce *B. poitrasii* biomass and cell wall chitosan

The software automatically calculated optimized process conditions on pooled ANOVA: (g/L): yeast extract 6; peptone 15; KH_2PO_4, 4; $MgSO_4$ 2; starch, 10; and 1 mL trace metal solution containing [(mg/mL): $FeSO_4.7H_2O$, 5; $MnSO_4$, 1.5; $ZnSO_4$, 3.34; $CoCl_2$, 2.0], pH 6.5, inoculum 10%, incubation temperature, 28 °C, incubation time, 48 h and agitation 180 rpm. The validation experiment was carried out under the above-optimized process conditions (Table 3.7).

Table 3.7 Yield of *B. poitrasii* chitosan from One Variable at Time and Validation experiment using Taguchi Design of experimental approach

Method	Biomass Dry wt. (g/L)		Chitosan (mg/g dry wt.)		% DDA (FTIR)		Molecular weight (kDa)	
	M	Y	M	Y	M	Y	M	Y
OVAT method	10.00±0.50	ND	51.00±0.52	ND	92.89	ND	42.82	ND
Taguchi Method	9.17±0.32	1.78±0.28	60.89±2.30	28.29±2.7	88.51	87.30	41.68	19.49

M, mycelia; Y,yeast cells; ND, not determined. From 1 L optimized medium, 10.95±0.3 g biomass and 608±2.5 mg of chitosan was obtained using Taguchi DOE approach.

It produced seven times more biomass (9.17±0.32 g/L of mycelial biomass and 1.78±0.28 g/L yeast biomass) than the biomass obtained in basal medium (1.28±0.10 g/L) (Mane *et al*., 2017a). The Taguchi optimized produced substantial amount of both mycelial and yeast biomass which increased the final yield of chitosan as compared to the OVAT approach (Table 3.7).

3.2.4b Degree of deacetylation of chitosan isolated from *B. poitrasii* (M and Y)

The degree of deacetylation (DDA) is one of the most important factors responsible for chitosan's physical and chemical properties. The solubility of chitosan is mainly dependent on the DDA. The fungal chitosan showed characteristic peaks for O-H stretching at 3450 cm^{-1}, for amide II at 1650 cm^{-1}, at 1420 cm^{-1} for C-H deformations, for amide III, C-N stretching at 1320 cm^{-1} and at 1035 cm^{-1} for O-bridge stretching in FTIR spectroscopy. These peaks are comparable with the FTIR spectra obtained for commercial chitosan from marine source (Figure 3.7).

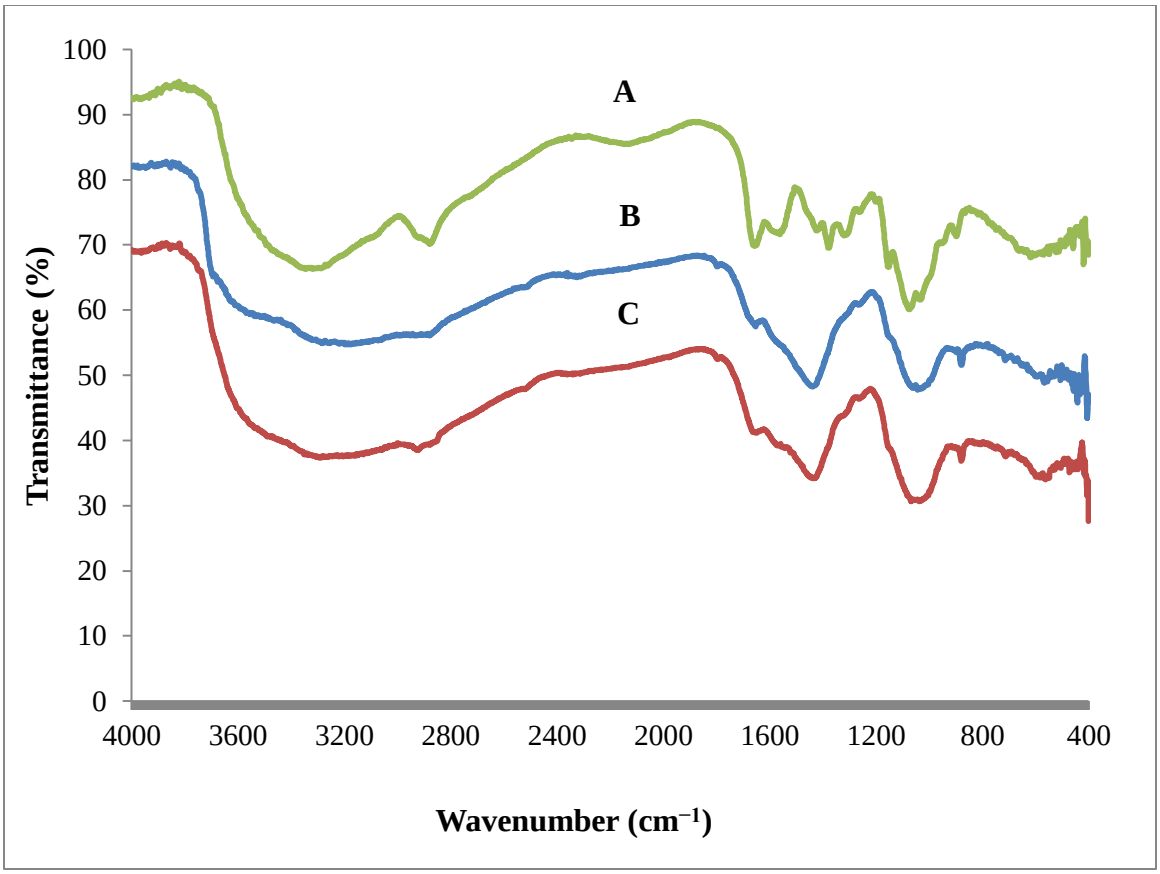


Figure 3.7 FT-IR profiles of *B. poitrasii* mycelium (B) and yeast (C) chitosans compared with chitosan obtained from marine source (A).

In validation experiment, % DDA of chitosan isolated from mycelia and yeast cells were 88.51% and 87.30%, respectively. The chitosan from marine source showed 79.26 % of DDA, lower as compared to the fungal chitosan.

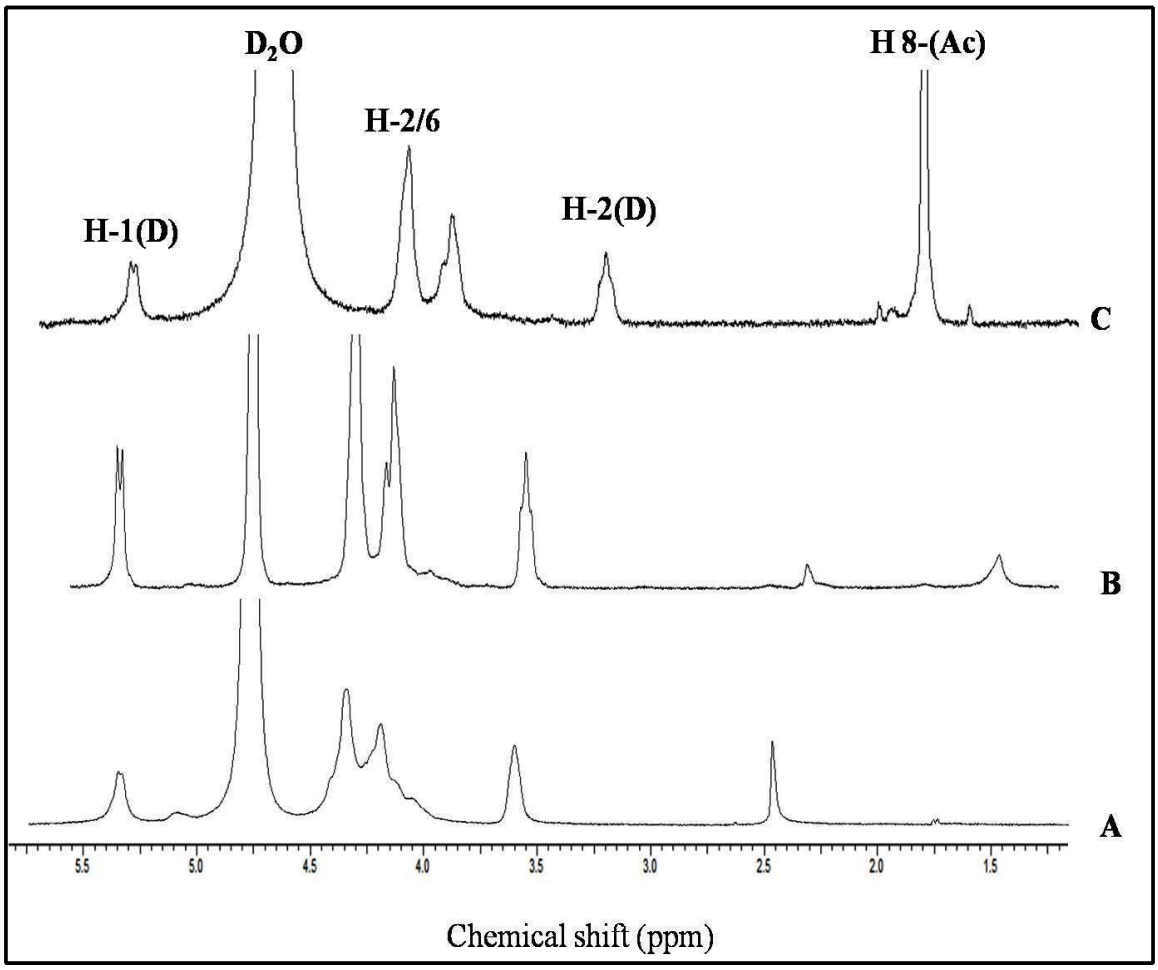


Figure 3.8 ^{1}H-NMR profiles of chitosan from marine source (A), *B. poitrasii* mycelium (B) and *B. poitrasii* yeast (C).

Further, chitosan samples from validation experiments were also analyzed by ^{1}H-NMR spectroscopy. The characteristic peak of H1 proton (a deacetylated monomer, H1D) and peak of three protons of acetyl group (H-Ac) for chitosan were observed. These peaks observed in fungal chitosan samples were comparable with the commercial chitosan from marine source (Figure 3.8). The % DDA as calculated from ^{1}H-NMR of marine chitosan was 86.51 whereas for *B. poitrasii* hyphal and yeast chitosan were 90.72 and 88.17, respectively.

3.2.5b Molecular weight of chitosan isolated from *B. poitrasii* mycelia and yeast cells by gel permeation chromatography

The molecular weight of chitosan samples from validation experiment was determined by gel permeation chromatography. The molecular weight of mycelial and yeast chitosan samples by GPC were 41.28 kDa and 21.72 kDa, respectively (Figure 3.9). These values were found to be comparable with the values obtained with viscometry method. The molecular weight of commercial chitosan from marine sources as determined by GPC was 251 kDa (Figure 3.9).

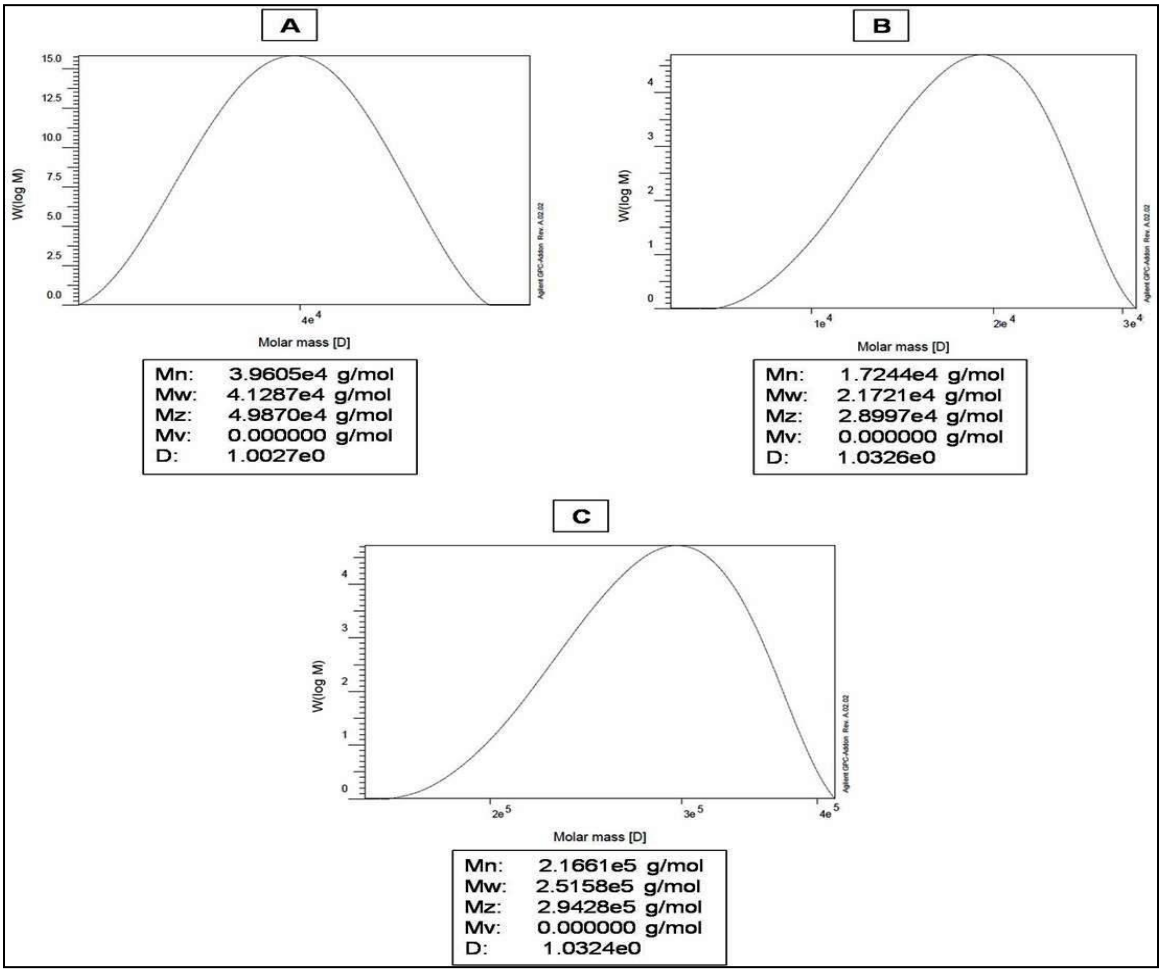


Figure 3.9 Gel permeation chromatography to determine the molecular weight of chitosan samples isolated from (A) *B. poitrasii* mycelia, (B) *B. poitrasii* yeast and (C) Marine sources.

3.2.6b Antifungal activity of *B. poitrasii* chitosan against plant pathogens

3.2.6.1b Disk diffusion assay to check inhibitory effect of fungal chitosan against plant pathogens

The *B. poitrasii* mycelial chitosan was tested at higher concentrations to check the inhibitory effect against two plant pathogenic fungi *viz*., *F. oxysporum* and *U. maydis*. The fungal chitosan showed mycelial growth inhibition at the lowest concentration that is 1 mg/mL as compared to the control plate by disk diffusion assay. Both mycelial and yeast chitosan showed the mycelial extension inhibition against the tested organisms (Figure 3.10).

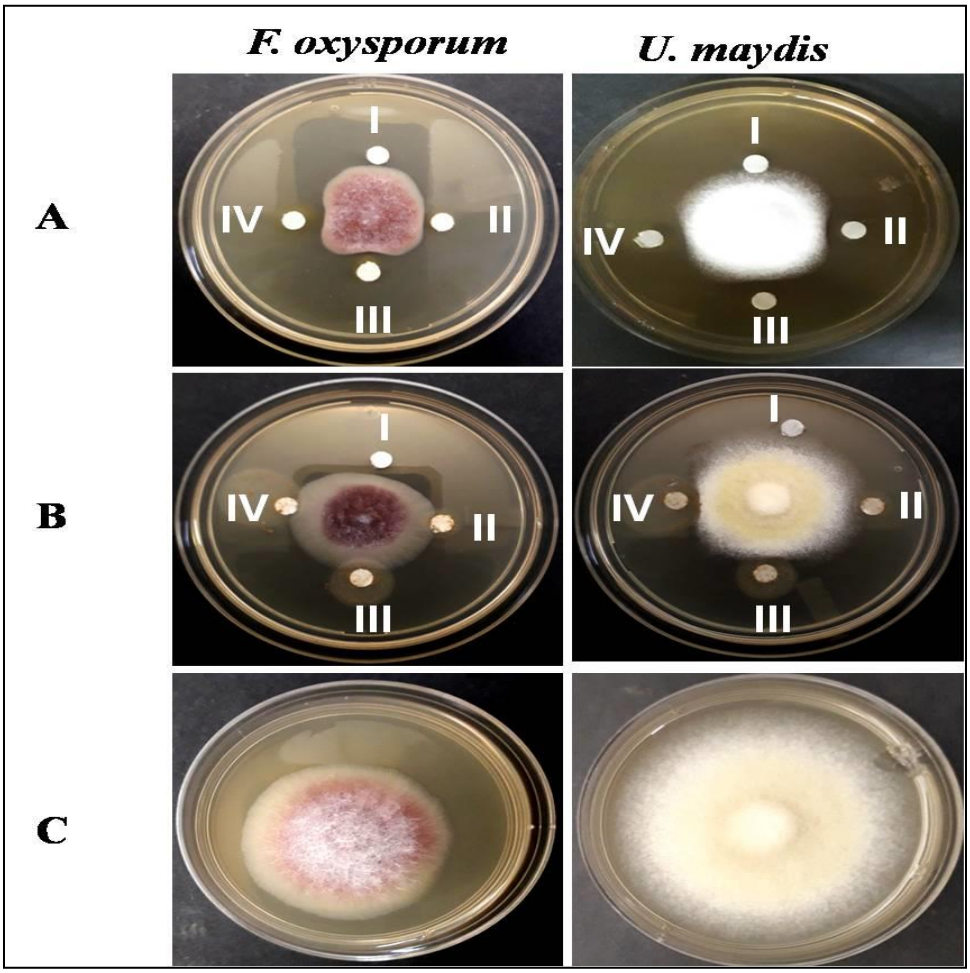


Figure 3.10 Mycelial extension inhibition assay for *B. poitrasii* chitosan with different concentrations against plant pathogenic *F. oxysporum* and *U. maydis*.

A. *B. poitrasii* mycelial chitosan; B, *B. poitrasii* yeast chitosan and C, Control plates. I Control (2% acetic acid); II 1 mg/mL; III 2 mg/mL and IV 3 mg/mL.

3.2.6.2b Antifungal activity of fungal chitosan by 96 well microdilution assay

The chitosan extracted from *B. poitrasii* yeast and mycelia were tested against plant pathogenic fungi *viz*., *F. oxysporum* and *U. maydis* to find out the MIC_{90} by using 96 well microdilution assay. The mycelial chitosan inhibited the growth of *F. oxysporum* (MIC_{90} 0.1 mg/mL) and *U. maydis* (MIC_{90} 0.4 mg/mL) more effectively than the yeast chitosan (MIC_{90} 0.4 and 0.8 mg/mL, respectively). The marine chitosan showed least effectiveness under studied conditions (MIC_{90} 0.8 and >1.6 mg/mL) (Table 3.8).

Table 3.8 Antifungal activity of chitosan polymers against plant pathogenic fungi

Chitosan source	**MIC_{90} (mg/mL)**	
	F. oxysporum	*U. maydis*
B. poitrasii (M)	0.1	0.4
B. poitrasii (Y)	0.4	0.8
Marine chitosan	0.8	>1.6
Control	ND	ND

ND, not detected; 2% acetic acid (used to dissolve chitosan polymer) was served as control; The antifungal assay was carried out using CLSI micro-broth dilution method; All the assays were carried out in triplicates and average values are presented.

3.3b Discussion

Media optimization is a crucial step in increasing the production of fungal biomass that ultimately increases the yield of extractable chitosan. Conventional optimization procedures involve altering one parameter at a time, keeping the rest of the parameters constant, enables understanding the impact of only those parameters on the process. Further, optimization by the conventional approach becomes erroneous. On the contrary, statistical optimization methods consider the interaction of variables in generating the process response. Chenthamarakshan *et al*., 2017 described the optimization of extracellular production of laccase from *M. palmivorus* LA1 by Taguchi method using L8 orthogonal array. The optimization improved the laccase yield by 17.6-fold. The process parameters for the growth of *Aspergillus sp.* to remove the copper and nickel were also optimized using the Taguchi method (Mario *et al*., 2008). In the present study, optimized conditions gave 7-fold higher biomass than the biomass obtained in the basal medium.

Previously, it was reported that peptone's source might affect the % germ tube formation in *B. poitrasii* (Doiphode, 2007). A similar effect was observed during the two optimization methods. Earlier, the concentration of peptone was less (10 g/L), resulting in more mycelial biomass (10 g/L). However, in the case of Taguchi media optimization, the higher peptone concentration (15 g/L) gave at-par mycelial biomass (9.17 g/L) with a substantial amount of yeast cells (1.78 g/L). So, ultimately the total yield of biomass was increased (10.95 g/L). The DDA is a crucial biophysical characteristic in deciding the quality and solubility of chitosan polymer. The chitosan extracted from yeast and mycelia of *B. poitrasii* showed ~90% DDA.

The chitosan isolated from another zygomycetous fungus *M. rouxii*, grown in three different media, showed variation (82.2-89.8%) in % DDA (Chatterjee *et al*., 2005). The chitosan's molecular weight varies depending on the source of isolation. Chitosans from crabs and shrimps

have high molecular weights as compared to fungal. The molecular weight of chitosan isolated from *C. elegans* was 27.2 kDa chitosans (Stamford *et al*., 2007). Munoz *et al*., 2015 reported the chitosan extraction from *A. niger* (molecular weight 190 kDa) to synthesize hydrogels for biomedical applications. The chitosan also has antiviral, antibacterial, and antifungal properties that can be used for different applications. Previously, Mane *et al*., 2017a showed hat *B. poitrasii* mycelial chitosan (molecular weight 42.82 kDa) inhibited the growth of *A. niger, C. albicans, C. glabrata,* and *C. neoformans* (MIC_{90} 0.025-0.8 mg/mL).

In the present study, *B. poitrasii* mycelial and yeast chitosan (molecular weights 41.68 and 19.49 kDa, respectively) were compared for their antifungal potential against dreadful plant pathogenic fungi *F. oxysporum* and *U. maydis*. The high molecular weight chitosan from the marine source was the least effective among tested chitosans against plant pathogens. It suggested that the molecular weight of chitosan along with % DDA is important factors in defining its antifungal potential.

3.4b Summary

The Taguchi DOE approach used in the present study for media optimization considers the interaction between multiple factors affecting the growth and cell wall chitosan content of zygomycetous fungus *B. poitrasii*. The biomass (yeast and mycelia) obtained under optimized growth conditions gave maximum extractable chitosan, which has low molecular weight and high %DDA, the properties that define its antifungal potential against plant pathogenic organisms.

Chapter 4

Exploring the potential of agriculturally important ascomycetous fungi *Metarhizium anisopliae* and *Myrothecium verrucaria* for chitosan production: A value addition to low cost-high volume products

Contents of chapter-4 have been published in a research article……

(Mane *et al*., 2017, Asian Chitin Journal, 13, 31-38)

4.1 Introduction

Chitin is a β-1, 4 -linked polymer composed of *N*-acetyl-D-glucosamine (GlcNAc) repeated units, commonly found in the exoskeleton or cuticles of many invertebrates and in the cell wall of the fungi (Deshpande, 1986). Chitosan, a deacetylated form of chitin, is primarily β- 1, 4- 2-amino-2-deoxy-D-glucose (or D-glucosamine) polymer. Chitosan has wide range of application in many areas, from agriculture to healthcare (Muzzarelli, 2000). The fungi, a second largest group on earth contains chitin and chitosan in varying concentrations, *viz.*, 2% in *S. cerevisiae* to about 60% in the walls of *Allomyces macrogynus* and *Sclerotium rolfsii* (Herrera, 1978). As compared to marine sources, for the production of chitosan, fungi are better in terms of constant supply, without contamination of heavy metals, and have extractable low molecular weight highly deacetylated chitosan (Ghormade *et al*., 2017). The fungal sources useful for chitosan production can be divided in to three groups, *viz*., (i) waste fungal biomass from Myco-tech industries (ii) fermentation of zygomycetous genera for biomass production and (iii) isolation of chitosan as value addition to existing mycotech-products (Cai *et al*., 2006; Mane *et al*., 2017b; Muzzarelli *et al*., 1994; Satari *et al*., 2015).

In view of harmful effects of chemical pesticides and fungicides, number of fungal organisms such as *Beauveria, Metarhizium, Verticillium* and *Trichoderma* are used for the control of insect pests and pathogens (Deshpande, 1999). In view of the parameters such as storage stability, shelf life in the and off the field, time required to control insect pests and pathogens, chemical pesticides have edge over mycopesticides. These factors add to the cost for mycoinsectcide use in the field. Earlier, to reduce the cost of production of mycopesticides and for value addition different potential applications such as plant growth promoting activity,

bioremediation of pesticide residues, use of enzymes for biotransformation were reported (Cocaign *et al*., 2013; Garcia *et al*., 2011; Inbar *et al*., 1994).

In this chapter for the first time the use of biomass after removal of conidia and extracellular enzymes of from agriculturally important *Metarhizium anisopliae* and *Myrothecium verrucaria* for the isolation of low molecular weight chitosan is reported. The morphological characteristics of these agriculturally important fungi is illustrated as below in Figure 4.1.

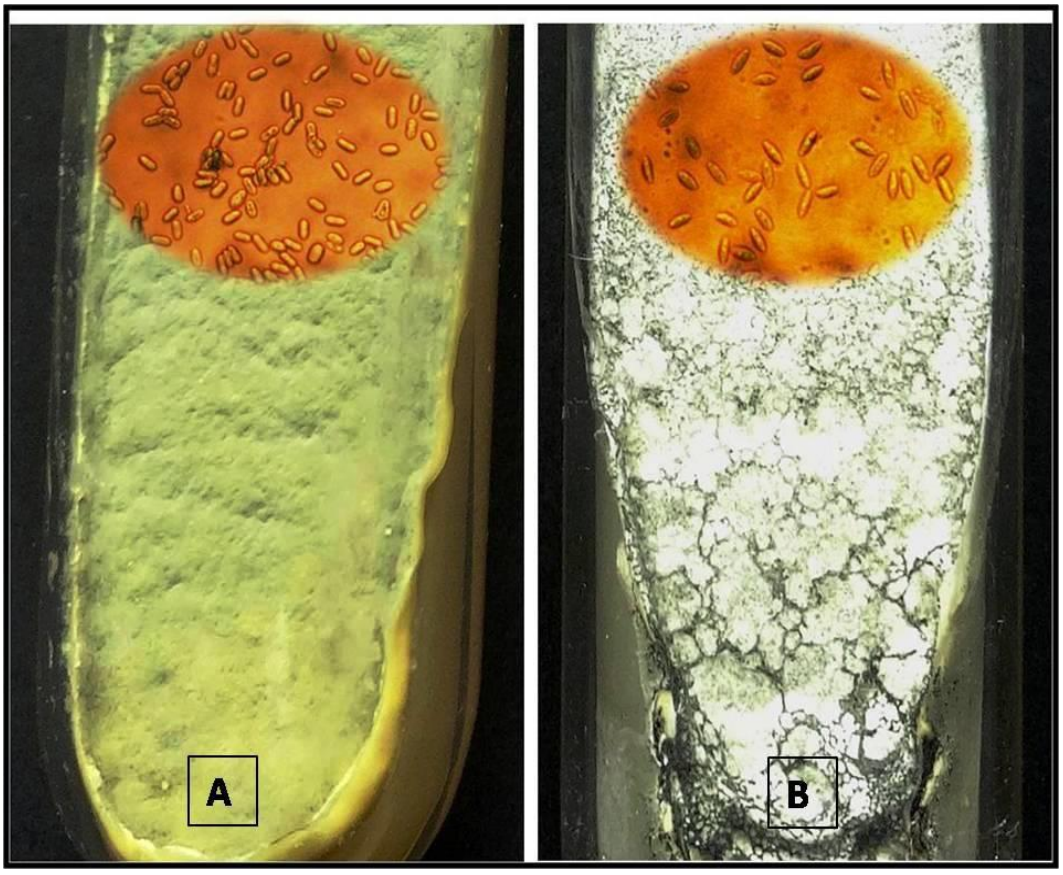


Figure 4.1 Morphological characteristics of agriculturally important fungi. A. *M. anisopliae*; B. *M. verrucaria.* Spore images were captured at 400X magnification.

3.2 Results

3.2.1 Isolation of fungal biomass

The mycelial biomass of three different fungal strains was used for chitosan isolation. (i) After solid state fermentation (SSF) of *M. anisopliae* MTCC5190 for 14 days, and separation of conidia (60 g/kg rice) using liquid extraction method, mycelial biomass (27.11±3.0 g/kg rice) was separated from the rice particles after repeated washings with distilled water. (ii) The mycelial biomass of *M. anisopliae* MCC1197, CDA producer (29.07±2.42 g/L) from YPG medium was separated after 72 h and washed with distilled water. (iii) *M. verrucaria* MTCC5191 mycelial biomass (28.85±2.5 g/L) was separated from extracellular enzyme mixture and unutilized chitin after 7 d.

Table 4.1 Amount of chitosan, degree of deacetylation and molecular weight of chitosan isolated from *M. anisopliae* and *M. verrucaria.*

Organism	Fermentation	Chitosan (mg/10g of dry biomass)	DDA (%) (FT-IR)	DDA (%) (^{1}H-NMR)	Molecular weight (kDa)
M. anisopliae MTCC5190	SSF	124.4±2.98	84.68	87.80	0.77
M. anisopliae MCC1197	SmF	180.8±4.79	84.70	82.05	1.36
M. verrucaria MTCC 5191	SmF	193.5±2.98	81.68	86.34	2.38
Marine chitosan (Commercial)	-	-	81.16	85.38	464.83

4.2.2 Chitosan extraction from fungal biomass

The yield of chitosan from *M. anisopliae* MTCC5190 mycelial biomass was 124.4±2.98 mg/10 g dry wt. (Table 4.1). In other words, per kg of substrate While CDA producer, *M. anisopliae* MCC1197 yielded 180.8±4.79 mg/10 g dry biomass in 72 h. From *M. verrucaria* MTCC 5191 the yield of chitosan was 193.5±2.98 mg/10 g of dry biomass obtained after 7 d (Table 4.1).

4.2.3 Degree of deacetylation of fungal chitosan

Pochanavanich & Suntornsuk measured degree of deacetylation by titration with NaOH which hydrolyzed the acetyl groups in chitosan which were measured. The %DDA for isolated fungal chitosans was in the range 83-90%. Interestingly, for crab shell chitosan %DDA was reported to be 97% which was high. While it was reported that %DDA of crab shell chitosan was in the range of 80-86 when measured using FTIR and ^{1}H NMR (Mane *et al.*, 2017a). Using FTIR, the DDA of the chitosans from *Metarhizium* and *Myrothecium* strains were found to be in the range 81-85% (Table 4.1; Figure 4.2). The chitosan isolated from *M. anisopliae* MTCC5190 grown in SSF was 84.68%. In case of hyphal form of *M. verrucaria* MTCC5191 the DDA was 81.68% (Table 4.1). With ^{1}H NMR the DDA of the chitosans from *Metarhizium* and *Myrothecium* strains were in the range 82-88% (Table 4.1; Figure 4.3).

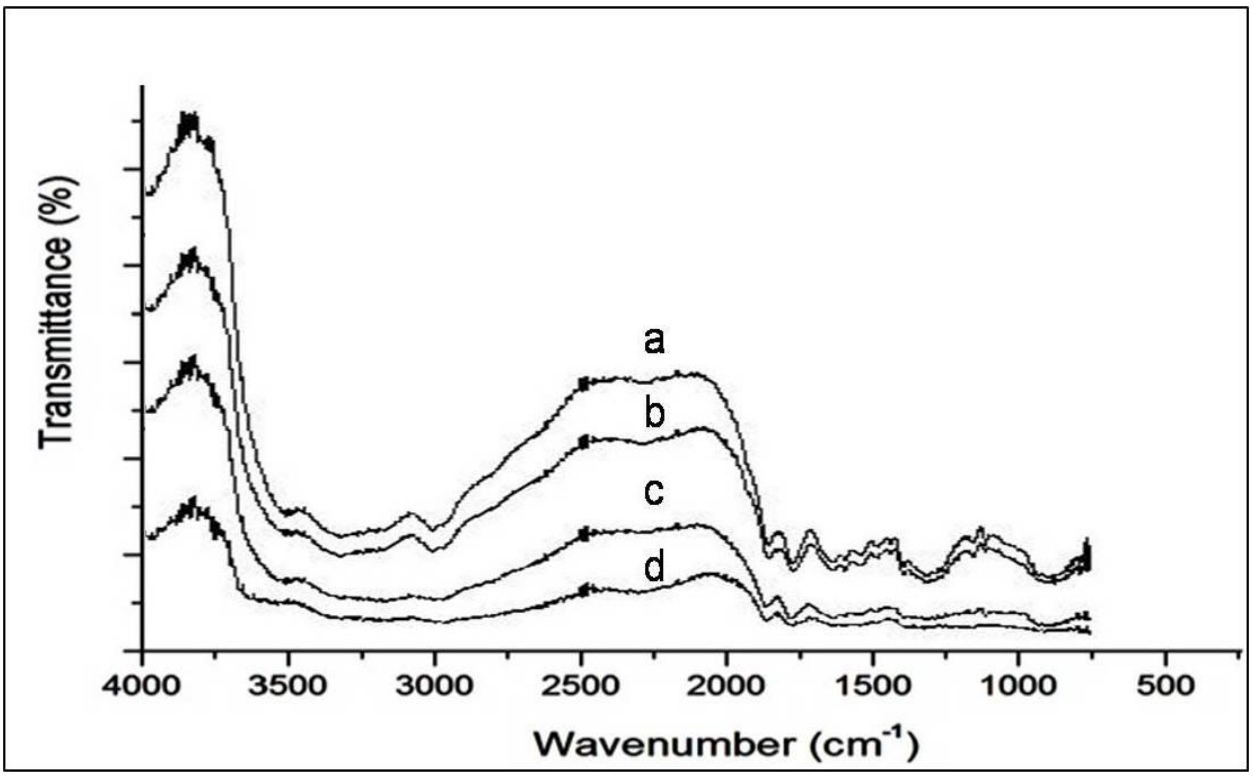


Figure 4.2 FTIR profiles of chitosan from *M. anisopliae* and *M. verrucaria.* a, chitosan isolated from *M. anisopliae* MTCC5190; b, chitosan isolated from *M. verrucaria* MTCC5191; c, chitosan isolated from *M. anisopliae* MCC1197 d, commercial chitosan from marine source.

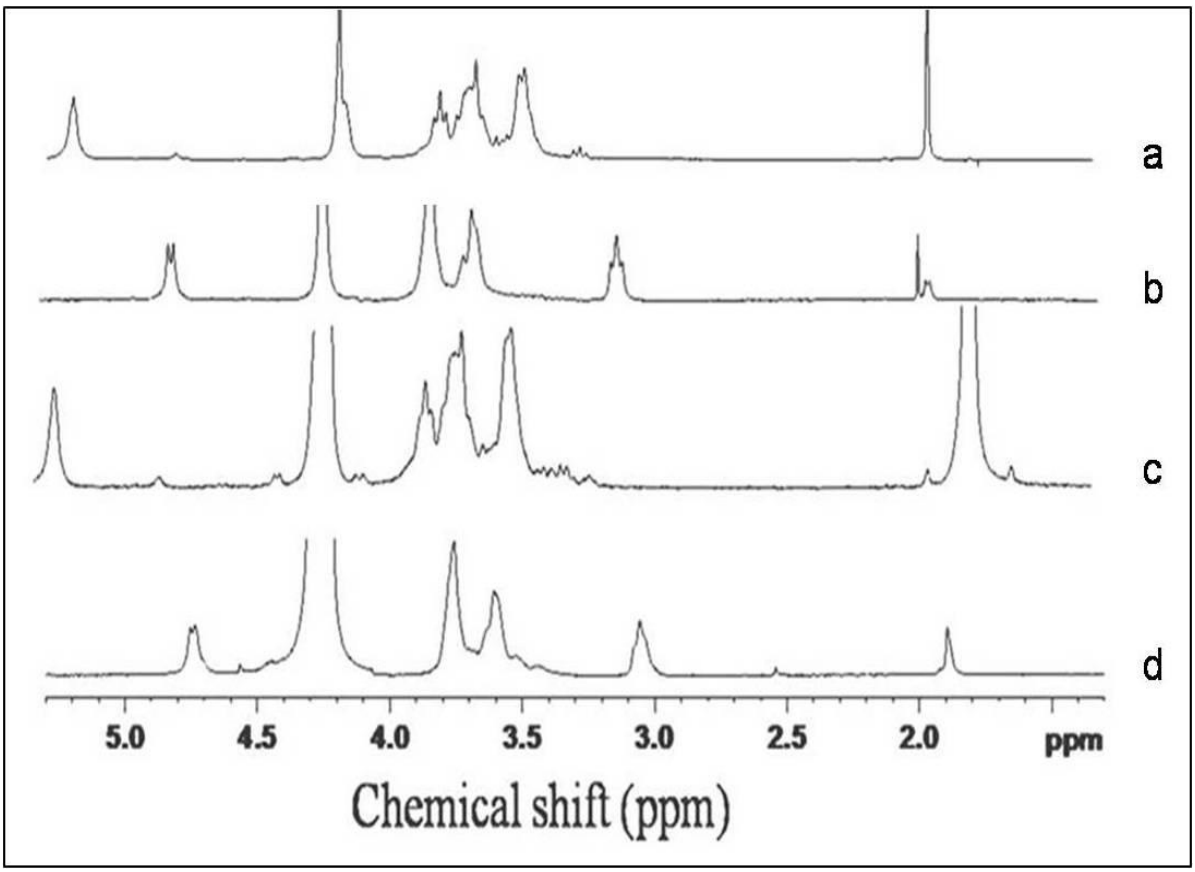


Figure 4.3 ^{1}H-NMR profiles of chitosan from agriculturally important fungi. a, chitosan isolated from *M. anisopliae* MTCC5190; b, chitosan isolated from *M. verrucaria* MTCC5191; c, chitosan isolated from *M. anisopliae* MCC1197 d, commercial chitosan from marine source.

4.2.4 Molecular weight determination of fungal chitosan

In the present investigations, chitosans isolated had low molecular weights (0.77- 2.38 kDa) (Table 4.1).

4.2.5 Antimicrobial activity of fungal chitosan

Usually low molecular weight chitosans have applications in human health care (Mane *et al*., 2017a). Kulikov *et al*., 2014 reported that chito-oligomers with molecular weights ranging from 2.09-19.99 kDa had high antifungal activity against several *Candida* strains (Kulikov *et al*., 2014). While Mane *et al*., 2017a demonstrated that *B. poitrasii* chitosan with 42.82 kDa

molecular weight had MIC_{90} in the range 0.025-0.8 mg/mL against human pathogens such as *A. niger*, *C. albicans*, *C. glabrata*, and *C. neoformans.* While chitosan from marine source with a DDA 79.6% and of low molecular weight (70 kDa) was found to be effective against plant pathogenic fungi such as *F. oxysporum* and others (Guerrero *et al*., 2007).

In view of this, potential of the chitosans isolated from *Metarhizium* and *Myrothecium* strains was tested for antibacterial and antifungal activities against 2 plant pathogens (Table 4.2). As compared to chitosan isolated from marine source (MIC_{90} 0.8 mg/mL), the fungal chitosans showed lesser MIC_{90} against *F. oxysporum* (0.05-0.2 mg/mL). Against *X. campestris*, MIC_{90} for chitosans were in the range 0.025-0.4 mg/mL.

Table 4.2 Antimicrobial activity of chitosan isolated from *M. anisopliae* and *M. verrucaria.*

	(MIC_{90}) mg/mL	
	X. campestris NCIM 5028	*F. oxysporum* CMI113138
Control (2% acetic acid)	-	-
M. anisopliae MCC1197	0.05	0.1
M. anisopliae MTCC5190	0.4	0.2
M. verrucaria MTCC5191	0.025	0.05
Commercial chitosan	0.4	0.8

4.3 Discussion

Fungi such as *Penicillium* from antibiotic industry and citric acid producing *Aspergillus* produce huge amount of waste mycelial biomass which can be used for the isolation of chitin and chitosan (Cai *et al*., 2006; Tan *et al*., 2002). Numbers of fungi used in mycotechnology are being

studied for value addition to their existing products (Ghormade *et al*., 2017). For example, Shiitake mushroom, *L. edode*s, produces a polysaccharide, lentinan, an immune-stimulant in the body. After its extraction the fungal biomass was reported to be used for the production of chitosan (Crestini & Giovannozzi-Sermanni, 1996). Using lignocellulosic substrates, *M. indicus* can produce ethanol and high amounts of polyunsaturated fatty acids such as γ-linolenic acid. The fungus was reported to produce 62-67% alkali insoluble material *i.e.* chitin and chitosan in hyphal form cell walls further reported use of citrus fruit waste to grow *M. indicus* and *R. oryzae* biomass for the production of chitosan (Satari *et al*., 2015, 2016). They reported that biomass contained approx. 0.6-0.7% chitin and chitosan per g of cell wall. Varelas *et al*., 2016 reported use of yeast lees for β-glucan production as value addition and to reduce the negative costs for wineries. It was suggested that the yeast lees can also be used to isolate chitosan which has DDA >75% (Ghormade *et al*., 2017). In view of the potential of use of agriculturally important fungi in the field, the quantities of mycelial biomass produced are high.

In view of the requirement of either conidia or enzymes in the fields to control different pests and pathogens, the amount of mycelial biomass generated will be high. It was reported that for effective control of *Helicoverpa armigera* in 4.2 ha field of pulses, 1.5 kg *M. anisopliae* conidia were used (Kulkarni *et al*., 2008). After harvesting conidia, 680 g mycelial biomass waste could be generated. Considering the area under pulses in India (25.23 million ha) this can be a better source for chitosan isolation. Similarly, total enzyme requirement for the control of pests and pathogens in grapes can generate almost 200 tons of mycelial biomass every year which can be used to reduce the cost of production (Vidhate *et al*., 2015).

In case of *L. edodes* wheat straw was used as a substrate for SSF and 6.8 g chitosan per kg of the substrate was extracted from the mycelial biomass (Crestini *et al*., 1996). Pochanavanich

and Suntornsuk reported chitosan extraction from different fungi such as *A. niger, L. edodes, P. sajo-caju, Z. rouxii* and *C. albicans*. The reported yield of chitosan was in the range of 10–140 mg/g cell dry weight.

According to Lago *et al*., 2011 the characterization of chitosan using FTIR method is more qualitative than ^{1}H NMR analysis of degree of deacetylation (Lago *et al*., 2011). In the present investigations, however, the values for % DDA were slightly higher with ^{1}H NMR. The chitosan isolated from the mycelial biomass of *M. anisopliae* MTCC5190 harvested after SSF showed unusually higher (87.8%) DDA which can be attributed to the interference of other polysaccharides from the substrates in ^{1}H NMR measurements.

Chitosan extracted from *A. niger* waste biomass from citric acid industry was very high, 267.97 kDa (Cai *et al*., 2006). Chitosans extracted from zygomycetous fungus *G. butleri* had molecular weight in the range 30-100 kDa (Nwe *et al*., 2002). It was reported further that the chitosan produced from mycelial biomass developed in SSF had lower mol wt. (30 kDa) as compared to the chitosan produced from biomass developed in SmF (100 kDa). It was in accordance with our investigations for *M. anisopliae* grown in SSF and SmF. Chatterjee *et al*., 2005 reported use of *M. rouxii* mycelial biomass grown in different media for chitosan isolation. The molecular weights were in the range from 24-50 kDa (Chatterjee *et al*., 2005). The molecular weight of chitosan isolated from *B. poitrasii* was 42.82 kDa while that from *A. coerulea* was 500 kDa (Muzzarelli *et al*., 1994).

4.4 Summary

Numbers of fungi are being used as myco-fertilizer and /or myco-pesticide in agriculture. One of the major concerns is the cost of the bio agents as agriculture products are of high volume low

cost. As fungi contain chitin and chitosan (5-60%) in their cell wall the ‘waste’ biomass was used as a raw material for the isolation of these polymers.

In the present study the agriculturally important fungi *viz.*, *M. anisopliae* conidia which were previously used extensively as mycoinsecticide for the control of insect pests in agriculture. The mycelial biomass after removal of conidia was used to isolate chitosan. The biophysical characterization using ^{1}H- NMR and viscometry of the isolated chitosan showed 85% degree of deacetylation and 1.30 kDa molecular weight. Furthermore, as *M. anisopliae* produces chitin deacetylase extracellularly, the same was found to be effective to increase the deacetylation level of chitosan from marine source. Another ascomycetous fungus, *Myrothecium verrucaria* which produces extracellularly cuticle degrading and mycolytic enzyme complex has been used as wide spectrum biocontrol agent to control both insect pests and pathogens. After the separation of crude enzyme complex, biomass was used for chitosan (DDA, 86%; MW 2.34 kDa) isolation. To evaluate the antifungal potential of this low molecular weight chitosan, the chitosans were tested against plant pathogenic organisms.

Chapter 5

Comparative analysis of chitosan from different fungi with special emphasis on zygomycetous dimorphic fungus *Benjaminiella poitrasii*: Evaluation of its chitosan nanoparticles for the inhibition of human pathogenic fungi

Contents of chapter-5 have been published in a research article……

(Mane *et al*., 2022, Biomacromolecules, https://doi.org/10.1021/acs.biomac.1c01248)

5.1 Introduction

Chitosan is known to be a natural glucosamine polymer [β- (1→4)-linked] synthesized by the deacetylation of chitin [β- (1→4)-linked] *N*-acetylglucosamine polymer. Landings of marine organisms such as crabs, lobsters, and shrimps are the primary commercial source of chitin/chitosan. Fungi by definition are the organisms which contain chitin as a main structural component in the cell walls (Ghormade *et al*., 2017). The possible fungal sources for chitin and chitosan extraction include: waste fungal biomass generated from biotech industries and fermentation of zygomycetous fungi (Ghormade *et al*., 2017). The use of fungi as a source of chitosan is advantageous due to its homogenous, highly deacetylated, nature and also available round the year.

Indeed, thousands of tons of waste fungal biomass is produced from Mycotech- industries every year and is subjected to land filling or incineration for easy disposal. Varelas *et al*., 2016 identified utilization of wine lees, primarily spent yeasts, to produce β-glucan, which has a wide range of applications in the food sector (Varelas *et al*., 2016). The same can also be used to extract chitosan. The main yeast genera of lees include: *H. guilliermondii, I. orientalis, P. membranifaciens* and *S. cerevisiae* (Chavan *et al*., 2009). As the available yeast lees is in huge quantities, this can also be a potential source for chitosan isolation.

As a potential source of chitosan zygomycetous fungi such as *A. coerulea* (Davoust *et al*., 1992), *C. elegans* (Valeria *et al*., 2001), *G. butleri* (Nwe *et al*., 2002), *M. rouxii* (Synowiecki & Al-Khateeb 1997), *M. indicus* (Satari *et al*., 2016) and *R. oryzae* (Tai *et al*., 2010) have been studied. The chitosan extraction has also been reported from other fungi from different classes such as Ascomycetes (*Metarhizium, Myrothecium*) (Mane *et al*., 2017b); Basidiomycetes (*A.*

bisporus, L. edodes, *P. sajor-caju*) to name a few (Pochanavanich & Suntornsuk, 2002; Wu *et al*., 2019).

Chitosan has several applications in cosmetics, pharmaceuticals, lowering of serum cholesterol, drug delivery, agriculture, and food additives (Muzzarelli, 2000). Chitosan shows antifungal activity due to its positive charge which interacts with negatively charged phospholipids of fungal cell membrane (Yien *et al*., 2012). The molecular weight and higher DDA (%) contribute significantly in the antifungal activity. Yien *et al*., 2012 reported that the chitosan nanoparticles showed higher antifungal activity against *C. albicans,* and *F. solani*. This inhibitory effect was attributed to the particle size and zeta potential.

In this chapter we have screened the fungal organisms which fall in to either of the above-mentioned categories like waste fungal biomass from wine and mushroom industries and fermentation of zygomycetous fungus *B. poitrasii.* Along with the chitosan production and characterization, synthesis of fungal chitosan nanoparticles for the determination of its antifungal potential against human pathogenic organisms were also carried out.

5.2 Results and Discussion

5.2.1 Biomass production using different class of fungi

Under similar growth conditions, the biomass obtained from the fungi used in the study was in the range of 1.2-9.2 g/L. The ascomycetous yeasts yielded higher dry biomass (3.90-5.16 g/L) when grown in 1% YPG medium. While the zygomycetous B. *poitrasii* gave 9.17±0.32 g of dry mycelial and 1.78±0.28 g of yeast biomass per liter of the medium when grown under the modified growth conditions as described earlier (Mane *et al*., 2017a). It has been reviewed extensively that the available waste biomass from different industries, including wine and mushroom industries, is in huge quantities (Ghormade *et al*., 2017). To carry out the

fermentation exclusively for the extraction of chitosan from the fungal biomass is relatively expensive (Ghormade *et al*., 2017). Nevertheless, the desired characteristics (molecular weight, %DDA and viscosity) for healthcare applications prompt the researchers to go for the cultivation of fungal biomass.

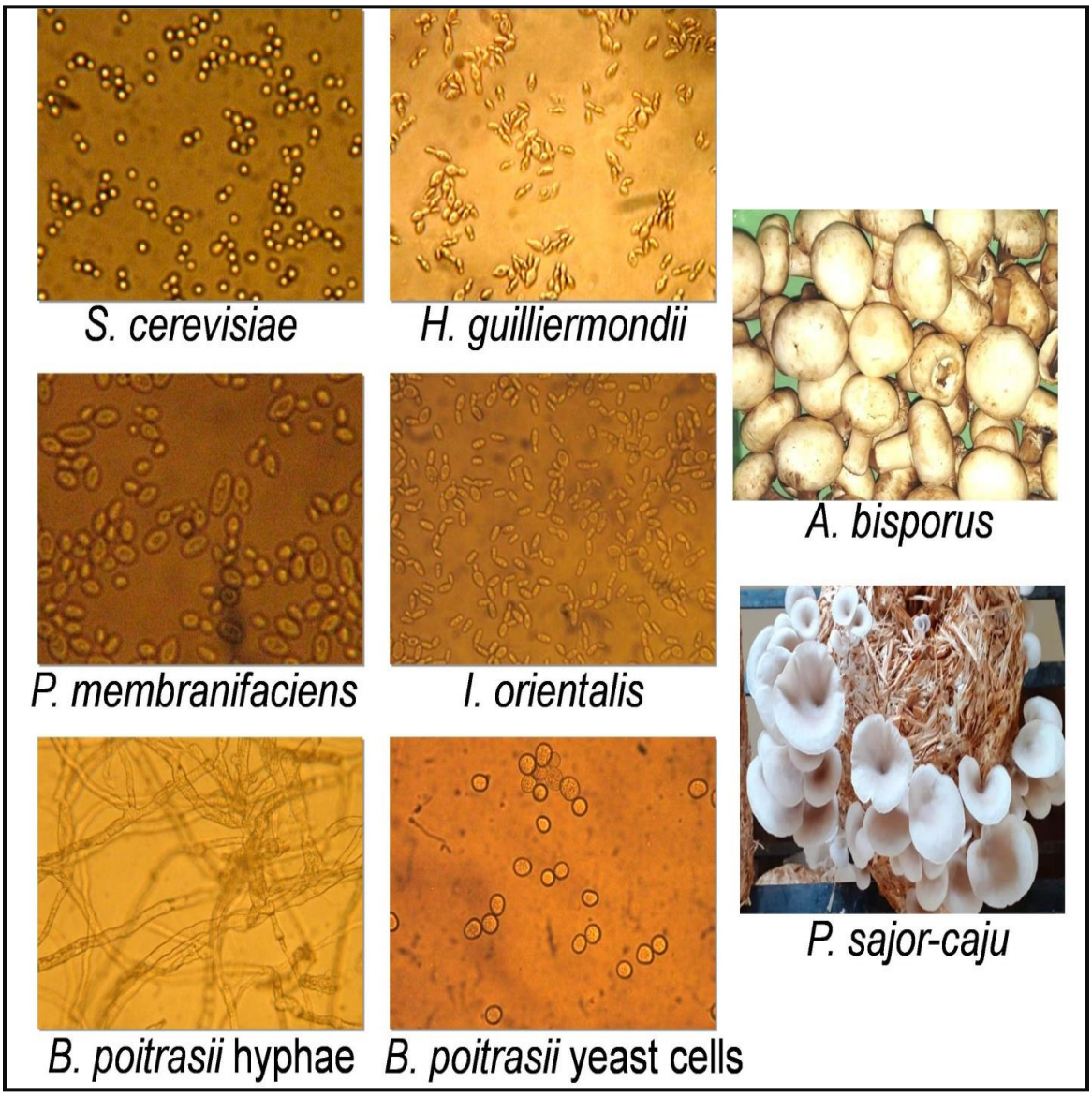


Figure 5.1 Schematic representation of different fungi screened for chitosan isolation. All images for ascomycetous and zygomycetous fungi were captured at 400X magnification.

5.2.2 Chitosan extraction from different fungi

Gooday reported the common wall constituents found in each division of fungi. The chitin/chitosan contents vary from 1-50% (Gooday, 1995). In the present investigation the chitosan isolation from winery yeasts such as *H. guilliermondii*, *I. orientalis*, *P. membranifaciens* and *S. cerevesiae* and fruit bodies of mushrooms, namely *A. bisporus* and *P. sajor-caju* (Basidiomycetes) and from *B. poitrasii* is depicted in Figure 5.1; Table 5.1.

Table 5.1 Extraction of chitosan from different fungi and their biophysical characterization

Fungal organisms	Chitosan (mg/g of dry biomass)	DDA (%) (^{1}H-NMR)	DDA (%) (FTIR)	Viscosity (cP)	Molecular weight (kDa)
		Zygomycetes			
B. poitrasii (H)	60.89±2.30	92.78	88.51	0.621	46.33
B. poitrasii (Y)	31.00±0.7	90.86	87.30	0.379	25.15
		Ascomycetes (Yeasts)			
H. guilliermondii	21.38±3.46	73.10	85.16	0.063	6.95
I. orientalis	15.00±0.91	70.32	83.52	0.013	6.21
P. membranifaciens	25.64±1.0	89.66	86.40	0.058	6.82
S. cerevisiae	31.00±0.70	83.59	86.68	0.073	6.55
		Basidiomycetes			
A. bisporus	15.92±0.85	ND	81.72	0.012	6.48
P. sajor-caju	13.52 ±1.22	ND	79.33	0.003	6.72
		Commercial source			
Marine chitosan	--	85.66	82.64	2.67	585*

ND: Not determined. *The molecular weight was determined by using viscometry data.

The chitosan yield in ascomycetous and basidiomycetous fungi was in the range of 13-31 mg/g of dry biomass. While in case of *B. poitrasii* grown in optimized medium the chitosan yield was 60.89±2.30 mg/g of dry mycelial biomass and 31.00±0.7 mg/g of dry yeast biomass (Table 5.1). Among the unicellular ascomycetous yeasts, *S. cerevisiae* showed highest content of chitosan (31.00±0.70 mg/g of dry biomass). Earlier Berger *et al*., 2018 isolated chitosan from mucorales fungi and the yield of chitosan was in between 5-15 mg/g of dry mycelial biomass.

5.2.3 Biophysical characterization of fungal chitosan

As mentioned in Table 1, the degree of deacetylation (DDA) of chitosan measured using ^{1}H-NMR was in the range of 70-89% for chitosan isolated from yeast and mushrooms. While DDA measured using FTIR was in the range of 79-87%. On the other hand, %DDA of chitosan isolated from *B. poitrasii* mycelial and yeast cells was >90% when measured by ^{1}H-NMR (Figure 5.2) while by FTIR (Figure 5.3) it was in the range of 87-88%.

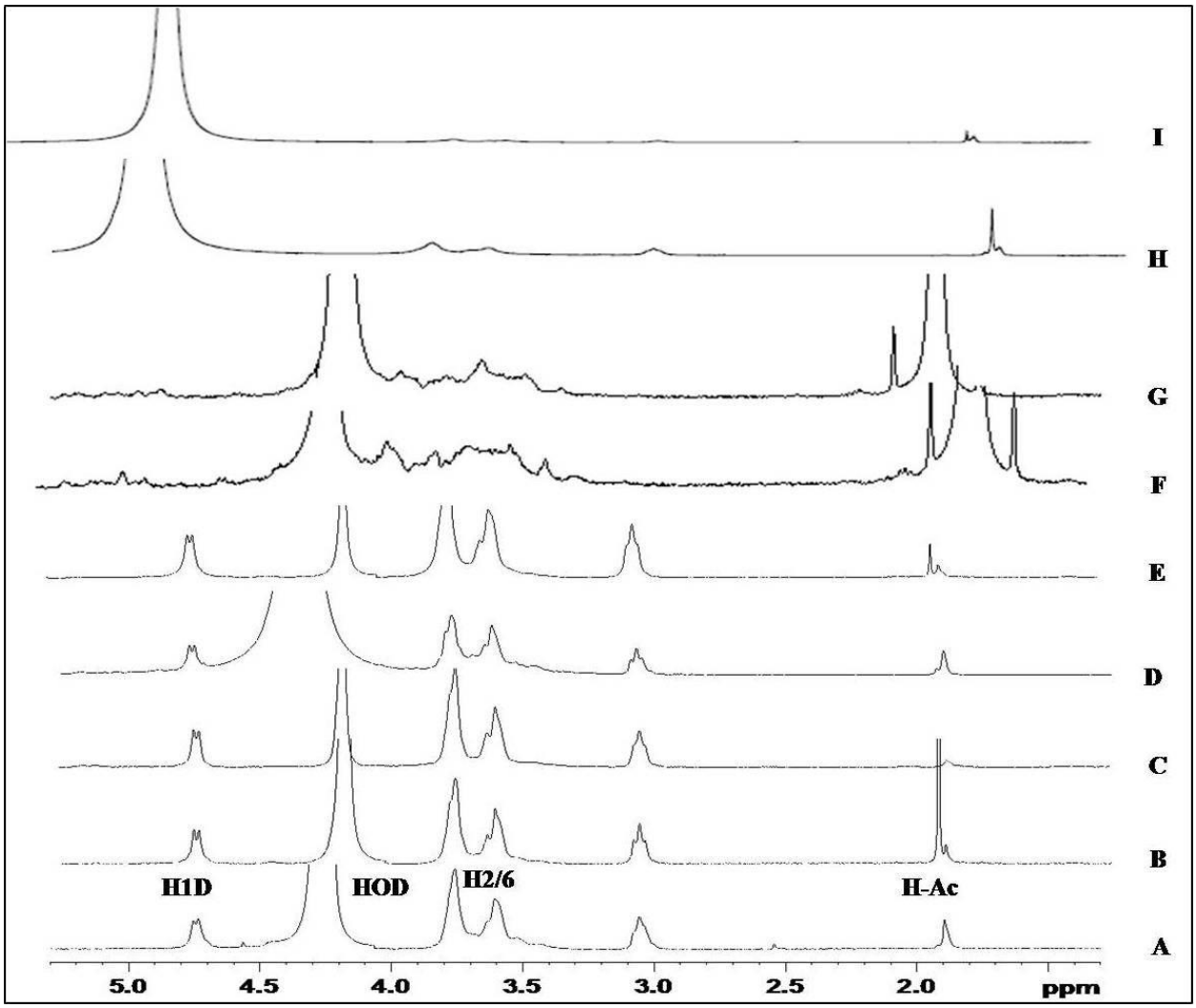


Figure 5.2 ^{1}H- NMR profiles of chitosan isolated from fungi of different classes. (Zygomycetes, B and C; Ascomycetes, D-G; Basidiomycetes, H and I) compared with chitosan from marine source (A). Chitosan isolated from: *B. poitrasii* hyphae (B); *B. poitrasii* yeast (C); *S. cerevisiae* (D); *P. membranifaciens* (E); *I. orientalis* (F); *H. guilliermondii* (G); *A. bisporus* (H); and *P. sajor-caju* (I).

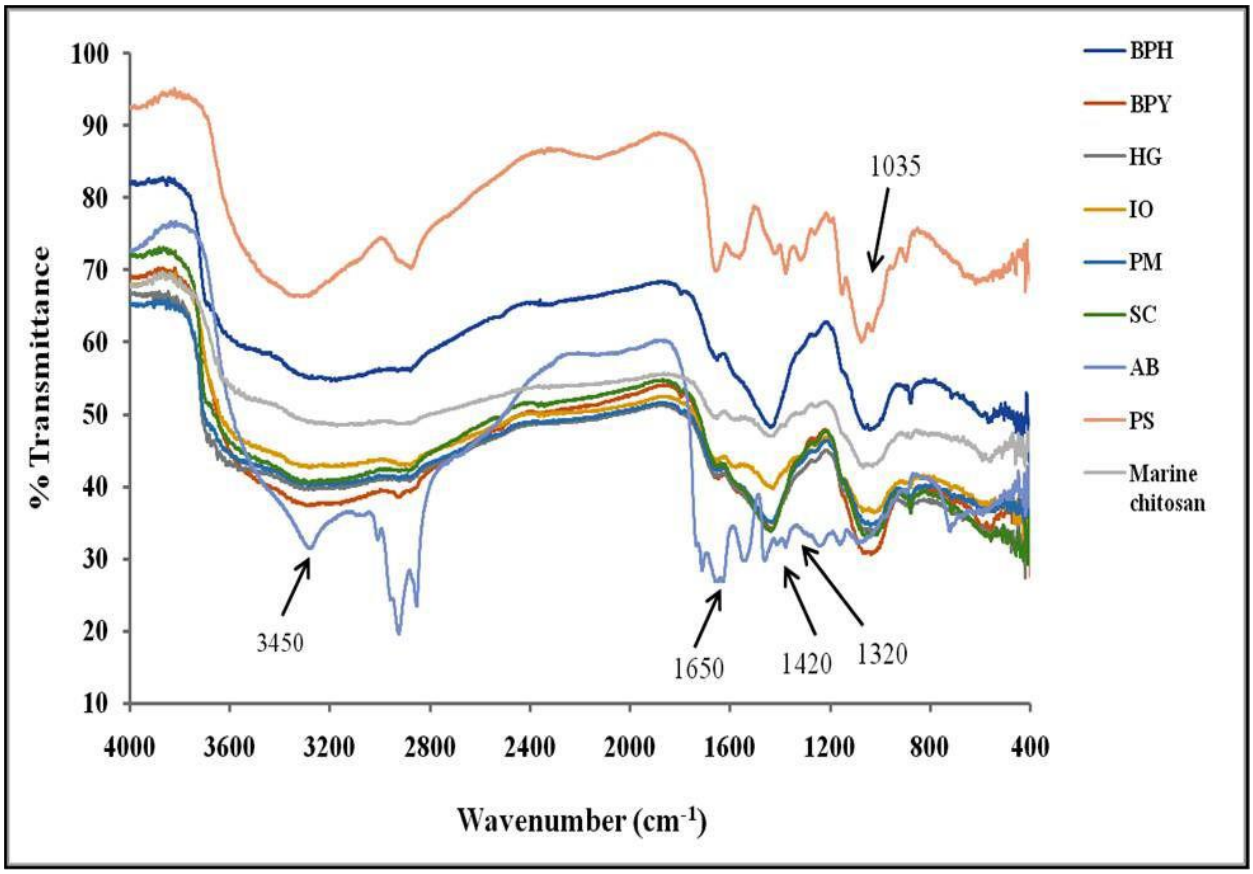


Figure 5.3 FTIR spectra of chitosan isolated from different fungi. *B. poitrasii* hyphae (BPH); *B. poitrasii* yeast (BPY); *H. guilliermondii* (HG); *I. orientalis* (IO); *P. membranifaciens* (PM); *S. cerevisiae* (SC); *A. bisporus* (AB); *P. sajor-caju* (PS) and commercial chitosan (Marine chitosan).

The characteristic absorption bands were observed at 3450 cm^{-1} (O-H stretching), 1650 cm^{-1} (amide II), 1420 cm^{-1} (C-H deformations), 1320 cm^{-1} (amide III, C-N stretching) and at 1035 cm^{-1} (O bridge stretching) by FTIR for all chitosans. Pochanavanich & Suntornsuk, 2002, isolated chitosan from fungi *Aspergillus, Rhizopus* and *Lentinus.* They reported %DDA measured by titration with NaOH which hydrolyzed the acetyl groups in chitosan in the range 86-90%. Moreover the %DDA of chitosan from crab shell was 97%. In the present study using ^{1}H-NMR and FTIR %DDA for crab shell chitosan was 80-86% (Table 5.1).

The viscometry method was used for the determination of viscous nature of fungal chitosan. The chitosan isolated from ascomycetous yeasts showed viscosity in the range of 0.013-0.073cP.The viscosity of chitosan from mushroom was in the range of 0.003-0.012 cP. In the present study, the viscosity of chitosan from zygomycetous fungus *B. poitrasii* hyphal and yeast was 0.621 and 0.379, respectively. The marine chitosan (2.67 cP) was more viscous as compared to fungal chitosan (Table 5.1).

The molecular weights of chitosan isolated from ascomycetous yeasts were low (6.21-6.95 kDa) (Table 5.1). Earlier Mane *et al*., 2017b reported chitosan isolated from 2 filamentous Ascomycetes *M. anisopliae* and *M. verrucaria*. The molecular weight was also low (0.77-2.38 kDa). While chitosan isolated from a zygomycetous *B. poitrasii* mycelium was higher 46.33 kDa and of yeast form cells was 25.15 kDa. The molecular weight of chitosan isolated from Basidiomycetes fungi, *A. bisporus* and *P. sajor-caju* was 6.48 and 6.72 kDa, respectively (Figure 5.4).

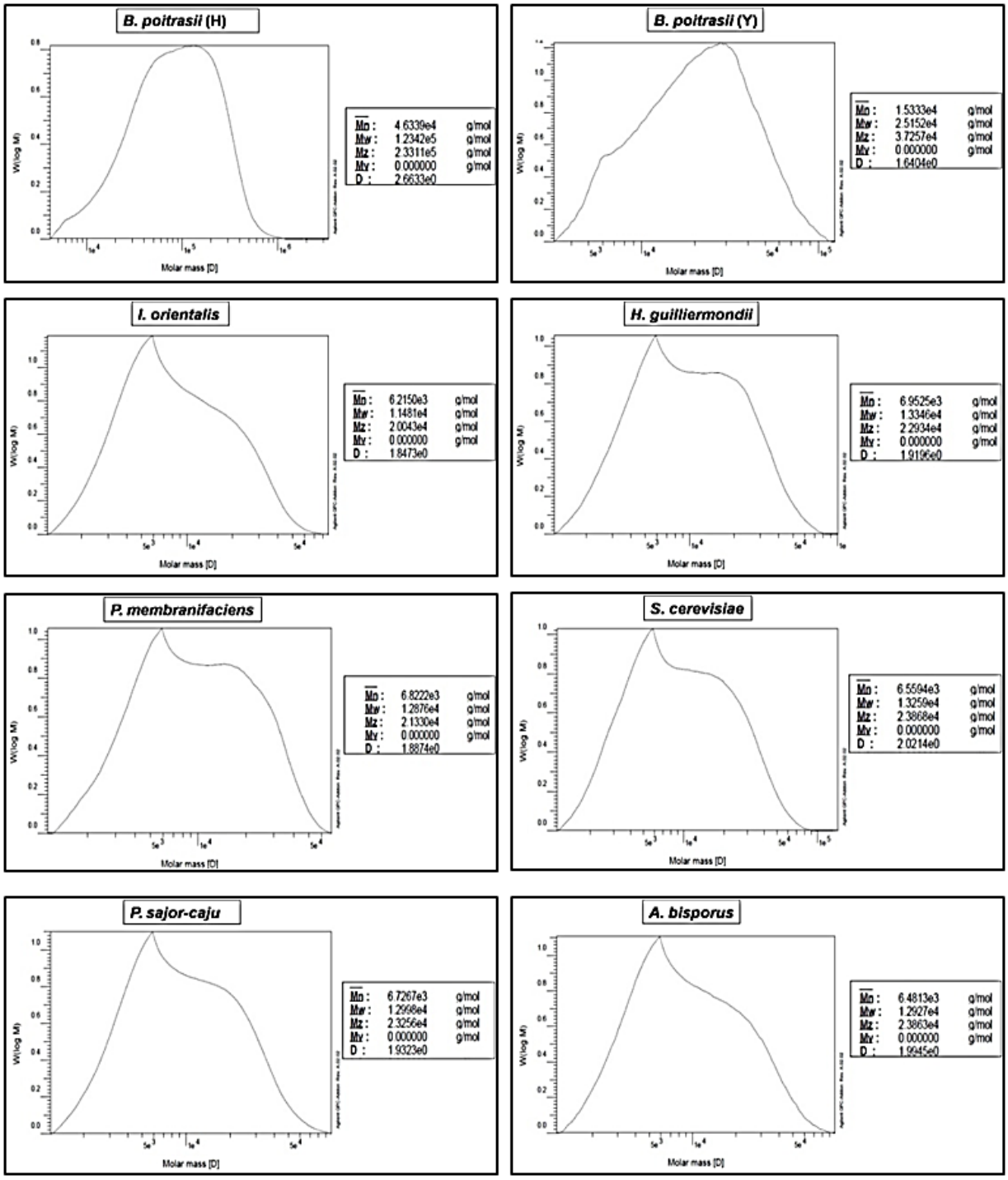


Figure 5.4 Gel permeation chromatography profiles of isolated fungal chitosans and reference marine chitosan for molecular weight determination.

5.2.4 Antifungal activity of fungal chitosan

The chitosan has antiviral, antibacterial, and antifungal properties and can be used in different application (Raafat & Sahl, 2009). The antimicrobial activity of chitosan is dependent on the properties like molecular weight and %DDA, which defines its solubility in water or dilute acid solutions (Yien *et al*., 2012). The chitosan isolated from ascomycetous and basidiomycetous fungi did not inhibit the growth of any of the tested human pathogenic yeasts and fungal strains. It can be attributed to the lower %DDA and very low molecular weights of these chitosan samples. As compared to the commercial chitosan from marine source (585 kDa, 85% DDA) (MIC_{90} 2.5-5.0 mg/mL), the *B. poitrasii* hyphal (46.33 kDa, 92.78% DDA) and yeast chitosans (25.15 kDa, 90.86% DDA) inhibited the growth of human pathogenic *C. albicans* ATCC 10231, *C. glabrata* NCYC 388, *C. tropicalis* ATCC 750, *C. neoformans* ATCC 34664 and *A. niger* ATCC 10578 at relatively lower concentrations (MIC_{90} 0.025-2.5 mg/mL) (Table 4.2). The antifungal activity of chitosan polymer against *A. niger*, *C. albicans*, *R. oryzae*, *Rhizopus stolonifera*, *Alternaria alternata* and others have been reported (Tayel *et al*., 2010; Yien *et al*., 2012; Zhong *et al*., 2007). These findings suggested that chitosan polymer properties *viz*., molecular weight, %DDA, types of fungal source, concentration and polycationic nature determines its antifungal potential (Tayel *et al*., 2010; Yien *et al*., 2012; Zhong *et al*., 2007).

Table 5.2 Antifungal activities of chitosan isolated from different fungi against human pathogens

	MIC_{90} (mg/mL)				
	C. albicans ATCC 10231	*C. glabrata* NCYC 388	*C. tropicalis* ATCC 750	*C. neoformans* ATCC 34664	*A. niger* ATCC 10578
Zygomycetes					
B. poitrasii (H)	0.025	0.2	0.8	0.8	0.8
B. poitrasii(Y)	0.8	2.5	1.6	0.8	0.8
Ascomycetes (Yeasts)					
H. guilliermondii	>5	>5	>5	>5	>5
I. orientalis	>5	>5	>5	>5	>5
P. membranifaciens	>5	>5	>5	>5	>5
S. cerevisiae	>5	>5	>5	>5	>5
Basidiomycetes					
A. bisporus	>5	>5	>5	>5	>5
P. sajor-caju	>5	>5	>5	>5	>5
Marine chitosan	5	2.5	2.5	2.5	>5
Control (2% acetic acid)	ND	ND	ND	ND	ND

MIC_{90}-Minimum inhibitory concentration; ND, not detected

The decreased MIC result owing to fungal resistance to chitosan is mostly attributed to the plasma membrane's free fatty acid (FFA) composition. A limitation of nutrients such as carbon and nitrogen are other elements that boost antifungal action. Nutrient deprivation alters cell wall design, which has an impact on fungal growth. Low fungal cell wall branching (glucan concentration) may also enhances chitosan sensitivity (Tayel *et al*., 2010). Therefore, to improve the activity of *B. poitrasii* chitosan, nanoparticles were prepared and tested against the human pathogenic organisms.

5.2.5 Characterization of fungal chitosan nanoparticles

The determination of hydrodynamic diameter and zeta potential of nanoparticles is mainly carried out by Dynamic Light Scattering (DLS). The average particle size of BpHCNp was found to be 196±2.32 nm with polydispersity index 0.388±0.002. For BpYCNp the average size was 178±4.09 nm with polydispersity index 0.341±0.03.While the marine chitosan nanoparticles (MCNp) showed more average particle size (689±57.82 nm) with polydispersity index 0.644±0.05 (Figure 5.7).

The morphology of nanoparticles prepared at different chitosan concentrations were determined by using field emission scanning electron microscopy (FE-SEM; Figure 5.5) and High-resolution transmission electron microscopy (HR-TEM; Figure 5.6). The morphology of chitosan nanoparticles was found to be spherical (Figure 5.6). The average size of BpHCNp, BpYCNp, and MCNp were found to be 63.58 nm, 51.34 nm, and 23.04 nm respectively.

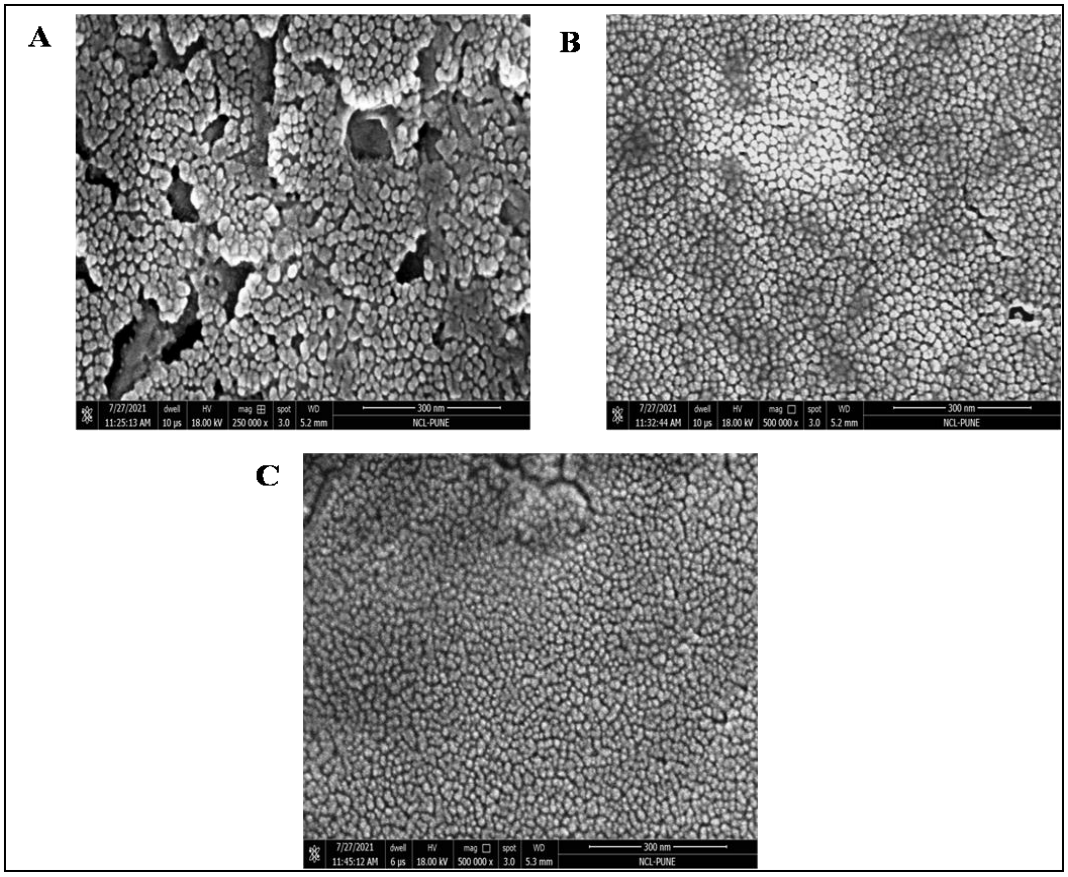


Figure 5.5 Field emission scanning electron microscopy to determine the morphologies of chitosan nanoparticles. *B. poitrasii* hyphal chitosan nanoparticles (A); *B. poitrasii* yeast chitosan nanoparticles (B); and marine chitosan nanoparticles (C).

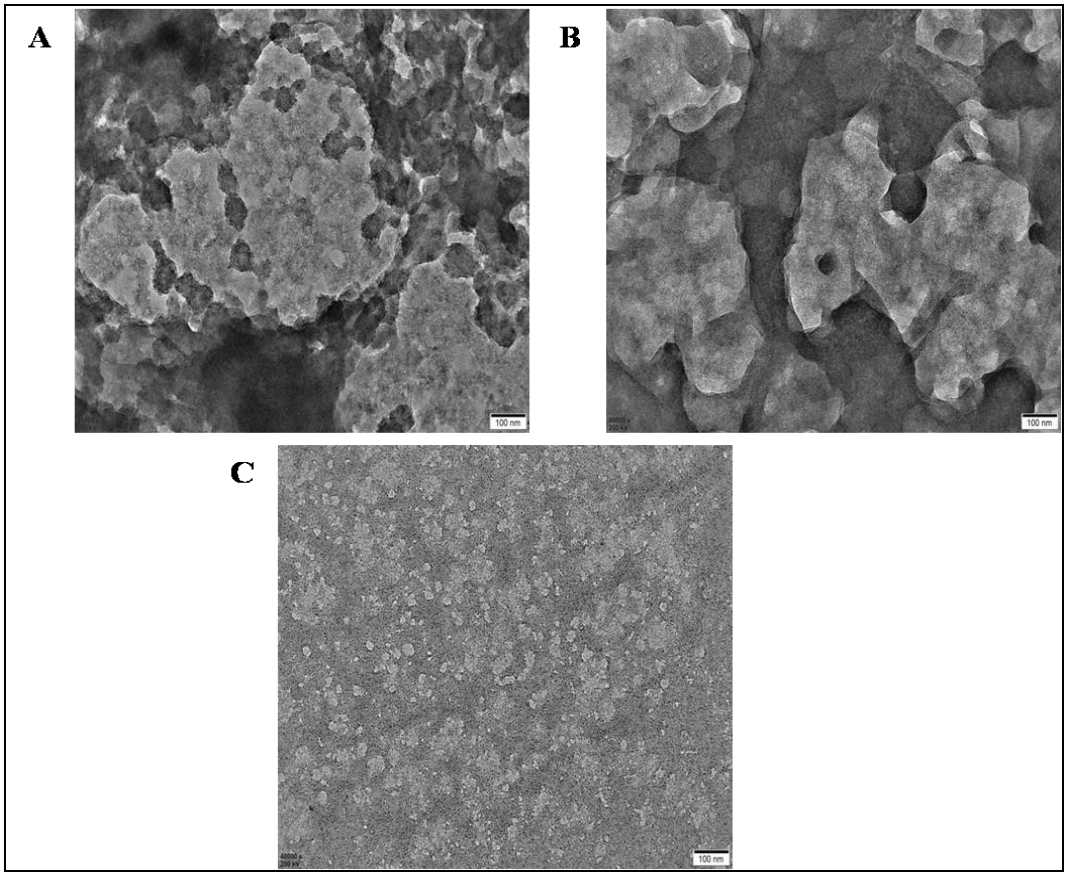


Figure 5.6 High resolution Transmission electron microscopy (HR-TEM) for size determination of chitosan nanoparticles. *B. poitrasii* hyphal chitosan nanoparticles (A); *B. poitrasii* yeast chitosan nanoparticles (B); and marine chitosan nanoparticles (C).

The zeta potential of BpHCNp, BpYCNp and MCNp was 24.48±1.58, 21.64±0.34 and 43.81±0.22 mV, respectively (Table 5.3).

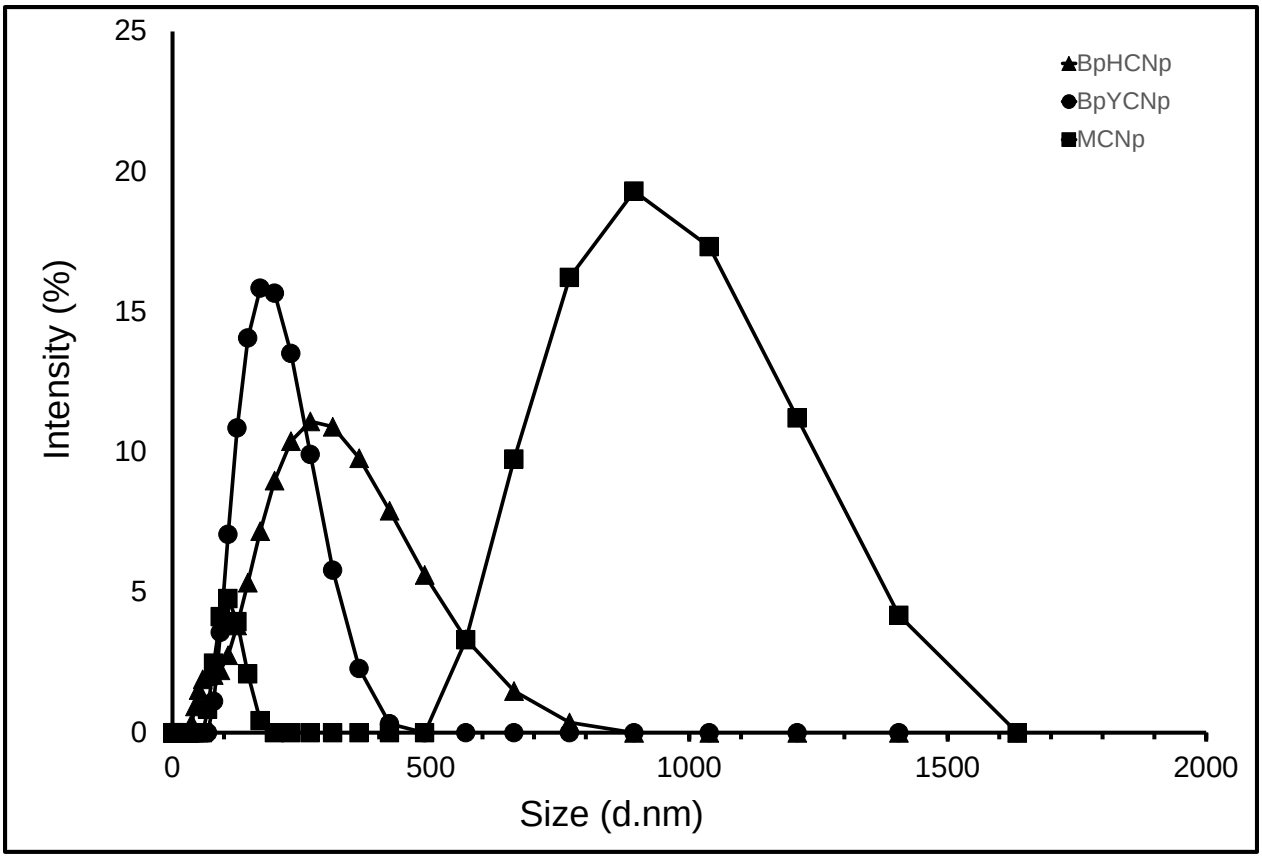


Figure 5.7 Size distribution of fungal chitosan TPP nanoparticles at 0.5 mg/mL concentration. BpHCNp- *B. poitrasii* hyphal chitosan nanoparticles; BpYCNp- *B. poitrasii* yeast chitosan nanoparticles; MCNp-Marine chitosan nanoparticles.

Table 5.3 Mean particle size, polydispersity index and zeta potential of chitosan nanoparticles

	Particle size (nm)	PDI	Zeta potential (mV)
BpHCNp	196±2.32	0.388±0.002	24.48±1.58
BpYCNp	178±4.09	0.341±0.03	21.64±0.34
MCNp	689±57.82	0.644±0.05	43.81±0.22

As shown in Table-5.3, the size and charge of MCNp differed significantly as compared to size and charge of BpHCNp and BpYCNp. This difference in the sizes and changes of synthesized nanoparticles are mainly due to the variations in molecular weight and % degree of

deacetylation of chitosan samples used for synthesis of nanoparticles. The other parameters *viz*., initial concentration of chitosan, chitosan to TPP ratio used, agitation speed, incubation temperature, choice of solvent and the presence or absence of different salts that affects the physicochemical properties of chitosan nanoparticles, were reported to contribute to their size and charge variations (Sreekumar *et al*., 2018).

5.2.6 Antifungal activity of fungal chitosan nanoparticles

Compared to the *B. poitrasii* hyphal chitosan, its nanoparticles showed better antifungal potential at the lower concentrations against human pathogenic *C. albicans* ATCC 10231 (MIC_{90}- 0.025 mg/mL), *C. glabrata* NCYC 388 (MIC_{90}- 0.1 mg/mL), *C. tropicalis* ATCC 750 (MIC_{90}- 0.4 mg/mL), *C. neoformans* ATCC 34664 (MIC_{90}- 0.4 mg/mL) and *A. niger* ATCC 10578 (MIC_{90}- 0.2 mg/mL). While the MIC_{90} for *B. poitrasii* yeast chitosan nanoparticles were in the range of 0.4-0.8 mg/mL against the tested organisms. However, the marine chitosan nanoparticles showed the higher MIC_{90} (0. 8->1.6 mg/mL) than the fungal chitosan nanoparticles (Table 5.4).

The chitosan nanoparticles (CNPs) showed improved antifungal potential against *C. albicans, Fusarium solani* and *A. niger* than the chitosan polymer (Yien *et al*., 2012). This improved antifungal potential of CNPs was attributed to the size and zeta potential of chitosan nanoparticles (Yien *et al*., 2012). The nano-formulation of chitosan and chitosan-silver nano composite also effectively inhibited the growth of plant pathogen *F. oxysporum* (Dhananjaya *et al*., 2017). In general, chitosan nanoparticles showed higher antifungal potential than the chitosan polymer (Lopez-Moya *et al*., 2019; Yien *et al*., 2012). Qi *et al*., 2004 attributed this improved antimicrobial potential of chitosan nanoparticles to their properties like small size, compact shape, and higher surface charge (Zeta potential). The small size and low molecular weight of CNPs reduces the viscosity and thereby improve their solubility in water or dilute acid solutions.

It led to protonation of more amino groups on CNPs allowing more efficient interaction between polycationic CNPs with the negatively charged host plasma membrane (Yien *et al*., 2012). Singh *et al*., 2008 also suggested that polycations primarily targets negatively changed plasma membrane. In this context, polycationic chitosan nanoparticles are more likely to interact with the fungal cells than the chitosan polymer. The small-sized, compact nanoparticles have a larger surface area, facilitating the adsorption of chitosan nanoparticles on the fungal cell and thereby disrupting membrane integrity.

Table 5.4 Antifungal activity of *B. poitrasii* chitosan nanoparticles against human pathogens

	MIC_{90} (mg/mL)				
	C. albicans ATCC 10231	*C. glabrata* NCYC 388	*C. tropicalis* ATCC 750	*C. neoformans* ATCC 34664	*A. niger* ATCC 10578
BpHCNp	0.025	0.1	0.4	0.4	0.2
BpYCNp	0.4	0.8	0.8	0.4	0.4
MCNp	0.8	1.6	1.6	0.8	0.8

5.2.7 Hemocompatibility of chitosan and chitosan nanoparticles

RBC hemolysis is important parameter for *in vivo* applications of any pharmaceutical agents as it can lead to anemia, jaundice, and other pathological conditions. Therefore, in the present study RBC hemolytic activities of chitosan samples and their nanoparticles were tested. The *B. poitrasii* yeast and hyphal chitosans as well as their nanoparticles were found to be hemocompatible as they showed <5% hemolysis at the highest tested concentration (5 mg/mL)

(Figure 5.8). The commercial chitosan from marine source however showed 6.53% hemolysis at 5 mg/mL, higher than the chitosan isolated from *B. poitrasii* hyphae (3.18% hemolysis) and yeast cells (3.06% hemolysis) under similar sets of conditions. The CNPs from marine source, *B. poitrasii* hyphae and yeast cells showed 3.30%, 2.96% and 1.49% hemolysis, respectively at the highest tested concentration (Figure 5.8). These observations agree with reports where chitosan and CNps did not show any cytotoxic effect against Human corneal epithelial (HCEC) and Hepatoma (HepG2) cell lines (de Campos *et al*., 2004; Jamil *et al*., 2016; Zhou *et al*., 2015). The study suggested that fungal chitosan and their nano-formulation are hemocompatible and therefore are safe for healthcare applications.

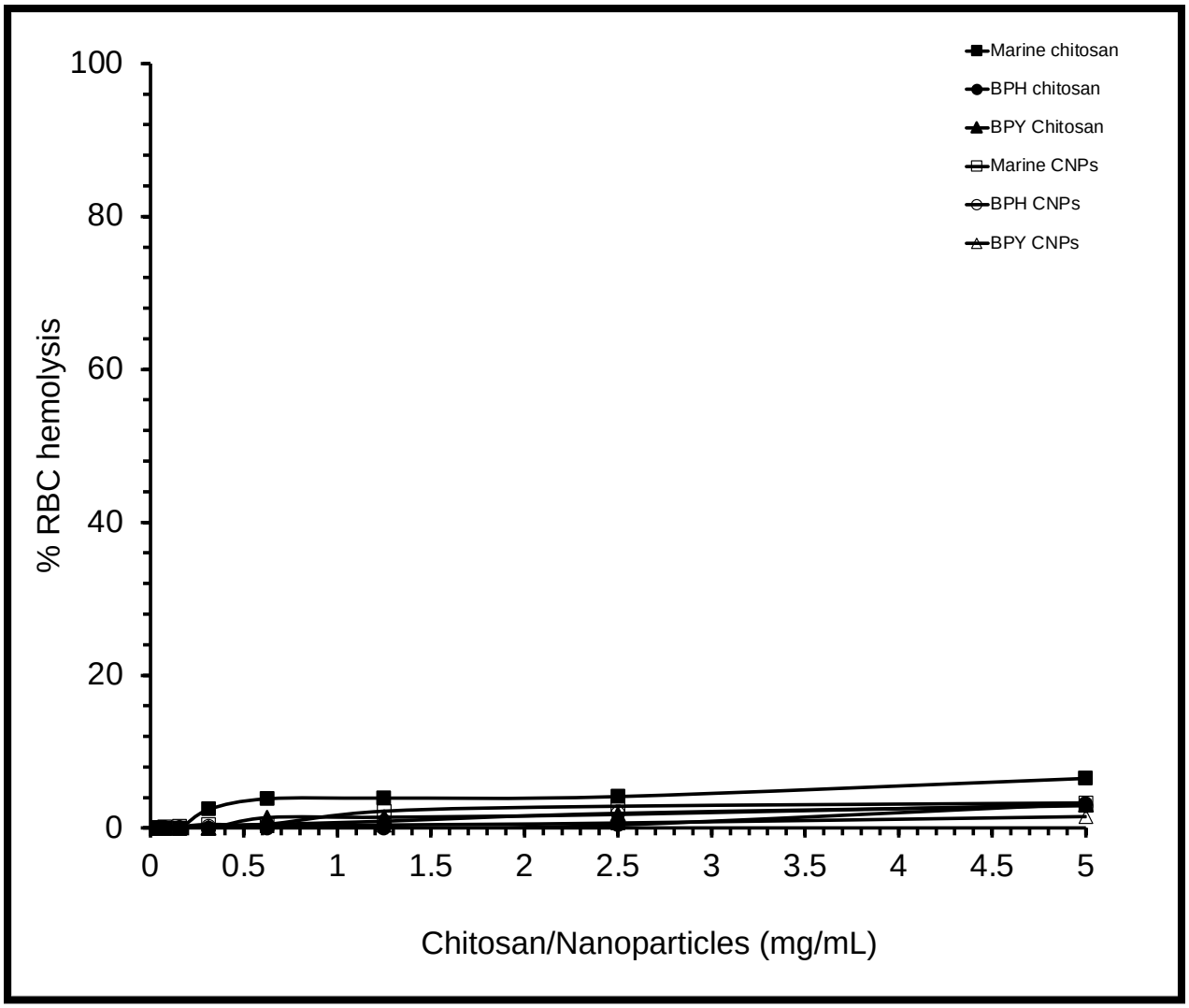


Figure 5.8 Red Blood Cells (RBC) hemolysis assay to check the hemocompatibility of chitosan polymers and their nanoparticles.

5.3 Summary

Though the chitosan contents in zygomycetous fungal cell walls are relatively higher than fungi from other classes, the availability of the waste biomass for other fungi from biotech industries is much more. In view of this isolation of chitosan from different industrially important fungi were studied. The chitosan from the ascomycetous yeast as well as basidiomycetous mushrooms showed low molecular weight as compared to the chitosan from *B. poitrasii*. However, they showed poor antifungal potential, which was attributed to their very low molecular weights and

low polycationic nature (*i.e.* low %DDA) as compared to the chitosan from *B. poitrasii*. The present investigations suggest that %DDA is more important in defining the antifungal potential of chitosan polymers than the molecular weight. Although the raw waste biomass available in case of ascomycetes and basidiomycetes fungi is higher but the isolated chitosan cannot be used for healthcare application due to the low cationic nature (*i.e.* low %DDA). On the other hand, chitosan isolated from the zygomycetous fungus *B. poitrasii*, was found to be promising (DDA- 92.78%, molecular weight- 46.33 kDa) source for healthcare applications, *per se.* Furthermore, the nanoparticles synthesized using *B. poitrasii* chitosan effectively inhibited the growth of human fungal pathogens at lower MIC_{90} than the chitosan. In the light of these results, it is suggested that chitosan nanoparticles prepared from *B. poitrasii* chitosan with high % DDA have the potential of becoming a powerful and safe natural antifungal agent. The chapter is summarised as depicted in Figure 5.9.

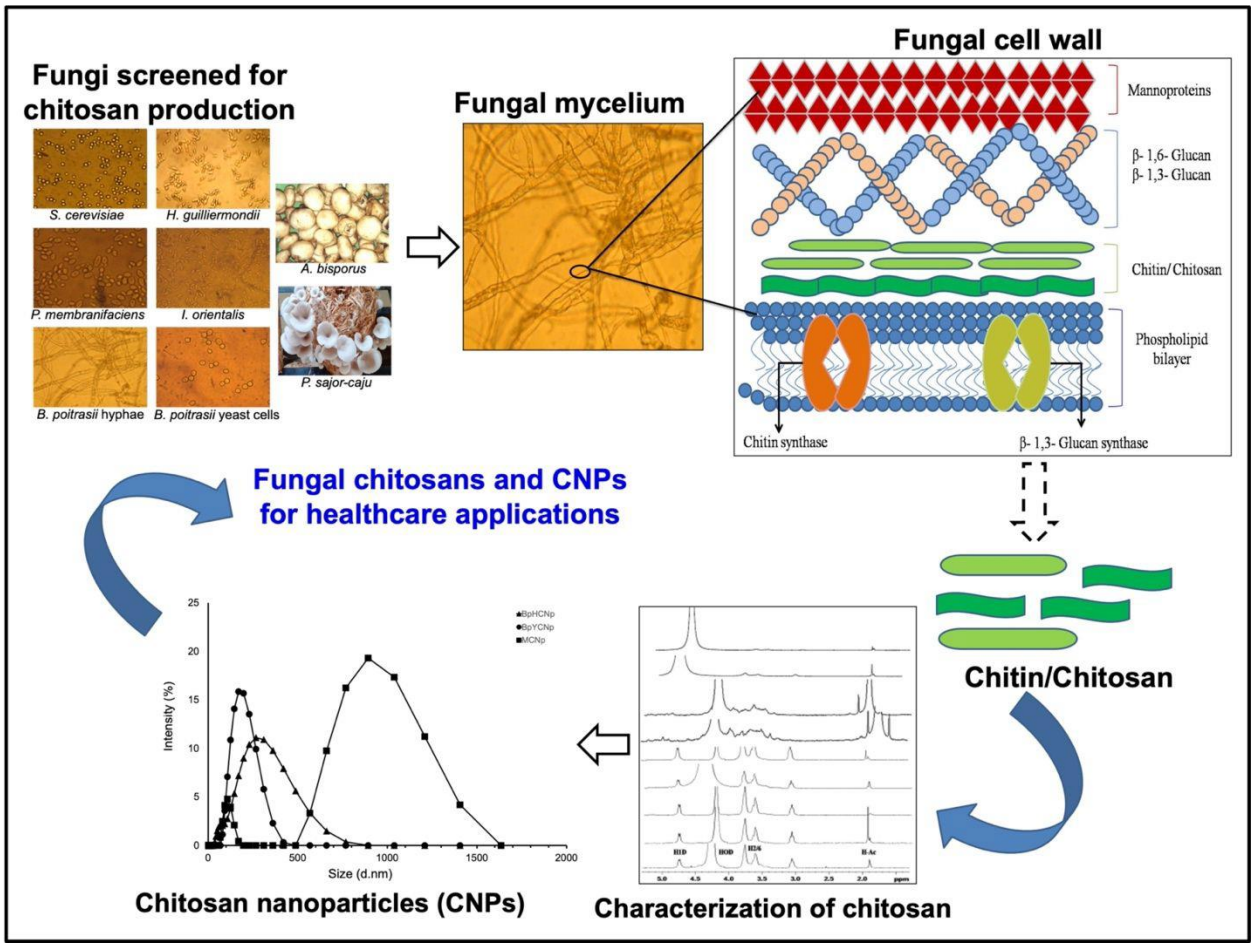


Figure 5.9 Schematic representation of summary of chapter 5

Chapter 6

Conclusions & Future prospects

6.1 Conclusions

The low molecular weight chitosan with a high degree of deacetylation can be isolated from the cell walls of zygomycetous fungi. One of the main challenges was the production of biomass sufficient to make the process cost-effective. The present study made efforts to achieve maximum biomass production by optimizing the media components and other parameters using OVAT and Taguchi DOE approach. For *B. poitrasii*, media optimized by OVAT approach produced 10.00±0.50 g/L (dry wt.) mycelial biomass, which was more than other zygomycetous sources reported so far. The yield of this extractable chitosan from *B. poitrasii* was increased by ~25% with CDA treatment. On the other hand, the Taguchi DOE approach considers the interaction between multiple factors affecting the growth and cell wall chitosan content of zygomycetous fungus *B. poitrasii*. The biomass (yeast and mycelia) obtained under optimized growth conditions gave maximum extractable chitosan, which has low molecular weight and high % DDA, the properties that define its antifungal potential.

Agriculturally important *M. anisopliae* strains (myco-pesticide) and *M. verrucaria* (industrial producer for cuticle degrading and mycolytic enzyme complex) were also explored for the isolation of chitosan. Chitosan isolated from these fungi would add value to their low cost- high volume agriculture-related products.

To explore the possibility of utilizing fungal waste biomass from biotech industries for chitosan production, the ascomycetous yeasts (*H. guilliermondii, I. orientalis, P. membranifaciens, S. cerevisiae*) and basidiomycetous mushrooms (*A. bisporus, P. sajor-caju*) were studied for their cell wall chitosan contents. The extractable amount of chitosan obtained from these strains was less than that obtained from *B. poitrasii*; however, they are of low molecular weight and lower % DDA than *B. poitrasii* chitosan. Although the raw waste biomass

available in the case of ascomycetes and basidiomycetes fungi is higher, the potential uses of this chitosan can be evaluated.

6.2 Future prospects

- In view of the higher % deacetylation and relatively lower molecular weight than marine source chitosan, fungal chitosan has applications in healthcare. The chitosan nanoparticles can be used as a wound healing agent.

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ABSTRACT

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Chitosan is a natural glucosamine polymer. Landings of marine organisms such as crabs, lobsters, and shrimps are the primary commercial source of chitin/chitosan. Fungi are organisms that contain chitin as a primary structural component in the cell walls. Therefore, the fungi can be an alternative to marine waste for isolating chitin/chitosan.

Benjaminiella poitrasii, a dimorphic zygomycetous fungus contains more chitosan in the mycelial cell wall than its yeast form. The optimization of suitable medium is necessary for the isolation of more chitosan. The optimization of *B. poitrasii* biomass was carried out with two different approaches, one variable at time approach (OVAT) and Taguchi design of experimental approach (DOE). The OVAT approach gave 10-12 g/L (dry wt.) of mycelial biomass in 48 h in a 2 L fermenter. Using the Taguchi DOE approach, interactions among eight different parameters showed that carbon source, incubation time, inoculum, yeast extract and peptone, were optimum for maximum biomass production.

The major concern for using entomopathogenic fungi as mycoinsecticide is the production cost, as it is a high volume, low-cost product. The mycelial biomass after removal of conidia can be used as a raw material for the isolation of chitosan, as value addition, and effective cost reduction. In this study, entomopathogenic *M. anisopliae* strains and another ascomycetous fungus, *M. verrucaria*, that produces cuticle degrading and mycolytic enzyme complex extracellularly were used for the isolation of low molecular weight chitosan.

The cell wall chitosan was also extracted from fungi belonging to different taxonomic classes *viz., B. poitrasii, H. guilliermondii, I. orientalis, P. membranifaciens, S. cerevisiae, A. bisporus,* and *P. sajor-caju*. The maximum chitosan yield was 60.89±2.30 mg/g of dry mycelial biomass of *B. poitrasii*. Further, to improve the efficacy of *B. poitrasii* chitosan, the nanoparticles were synthesized, the average particle size was <200 nm. The *B. poitrasii* chitosans and their nanoparticles (yeast and hyphal) were further evaluated for antifungal activity against human pathogenic organisms. BpHCNps showed lower MIC_{90} values (0.025-0.4 mg/mL) than the chitosan polymer against the tested human pathogens. The study suggested that nano-formulation of fungal chitosan, which has low molecular weight and high %DDA, is desirable for antifungal applications against human pathogens. Moreover, chitosan and its nanoparticles were hemocompatible and are therefore safe for healthcare applications.

List of publications

Research Paper(s):

- **Mane S. R.,** Pathan E. K., Kale D., Ghormade V., Gadre R.V., Rajamohanan P. R., Badiger M.V., and Deshpande M.V. (2017) Optimization for the production of mycelial biomass from *Benjaminiella poitrasii* to isolate highly deacetylated chitosan. **Journal of Polymer Materials,** 34, 145-156.
- **Mane S. R.,** Ghormade V., Rajamohanan P. R., Badiger M. V., and Deshpande M.V. (2017) Isolation of low molecular weight chitosan from agriculturally important ascomycetous fungi *Metarhizium anisopliae* and *Myrothecium verrucaria*. **Asian Chitin Journal**, 13, 31-38.
- **Mane S. R.,** Pathan E., Patil G., Tupe S. G., Ghormade V., Chaudhari B. P., and Deshpande M. V. (2022) Taguchi design of the experimental approach to increase the biomass and cell wall chitosan contents of zygomycetous dimorphic fungus *Benjaminiella poitrasii*. **Journal of Polymer Materials (Accepted in press)**.
- **Mane S.,** Pathan E., Tupe S., Deshmukh S., Kale D., Ghormade V., Chaudhari B. P., and Deshpande M.V. (2022) Isolation and characterization of chitosans from different fungi with special emphasis on zygomycetous dimorphic fungus *Benjaminiella poitrasii*: Evaluation of its chitosan nanoparticles for the inhibition of human pathogenic organisms. **Biomacromolecules**, **DOI: 10.1021/acs.biomac.1c01248.**

List of posters

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Mane S. R., Ghormade V., Rajamohanan P. R., Badiger M. V., Chaudhari B. P., and Deshpande M. V. "Bioprospecting of entomopathogenic fungus *M. anisopliae* for value addition". A poster was presented on National science day celebration held during February, 2018 at CSIR-National Chemical Laboratory, Pune, India.

Deshpande M. V., Pathan E.K., **Mane S. R.,** Panmei R., Patil A. V., Chandekar S., Chaudhari B.P., and Giri A. P. "Fungal Realm in Chitin/Chitosan Industry: Aspects and Prospects". Best Poster Award in the 7th Indian Chitin and Chitosan Society Meeting (ICCS-2018) held during October, 2018 organized by CSIR-National Chemical Laboratory, Pune, India.

Optimization for the Production of Mycelial Biomass from *Benjaminiella poitrasii* to isolate Highly Deacetylated Chitosan

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ABSTRACT

Benjaminiella poitrasii, a dimorphic zygomycetous fungus contains more chitosan in the mycelial cell wall than the cell wall of its yeast form. The optimized medium containing yeast extract, peptone, $MgSO_4$, KH_2PO_4, trace metals (Fe^{2+}, Mn^{2+} Zn^{2+} and Co^{2+}) solution and 1% starch produced 10-12 g/L(dry wt.) of mycelial biomass in 48 h in a 2L fermenter. Using 1N NaOH treatment from 1 g of dried biomass 51.00 ± 0.52 mg of chitosan of 42.82 KDa molecular weight and 94.24 % degree of deacetylation was extracted. With Metarhizium anisopliae chitin deacetylase (CDA), chitosan yield was 59.00 ± 0.84 mg while treatment with CDA of B. poitrasii it was 78.05 ± 0.58 mg/g of dry wt. of biomass. The chitosan dissolved in 2% acetic acid showed higher antifungal activity against Candida albicans (MIC_{90} 0.025 mg/mL) and Candida glabrata (MIC_{90} 0.2 mg/mL) than chitosan extracted from marine source (MIC_{90} >1.6 mg/mL) suggesting use of fungal chitosan in healthcare.

KEYWORDS: *Biophysical characterization, Chitin deacetylase, Chitosan, Degree of deacetylation, Media optimization, ^1H NMR.*

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1. INTRODUCTION

Chitosan, a β (1-4) 2- amino-2-deoxy-D-glucose (or D-glucosamine) polymer formed by deacetylation of chitin by the action of enzyme chitin deacetylase (CDA, EC 3.5.1.41). It has a wide range of applications from agriculture to human health care products [1-3]. Currently, chitosan is commercially produced from shellfish, shrimp waste, crab and lobster processing using strong alkalis at high temperatures for long periods of time [4]. However, the supply of marine waste is seasonal and limited, resulting in variability in source material. Furthermore, physico-chemical properties of chitosan derived from such processes are heterogeneous [2,5]. Since chitosan is required on large scale for variety of applications, it was proposed that it would be difficult to replace the marine sources with other organism in near future [6,7]. Ghormade *et al* [8] reviewed the potential of fungal waste from mycotech industries as an alternate source for chitosan. Furthermore, other than waste biomass utilization for value addition, number of zygomycetous fungi can be employed for chitosan production, as they contain relatively more chitosan in their cell walls than fungi from other classes [8,9]. In addition, the quality of fungal chitosan in terms of homogenous polymer length, degree of deacetylation (DDA) and solubility offers advantages over the crustacean sources [8,10]. Extraction of chitosan was reported mainly from zygomycetous fungi like *Absidia coerulea, Cunninghamella elegans, Gongronella butleri, Mucor rouxii*, *Mucor racemosus*, *Mucor indicus* and *Rhizopus oryzae* [11-15]. In case of *G. butleri*, Nwe *et al* [14] used both solid state fermentation (SSF) as well as submerged fermentation (SMF) for biomass production. Suntornsuk *et al* [16] reported use of soyabean, mung bean, molasses, corn-steep liquor, or sweet potato extract as cheaper alternate substrates to grow *R. oryze* and others for biomass development.

Benjaminiella poitrasii is dimorphic, zygomycetous NCL isolate, extensively studied for its cell wall chemistry. It has been reported that mycelia form cells contain 35% chitin/chitosan the cell wall [17]. Since, extractable chitin/chitosan contents are much higher in the cell wall of *B. poitrasii* as compared to other fungi, in the present investigations optimization of *B. poitrasii* mycelial biomass production for the extraction of highly deacetylated chitosan is carried out. Furthermore, use of CDA enzyme to increase the chitosan contents and antifungal activity of fungal chitosan are reported.

2. EXPERIMENTAL

2.1 Microorganisms

The parent strain of *Benjaminiella poitrasii* isolated at CSIR-National Chemical Laboratory, Pune, was maintained on 1% YPG agar (0.3%, yeast extract; 0.5%, mycological peptone; 1%, glucose; 2%, agar, pH adjusted to 6.5) slants. *Metarhizium anisopliae,* MCC 1197 source for extracellular CDA was maintained on potato dextrose agar (containing 2% glucose) slants at 28 °C and routinely sub-cultured every week.

2.2 Optimization of fungal biomass production

Yeast cell inoculum of *B. poitrasii* was developed by inoculating 1.6 x10^7 spores in 100 mL of 1% YPG broth from 3 d old slant and incubating at 37 ºC under shaking condition for 24 h. Before inoculation the yeast cells were washed with sterile distilled water to remove traces of glucose. The following media were used to obtain mycelial biomass: (A) YP (g/L), yeast extract 3, peptone 5; (B) YP modified: YP with (g/L): KH_2PO_4, 5.0; $MgSO_4$, 2.0 and 1.0 mL trace metal solution (mg/

mL): $FeSO_4$, $7H_2O$, 5; $MnSO_4$, 1.5; $ZnSO_4$, 3.34; $CoCl_2$, 2.0; (C) YP modified with 2% corn steep liquor (CSL); (D) YP modified with 0.5% soluble starch; (E) YP modified with 0.5% maltodextrins; (F) YP modified with 1% starch; (G) Optimized medium containing (g/L): yeast extract, 6.0; peptone, 10.0; soluble starch,10.0 and inorganic salts and trace metal solution as mentioned above. The YP medium along with 20% sweet potato extract was also used for biomass production. The medium was inoculated with 1.6×10^8 yeast cells/L and incubated at 28 ºC for 48 h. The mycelial biomass was separated by filtration using Whatman filter paper no. 1, washed, dried and stored in refrigerator until use.

The optimized medium in shake flask was further used in 2L fermenter for scale up. The development of an inoculum in optimized medium for fermentation studies is described under Section 3.2. For fermentation agitation was programmed from 250-400, aeration 2.5 lpm, initial pH 6.5 and was carried out 28 ºC for 48 h.

2.3 Isolation of cell walls

All preparations were carried out between 0-4 °C, unless otherwise mentioned. The biomass harvested as described above was washed and re-suspended in cold distilled water. The mycelial form cell walls were isolated using Braun homogenizer as described earlier [17]. The cell wall pellet after centrifugation was washed 5-6 times with cold distilled water then twice with decreasing concentration of cold NaCl solution (5.0%, 2.0% and 1.0%, w/v) followed (10-12 times) by cold distilled water. The cell wall preparations were clean and free from cytoplasmic material as determined by light microscopy. The purified cell wall samples were lyophilized and stored at -20 °C till further use.

2.4 Chitosan extraction

Chitin and chitosan were extracted from the isolated cell wall as described earlier [17, 18]. The cell wall pellet was re-suspended in 1N NaOH (1:40 w/v) followed by autoclaving at 121 °C for 15 min. Alkali insoluble mass was washed thoroughly with distilled water, further with ethanol followed by centrifugation at 12000 x g for 10 min. The residue was refluxed in 100 volumes of 2% acetic acid at 95 °C for 24 h. The slurry was centrifuged at 12000 x g at 4 °C for 45 min. Chitosan was precipitated out from the supernatant by adjusting the pH to 8.5-10 with 1N NaOH. The precipitate was washed several times with chilled distilled water. It was further washed with acetone and dried in oven at 60 °C, the sample were stored at -20 °C till further use. In another experiment, instead of cell wall isolation, the washed *B. poitrasii* mycelial biomass was directly used for the extraction of chitosan.

2.5 Production of extracellular chitin deacetylase by *Metarhizium anisopliae* and intracellular chitin deacetylase by *B. poitrasii*

The extracellular constitutive production of chitin metabolizing enzymes particularly CDA by *M. anisopliae* strain was carried out in 1% YPG medium at 28 °C for 72 h [19]. The extracellular supernatant was further ten times concentrated by ultra-filtration (UM 30 Amicon membrane) and used for deacetylation of *B. poitrasii* cell wall.

To obtain intracellular crude CDA from *B. poitrasii,* the fresh mycelial biomass obtained in optimized medium, was homogenized with glass beads in 50 mM sodium tetraborate buffer (pH 8.5), in Braun's homogenizer for 5 cycles of 15 sec each and then centrifuged at 12,000 g for 15 min. The supernatant was transferred to new microfuge tube. This supernatant was used as intracellular crude CDA.

2.6 Enzymatic deacetylation of *B. poitrasii* cell wall

The cell wall pellet isolated as mentioned under section 2.3 was treated with 10 mL of *M. anisopliae* extracellular and *B. poitrasii* intracellular CDA (100 mU each), separately at 37 °C for 24 h with constant shaking. After 24 h, the pellet was collected by centrifugation and further processed for chitosan extraction. In the control sample, cells were homogenized and chitosan was extracted by 1N NaOH treatment as described in section 2.4. The supernatant obtained after CDA and chemical treatment was used to quantify the released acetate by Bergmeyer's method using acetic acid (ACS manual format) assay kit (Megazyme, Ireland) according to manufacturer's instructions. Based on the concentration of acetate released due to each treatment, the chitosan contents were calculated.

2.7 Antifungal assay

B. poitrasii chitosan was solubilized in 2% acetic acid solution to make a 1% solution (10 mg/mL). The solution was kept for stirring overnight for dissolution of chitosan and pH was adjusted to 5 with 1N NaOH. Antifungal activity of isolated fungal chitosan against human pathogenic *Candida albicans* ATCC 10231, *Candida glabrata* NCYC 388, *Candida tropicalis* ATCC 750, *Cryptococcus neoformans* ATCC 34664 and *A. niger* ATCC 10578, by Clinical Laboratory Standards Institute's (CLSI) broth micro-dilution assay (CLSI document M27-A3 and CLSI M38-A2) were carried out as described earlier [20]. Overnight grown yeast cells were diluted (~2 x 10^3 cfu/mL) in RPMI 1640 medium and inoculated (100 ìL) in the wells of the microtitre plate. For filamentous fungi, 2 x 10^4 spores/mL were used as an inoculum. The microtitre plates were incubated at 28 ºC for 24-36 h for yeasts and 48 h for filamentous fungi. Acetic acid (2%) was used as a negative control whereas fluconazole was used as positive control. After incubation the minimum inhibitory concentration (MIC) was defined as the lowest concentration exhibiting >90% inhibition of visible growth as compared to growth of the control.

2.8 Biophysical characterization of chitosan

2.8.1 Fourier Transform Infrared (FT-IR) spectroscopy

The Fourier transform infrared (FT-IR) spectra were taken on a Bruker Optics ALPHA-E spectrometer with a universal Zn-Se ATR (attenuated total reflection) accessory in the 400-4000 cm^{-1} region or using a Diamond ATR (Golden Gate) [21]. The chitosan sample was dried at 105 °C for 1 h and consequently grinded for 5 min. It was mixed with KBr and was kept at 105 °C for 1 h and after grinding for overnight. The FT-IR was taken using KBr pellet as a reference as described by Van de Velde and Kiekens [21]. The DDA was determined by means of the absorbance ratio A_{1320}/A_{1420}. The following formula was used for the % Degree of acetylation (DA) calculation = 31.198 (A_{1320}/A_{1420}) - 12.20. The % DDA was calculated by using formula: % DDA= 100 - % DA [21].

2.8.2[1] H-*Nuclear Magnetic Resonance* Spectroscopy

The samples were prepared by dissolving 10 mg of chitosan in a solution containing 1.96 mL of D_2O and 0.04 mL of concentrated HCl with continuous stirring for 30 min. Samples were lyophilized and dissolved in 1 mL of D_2O. The 600 µL of sample was used for the NMR spectral analysis [22]. All the NMR spectra were taken on Bruker AV400 NMR spectrometer. The experiments were carried out at 70 °C at which the solvent (D_2O) peak does not interfere with any of chitosan's peaks. DDA was calculated using formula 1 and 2 integrals of proton ^1H of deacetylated monomer (H1-D) and the peak of the three protons of acetyl group (H-Ac) [23].

% DDA = H1D/H1D+H1A × 100….. (1)

% DDA = H1D/H1D+HAc/3 × 100… (2)

The signal from protons H2, H3, H4, H5, H6, H6' of both monomers and the peak of acetyl group (H-Ac) was also used for DDA calculation [23].

% DDA = [1- (1/3 HAc /1/6 H 2-6)] × 100….. (3)

2.8.3 Determination of molecular weight

Viscosity measurements were carried out at 25 °C using Ubbelohde-type viscometer (Fisher, Germany). Viscometer was connected to a visco-clock (Schott Visco clock), which automatically recorded the time of flow of solution through the two marks of the viscometer. Every value recorded was an average of 3 measurements. Chitosan samples (0.1%) were prepared in 0.5M acetic acid and 0.5M sodium acetate [24]. The flow time was determined in sec. The average molecular weight was then calculated using the Mark- Houwink equation:

$[\eta] = KMv^{\alpha}$, Where K and α for HAc/NaAc is $119\times10^5$and 0.5, respectively [24].

2.9 Chitin deacetylase assay

Chitin deacetylase (CDA) activity was estimated using ethylene glycol chitin (EGC) as a substrate prepared as described earlier with slight modification [19]. The reaction mixture contained: 100 µL 50 mM sodium tetraborate buffer, pH 8.5, 100 µL EGC (1 mg/mL), and 50 µL

enzyme, incubated at 37 °C for 30 min. The reaction was terminated by heating at 100 °C for 15 min. The acetate molecules released were measured by Bergmeyer's method using acetic acid (ACS manual format) assay kit according to manufacturer's instruction. The acetate released was determined based on NADH formation that was measured by increase in the absorbance at 340 nm. One unit of enzyme released 1 µmol of acetate from ethylene glycol chitin per min [19].

3. RESULT AND DISCUSSION

3.1 Media optimization for the production of *B. poitrasii* mycelial biomass in shake flasks

Different media were used to obtain maximum mycelial biomass of *B. poitrasii*. As shown in Fig. 1 the biomass yield increased 3.34 fold in shake flask when grown in medium G (optimized medium, 44.50 ± 0.80 g/L) as compared to medium B (YP modified medium, 13.30 ± 0.60 g/L) within 48 h. The addition of 2% CSL (medium C), 0.5% soluble starch (medium D), 0.5% malto-dextrins (medium E) or 1% soluble starch (medium F) in YP modified medium did not increase biomass appreciably.

The following media were used: A, YP; B, YP modified i.e. with inorganic salts; C, YP modified with 2% CSL; D, YP modified with 0.5% starch; E, YP modified with 0.5% maltodextrins; F, YP modified with 1% starch and G, optimized medium.

The addition of inorganic salts and trace metals in YP medium increased biomass 2 times *i.e* 6.44 ± 0.90 g/L in YP medium increased to 13.30 ± 0.60 g/L in YP modified medium. Furthermore, the yield of biomass increased 2.69 fold (17.33 ± 0.80 g/L) with 20% sweet potato extract (Table 1).

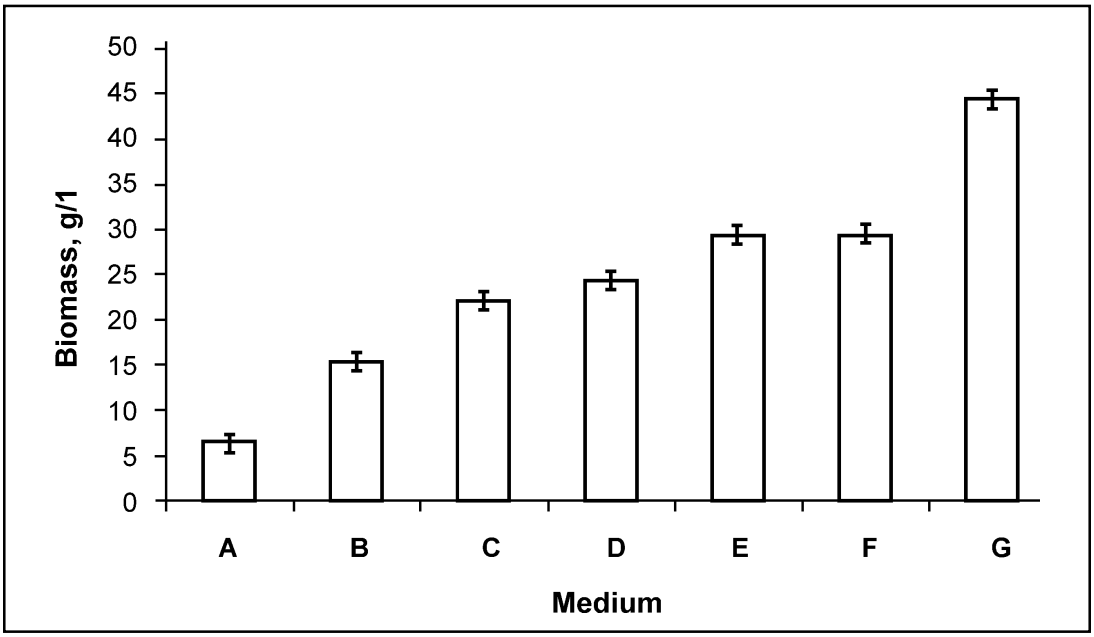

Fig. 1. *B. poitrasii* biomass production in different nutritional media under shaking condition after 48 h.

TABLE 1. The yield of biomass and chitosan of *B. poitrasii* grown in different media in shake flasks

Medium	Biomass wet wt (g/L)	Biomass dry wt (g/L)	Chitosan (x 10^{-3} g)/L
YP (control)	6.44 ± 0.90	1.28 ± 0.10	83.52 ± 5.00
YP modified	13.30 ± 0.60	2.66 ± 0.25	151.67 ± 3.10
YP+SP extract	17.33 ± 0.80	3.46 ± 0.30	226. 00 ± 4.00
Optimized medium	44.50±0.80	8.72±0.45	443.24 ±4.10
Optimized medium*	46.20 ± 1.00	9.25 ± 0.50	460.47 ± 5.00
Optimized medium**	51.20 ± 1.00	10.00 ± 0.50	510.00 ± 5.20

The compositions of different media are described under Materials and Methods.
The yeast cells (1.6 x 10^8/L) were inoculated and flasks were incubated under shaking conditions at 28 ºC for 48 h.

* The 10% (v/v) inoculum developed in optimised medium as described under Materials and Methods was used in shake flask.

** The inoculum (10% v/v) developed in the optimized medium was used for 2L fermenter.

The inoculum developed in optimized medium (100 mL) by inoculating1.6 x10^7 spores from 3 d old slant and incubating at 28 ºC under shaking condition for 24 h was used for biomass production in optimized medium. With 10% inoculum containing both yeast and hyphal cells in equal proportion, the biomass produced in shake flask was marginally higher (Table 1).

Microscopically the mycelial morphologies produced in different media were comparable However, the mycelia were thicker on sweet potato medium as compared to other media used (data not shown).

3.2 Production of *B. poitrasii* mycelial biomass in fermenter

The production of mycelial biomass optimized in shake flask was successfully up scaled to 2L fermenter. The inoculum (10% v/v) developed in optimized medium was used. The biomass was produced in the optimized medium in 2L fermenter maintaining pH 6.5, air flow 2.5 lpm and agitation at 250-400 rpm. After 48 h the biomass yield was 51.20 ± 1.00 g (wet wt.)/L (Table 1).

After media optimization studies, the amount of biomass obtained was increased by 7.95 fold with simultaneous increase in the yield of extractable chitosan by 6.1 fold *viz.* from 83.52 ± 5.00 to 510.00 ± 5.20 mg/L (Table 1).

3.3 Biophysical characterization of chitosan

3.3.1 Fourier Transform Infrared (FT-IR) spectroscopy

IR spectra were used to identify the presence of chitosan extracted from mycelial biomass of *B. poitrasii.* The typical peaks at 1452 and 1556 cm^{-1} for purified chitosan (Sigma Chem. Co., USA) and also for *B. poitrasii* samples were used to confirm the presence of chitosan. The degree of deacetylation calculated from FT-IR was 92.89% for *B. poitrasii* chitosan while for commercial chitosan (Sigma), it was 80.22% (Fig. 2; Table 2).

TABLE 2. Degree of deacetylation and molecular weight of chitosan isolated from *B. poitrasii* mycelia grown in different media

Isolated chitosan from *B. poitrasii* grown in media	DDA (^{1}H-NMR) (%)	DDA (FT-IR) (%)	Molecular weight (KDa)
YP	95.15	93.87	42.95
YP modified	94.76	92.77	43.57
Optimized medium	94.24	92.89	42.82
YP Modified with 20% Sweet potato extract	ND	ND	37.31
Purified chitosan (Sigma Chem. Co., USA)	85.66	80.22	464.83

ND, Not determined

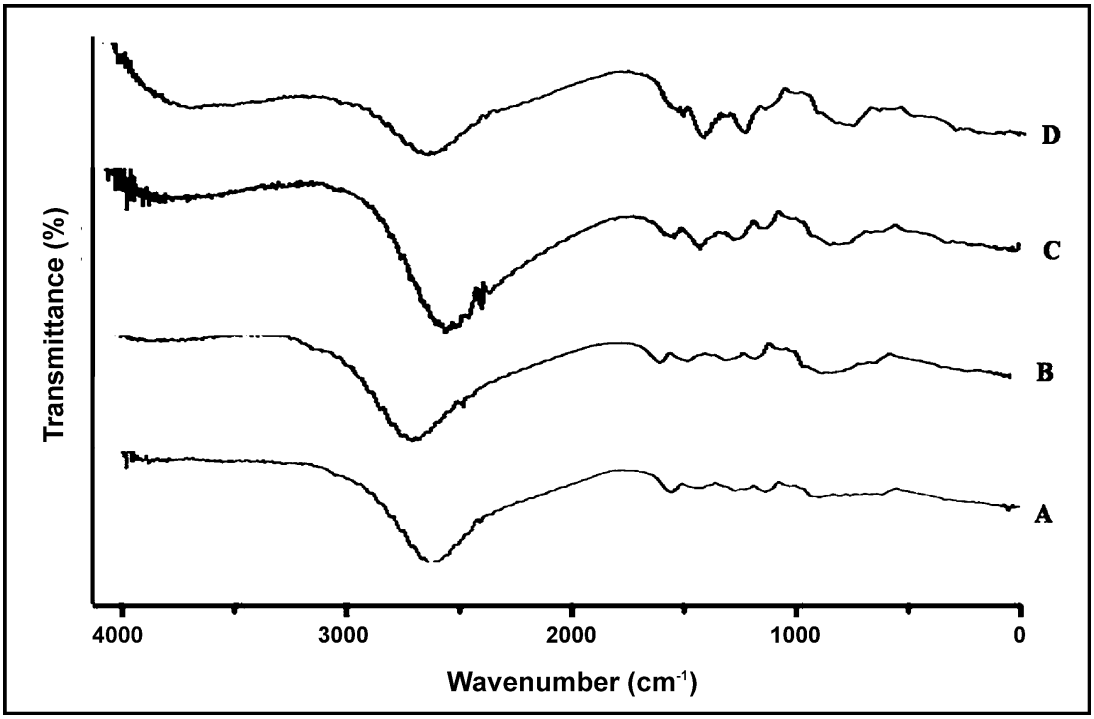

Fig. 2. FT-IR profiles of chitosan from different sources.

A, purified chitosan from Sigma Chem. Co., USA; B, chitosan isolated from *B. poitrasii* grown in YP medium; C, chitosan of isolated from *B. poitrasii* grown in YP modified medium; D, chitosan isolated from *B. poitrasii* grown in optimized medium.

Fig. 3. [1]H-NMR profiles of chitosan from different sources.

3.3.2 [1] H- *Nuclear Magnetic Resonance* Spectroscopy

[1]H- NMR was *used to calculate t*he degree of deacetylation. *B. poitrasii* chitosan isolated from biomass grown in different media had 94.24% DDA while the chitosan procured from Sigma showed 85.66% DDA (Fig. 3; Table 2). The % DDA values calculated using FT-IR were marginally lesser than values obtained by [1]H- NMR.

A, purified chitosan from Sigma Chem. Co., USA; B, chitosan isolated from *B. poitrasii* grown in YP medium; C, chitosan of isolated from *B. poitrasii* grown in YP modified medium; D, chitosan isolated from *B. poitrasii* grown in optimized medium; E, chitosan isolated from *B. poitrasii* grown in sweet potato medium.

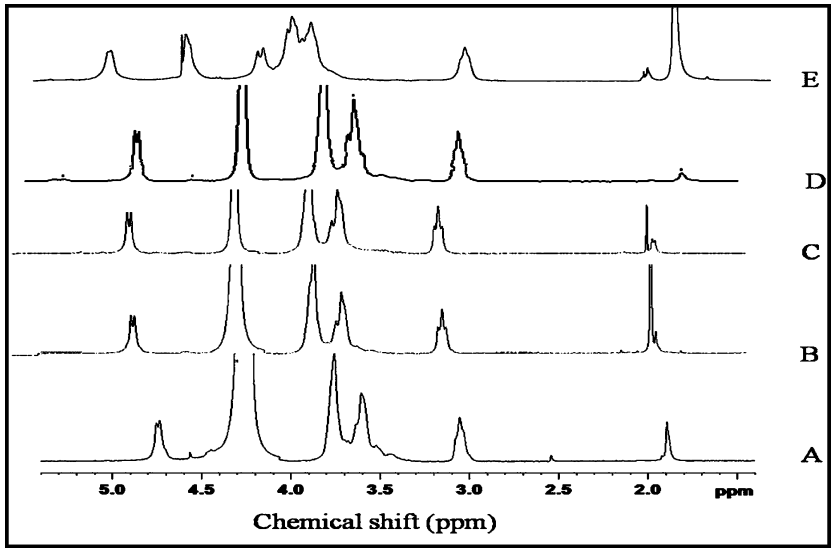

Fig. 3. [1]H-NMR profiles of chitosan from different sources.

TABLE 3. Chemical and enzymatic deacetylation of *B. poitrasii* mycelia biomass

Sr. No.	Treatment	Chitosan (mg/g dry wt.)	Fold increase
1.	1N NaOH (Control)	51.00 ± 0.52	-
2.	Extracellular CDA of *M. anisopliae*	59.00 ± 0.84	1.16
3.	1N NaOH followed by *M. anisopliae* CDA	70.70 ± 0.72	1.39
4.	Intracellular CDA of *B. poitrasii*	*78.05 ± 0.58	1.53

B. poitrasii mycelial biomass (1 g, dry weight) was treated as described under Material and Methods.
*Measured by acetate release as described under section 2.6

3.3.3 Viscometry

The average molecular weight of *B. poitrasii* chitosan was 42.82 KDa while of commercial chitosan (Sigma) from marine source was almost ten times *viz.* 464.83 KDa (Table 2).

3.4 Enzymatic deacetylation of *B. poitrasii* biomass with *M. anisopliae* chitin deacetylase

As compared to the NaOH treatment, chitosan content was increased by 1.16 fold (59.00 ± 0.84 mg/g of biomass) with *M. anisopliae* CDA treatment. When biomass was treated with 1N NaOH followed by *M. anisopliae* crude CDA, chitosan content was increased to 70.70 ± 0.72 mg/g of biomass *i.e*. 1.39 fold as compared to the chemical treatment. However, the treatment with *B. poitrasii* mycelial intracellular crude CDA yielded 78.05 ± 0.58 mg chitosan/g of biomass i.e. 1.53 fold more as compared to the NaOH treatment (Table 3).

3.5 Antifungal activity of chitosan

B. poitrasii chitosan showed better antifungal activity against *C. albicans* (MIC_{90} 0.025 mg/mL) and *C. glabrata* (MIC_{90} 0.2 mg/mL) than against *C. neoformans, C. tropicalis* and *A. niger* though not comparable with fluconazole. Commercial chitosan from marine source showed MIC_{90} > 1.6 mg/mL against all the fungi tested (Table 4).

TABLE 4. Antifungal activity of chitosan against different human pathogens

	Minimum Inhibitory Concentration (MIC_{90}) mg/mL				
	Candida albicans ATCC 10231	*Cryptococcus neoformans* ATCC 34664	*Candida glabrata* NCYC 388	*Candida tropicalis* ATCC 750	*Aspergillus niger* ATCC 10578
B. poitrasii chitosan	0.025	0.8	0.2	>0.8	0.8
Commercialchitosan	>1.6	>1.6	>1.6	>1.6	>1.6
Fluconazole	0.008	0.032	0.128	>0.128	>0.128

*Antifungal assay was carried out in 96 well plates as described under Materials and Methods

4. DISCUSSION

The chitosan with high degree of deacetylation and low molecular weight can be isolated from cell walls of zygomycetous fungi [25, 26]. One of the main challenges was the production of biomass sufficient enough to make the process cost-effective.

The simple yeast extract-peptone and glucose medium was used for the biomass production of *A. coerulea* [1]. The yield reported was 6.2 g/L (dry wt.) while Nwe *et al*. [14] obtained 5.63 g/L dry wt. of biomass of *G. butleri* with 10% sweet potato extract medium containing minerals and urea as a nitrogen source. In case of *B. poitrasii* in optimized medium, the yield was 10.00 ± 0.50 g/L (dry wt.), which was more than other zygomycetous sources reported so far (Table 1).

Among different zygomycetes screened, Tan

et al [27] reported that *Cunnighamella echinulata* showed high chitosan contents (7.14%) of dry wt. of biomass as compared to other zygomycetes such as *Rhizopus* species, *Mucor hiemalis, A. glauca, G. butleri* and others (2- 6%). While Nwe *et al.* [14] obtained 11% chitosan from *G. butleri* biomass. In case of *B. poitrasii* the extractable chitosan was 5.1- 6.5% (Table 1). With *B. poitrasii* CDA treatment it was increased to 7.8% (Table 3). On equal activity basis, the *M. anisopliae* CDA treatment increased to only 5.9% (Table 3). Nahar *et al* [19] reported that different isozymes of CDA having different roles, such as growth, interaction with insect host and self defense from insect chitinase, were produced extracellularly in *M. anisopliae*. The isozyme which is involved in self defense, i.e. modification of cell wall from chitin to chitosan, might be important to increase chitosan contents after CDA treatment.

Different extraction procedures were designed to obtain more chitosan in different organisms. In the present investigations the treatment of *B. poitrasii* biomass with 1N NaOH at 121 °C for 15 min and further with 2% acetic acid (0.3M) at 95 °C for 24 h was found to be effective. Kolodziejska et al.[28] observed that deacetylation level of chitin from marine source increased after two stage, *viz.* chemical followed by *M.rouxii* CDA treatment. Similarly, in the present study chitosan yield was increased in 2 stage process, *i.e.* 1N NaOH treatment followed by *M. anisopliae* CDA treatment by 1.39 fold as measured by acetate release (Table 3). The *B. poitrasii* CDA treatment increased the release of acetate (1.53 fold) from dry mycelial biomass as compared to the 1N NaOH treatment (Table 3).

Rane and Hoover [29] reported higher chitosan contents with high deacetylation in *A. coerulea* when grown in a medium containing high glucose and protein contents supplemented with minerals as compared to minimal medium, in absence of minerals. Rice and corn as carbon sources supported growth of *R. oryzae* with 601 mg/L chitosan yield [30]. While Amorim *et al* [31] isolated 580 mg/L chitosan from *Cunnighamella bertholletiae* using sugar cane juice. Yokoi *et al*. [32] observed that sweet potato medium was better for *G. butleri* than buckwheat (*Fagopyrum esculentum*) medium. The chitosan yield was 730 mg/L. While Maw *et al.* [33] obtained 470 mg/L chitosan with another strain of *G. butleri.* From Table 1 and 3 it can be suggested that in case of *B. poitrasii* maximum possible yield of chitosan could be 780 mg/L.

Most of zygomycetous fungi are reported to have chitosan with 80-90% DDA. The % DDA of *B. poitrasii* chitosan extracted from mycelial cells grown in different media was in the range of 94-95% (Table 2).

Generally low molecular weight chitosans were observed to have high antifungal activity [34]. Kulikov *et al* [35] demonstrated that chito-oligomers with molecular weights ranging from 2.09-19.99 KDa had high antifungal activity against several *Candida* strains. *B. poitrasii* chitosan exhibited low molecular weight (42.82 KDa). It showed MIC_{90} in the range 0.025- 0.8 mg/mL against human pathogenic yeasts such as *C. albicans, C. neoformans, C. glabrata* and filamentous *A. niger* (Table 4).

Young-Ju *et al* [36] reported that *Mortierella* species produced highly active chitin deacetylase which could deacetylate chito-

oligomers too. The metabolic engineering in the fermenter for the production of mycelia with more % of chitosan in the cell wall and also an enhanced intracellular CDA activity in *B. poitrasii* could be the next step for the production of highly deacetylated chitosan.

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Isolation of low molecular weight chitosan from agriculturally important ascomycetous fungi *Metarhizium anisopliae* and *Myrothecium verrucaria*

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ABSTRACT

One of the major concerns for the use of entomopathogenic fungi as mycoinsecticide is the production cost, as it is a high volume low cost product. The mycelial biomass after removal of conidia can be used as a raw material for the isolation of chitosan, as a value addition and effective cost reduction. In this study, entomopathogenic Metarhizium anisopliae strains and Myrothecium verrucaria another ascomycetous fungus which produces extracellularly cuticle degrading and mycolytic enzyme complex, were used for low molecular weight chitosan isolation. From 10 g of dried biomass (i) Metarhizium anisopliae MTCC5190 124.4 ± 2.98 mg of chitosan of 0.77 KDa molecular weight and 87.80% degree of deacetylation (DDA measured by ^{1}H NMR) was extracted; (ii) of an extracellular chitin deacetylase (CDA) producer M. anisopliae strain MCC1197, yielded 180.8 ± 4.79 mg of 1.36 KDa molecular weight and 82.05% DDA; (iii) after the separation of crude enzyme complex Myrothecium verrucaria MTCC5191 the 193.5 ± 2.98 mg of chitosan of 2.38 KDa molecular weight and 86.34% DDA was isolated.

Key Words: *Agriculture, Chitosan, Degree of deacetylation, FTIR, ^{1}H-NMR, Molecular weight*

INTRODUCTION

Chitin is a β-1, 4 -linked polymer composed of *N*-acetyl-D-glucosamine (GlcNAc) repeated units, commonly found in the exoskeleton or cuticles of many invertebrates and in the cell wall of the fungi[1]. Chitosan, a deacetylated form of chitin, is primarily β- 1, 4- 2-amino-2-deoxy-D-glucose (or D-glucosamine) polymer. Chitosan has wide range of application in many areas, from agriculture to healthcare[2].

The fungi, a second largest group on earth contains chitin and chitosan in varying concentrations, *viz.* 2% in *Saccharomyces cerevisiae* to about 60% in the walls of *Allomyces macrogynus* and *Sclerotium rolfsii*[3]. Generally the zygomycetous fungal genera such as *Absidia, Benjaminiella, Gongrenella, Mucor* and *Rhizopus* contain high proportion of chitosan[4-6]. As compared to marine sources, for the production of chitosan, fungi are better in terms of constant supply, without contamination of heavy metals, and have extractable low molecular weight highly deacetylated chitosan[5]. The fungal sources useful for chitosan production can be divided in to three groups, *viz.* (i) waste fungal biomass from Myco-tech industries[7] (ii) fermentation of zygomycetous genera for biomass production[6, 8] and (iii) isolation of chitosan as value addition to existing mycotech-products[9]. In view of harmful effects of chemical pesticides and fungicides, number of fungal organisms such as *Beauveria, Metarhizium, Verticillium* and *Trichoderma* are used for the control of insect pests and pathogens[10]. In view of the parameters such as storage stability, shelf life in the and off the field, time required to control insect pests and pathogens, chemical pesticides have edge over mycopesticides. These factors add to the cost for mycoinsectcide use in the field. Earlier, to reduce the cost of production of mycopesticides and for value addition different potential applications such as plant growth promoting activity, bioremediation of pesticide residues, use of enzymes for biotransformation were reported[5, 11-13].

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Metarhizium anisopliae conidia can be used successfully to control different insect pests in agriculture fields[14]. While *M. ansiopliae* strain which produces chitin deacetylase extracellularly is useful singly or in combination in field to control insect pests[15]. Similarly, cuticle degrading and mycolytic enzyme (CDE/ME) complex of *M. verrucaria* was reported to be effective to control pests and pathogens in grapes [10, 16]. In the present investigations for the first time the use of biomass after removal of conidia and extracellular enzymes from these agriculturally important strains for the isolation of low molecular weight chitosan is reported.

EXPERIMENTAL

Microorganisms

Metarhizium anisopliae MCC1197 and *Metarhizium anisopliae* MTCC5190 were maintained on 1% YPG (0.3% yeast extract, 0.5% mycological peptone, and 1% D-glucose, and pH 6.5) agar slants. *Myrothecium verrucaria* MTCC 5191 was maintained on natural potato dextrose (20% potato and 2% D-glucose, pH 6.5) agar (PDA) slants. All the slants were stored at 4-8°C. The plant pathogens, *Xanthomonas campestris* NCIM 5028 and *Fusarium oxysporum* NCIM 1043 were maintained on nutrient agar and on PDA, respectively.

Solid state fermentation for conidia production by M. anisopliae

The mycelial inoculums of *M. anisopliae* MTCC5190 was developed in YPG medium for 48 h. Further the production of conidia by solid state fermentation using rice grains as a substrate in Unicorn bags (autoclavable, type/14 with single microvented filter of 0.2 µm, 2 kg capacity, 64 x 36 cm, Unicorn Imp & Mfg Corp, USA) at 28° C and 70-80% RH for 14 days was carried out as described earlier[17]. After harvesting of conidia using liquid extraction (0.1% Tween 80) 14 days, the solid substrate along with mycelial biomass was suspended in distilled water and centrifuged at 10000 x g for 10 min. The supernatant containing mycelial fragments were separated by decantation, washed 3 times with distilled water and centrifuged 1000 x g for 10 min.

Submerged fermentation for the production of extracellular enzymes by M. anisopliae and M. verrucaria

The submerged fermentation of *M. anisopliae* MCC1197 was carried out in 1% YPG medium for extracellular, constitutive, chitin deacetylase(s) (CDA) for 72 h[15]. *M. anisopliae* MCC1197 biomass was separated from the crude CDA by centrifugation at 10000 x g for 10 min and washed repeatedly before its use. The production of cuticle degrading and mycolytic enzyme (CDE and ME) mixture by *M. verrucaria* MTCC5191 was carried out in submerged fermentation using chitin as a sole carbon source for 7 days as described earlier [16]. After separation of CDE/ME enzyme mixture from *M. verrucaria* biomass and unutilized chitin by centrifugation at 10000 x g for 10 min. The biomass was separated from the unutilized chitin at 1000 x g for 10 min, after repeated washing with distilled water.

Isolation of cell wall

All preparations were conducted between 0-4°C, unless otherwise mentioned. The cells were washed and suspended in cold distilled water and broken by mechanical shaking with glass beads (0.45-0.5mm) in a Braun homogenizer for 90s (3 cycles of 30s each). The homogenate was made free from glass beads by decantation. The cell walls were sedimented by centrifugation at 10000 x g for 10 min and the pellet was washed 5-6 times with cold distilled water until the supernatant became clear and then twice with decreasing concentration of cold sodium chloride solution (5.0%, 2.0% and 1.0%, w/v) followed (10-12 times) by cold distilled water. The cell wall preparations were clean and free from cytoplasmic material as determined by light microscopy that showed absence of cytoplasmic material and ultraviolet absorbing material[18]. The purified cell wall samples were lyophilized and stored at -20°C.

Chitosan extraction

Chitin and chitosan were extracted from the isolated cell wall according to Mane *et al*[6]. The cell wall pellet was re-suspended in 1N NaOH (1:40 w/v) followed by autoclaving at 121°C for 15 min. The alkali insoluble fraction (AIF) was separated by centrifugation at 8000 x g for 15 min and neutralized by washing with cold distilled water to pH 7.0. The pellet obtained was treated with 2% acetic acid for 30 min at 95°C, followed by centrifugation at 8000 x g and supernatant was collected. Chitosan was precipitated from the supernatant using 1N NaOH. A white precipitate of chitosan obtained at pH 8.5-10, was washed with acetone, and dried at 60°C to a constant weight.

Biophysical characterization of chitosan
Fourier transform infrared (FT-IR) spectroscopy

Bruker Optics ALPHA-E spectrometer with a universal Zn-Se ATR (attenuated total reflection) accessory was used to take the Fourier transform infrared (FT-IR) spectra of chitosan in the 400-4000 cm^{-1} region [19]. The sample dried at 105°C for 1 h and then powdered was mixed with KBr with appropriate control. The degree of deacetylation was determined by means of the absorbance ratio A_{1320}/A_{1420}. The following formula was used for the % Degree of acetylation (DA) calculation = 31.198 (A_{1320}/A_{1420})-12.20. The % DDA (degree of deacetylation) was calculated using formula: % DDA= 100- % DA.

^1H -Nuclear magnetic resonance *spectroscopy (^1H-NMR)*

The samples were prepared by dissolving 10 mg of chitosan in a solution containing 1.96 ml of D_2O and 0.04 ml of concentrated HCl with continuous stirring for 30 min. Samples were lyophilized and dissolved in 1 ml of D_2O. The 600 µl of sample was used for the NMR spectral analysis [20]. All the NMR spectra were taken on Bruker AV400 NMR spectrometer. The experiments were carried out at 70°C at which the solvent (D_2O) peak does not interfere with any of chitosan's peaks. Degree of deacetylation (DDA) was calculated using formula 1 and 2 integrals of proton ^1H of deacetylated monomer (H1-D) and the peak of the three protons of acetyl group (H-Ac).

$$\% \text{ DDA} = \text{H1D/H1D+H1A} * 100 \ldots\ldots (1)$$

$$\% \text{ DDA} = \text{H1D/H1D+HAc/3} * 100 \ldots (2)$$

The signal from protons H2, H3, H4, H5, H6, H6'of both monomers and the peak of acetyl group (H-Ac) was also used for DDA calculation.

$$\% \text{ DDA} = [1 - (1/3 \text{ HAc} / 1/2 \text{ H 2-6})] \times 100 \ldots\ldots (3)$$

Viscosity measurement

Viscosity measurements were carried out using Ubbelohde-type viscometer at 25°C. Chitosan (0.1%) samples were prepared in 0.5 Macetic acid and 0.5 M sodium acetate. The flow time was determined in seconds. The average molecular weight was calculated using the intrinsic viscosity method curves for ηsp /concentration versus concentration (where by η sp – specific viscosity) were plotted and extrapolated in order to obtain the intrinsic viscosity [η], ($[\eta] = [\eta_{sp}/c]$ $(c \rightarrow 0)$.

The average molecular weight was then calculated using the Mark- Houwink equation:

$[\eta] = KM^{\alpha}$, where K and α for HAc/NaAc is $119 \times 10^5$and 0.5, respectively [21]

Antimicrobial assay

Chitosans extracted fromdifferent sources were solubilized in 2% acetic acid solution to make a stock of 10 mg/ml. The solutions were kept for stirring overnight for dissolution of chitosan. Finally, the pH was adjusted to 5 using 1 N NaOH.

Antibacterial activity of the fungal chitosan was checked against *X. campestris*NCIM 5028 and antifungal activity was checked against *F. oxysporum*NCIM 1043 byClinical Laboratory Standards Institute's (CLSI) broth micro-dilution assay (CLSI documentM27-A3 and CLSI M38-A2) as described earlier [22]. The stock was diluted in RPMI 1640medium (32 µl in 200 µl) and added to the first row of a 96-well microtitre plate. Thechitosan samples were serially diluted using RPMI 1640medium in successive wells to get final concentration in therange of 0.0125-1.6 mg/ml. *X. campestris*was grown for 24 h in Luria Bertani (LB) broth. The OD of the culture was adjusted to 0.12-0.14 using RPMI and inoculated (100 µl) in the wells of themicrotitre plate. The microtitre plates wereincubated for 24h. *F. oxysporum* spores ($2x10^4$/ml) were suspended in the medium and inoculated (100 µl) in the wells of themicrotitre plate. The microtitre plates wereincubated for 48 h.

Acetic acid (2%) was used as a control. In both the cases, after incubation theabsorbance was measured at 600 nm by using microtitre plate reader (Epoch, BiotechInstruments) to assess cell growth. The MIC was defined as the lowest concentrationexhibiting >90% inhibition of growth as compared to the control.

RESULTS AND DISCUSSION

Fungi such as *Penicillium* from antibiotic industry [23] and citric acid producing *Aspergillus*[7] produce huge amount of waste mycelial biomass which can be used for the isolation of chitin and chitosan. Number of fungi used in mycotechnology are being studied for value addition to their existing products [5]. For example, Shiitake mushroom, *Lentinusedodes*, produces a polysaccharide, lentinan, an immune-stimulant in the body. After its extraction the fungal biomass was reported to be used

for the production of chitosan [24]. Using lignocellulosic substrates, *Mucorindicus* can produce ethanol and high amounts of polyunsaturated fatty acids such as γ-linolenic acid. The fungus was reported to produce 62-67% alkali insoluble material *i.e.* chitin and chitosan in hyphal form cell walls [9, 25] further reported use of citrus fruit waste to grow *M. indicus* and *Rhizopusoryzae* biomass for the production of chitosan. They reported that biomass contained approx. 0.6-0.7% chitin and chitosan per g of cell wall. Varelas*et al.* reported use of yeast lees for β-glucan production as value addition and to reduce the negative costs for wineries [26]. *Hanseniasporaguilliermondii, Issatchenkiaorientalis, Pichiamembranifaciens*and *Saccharomyces cerevisiae* are the reported main genera of yeast lees[27]. It was suggested that the yeast lees can also be used to isolate chitosan which has DDA >75% [5]. In view of the potential of use of agriculturally important fungi in the field, the quantities of mycelial biomass produced are high. The details of chitosan isolation and characterization from *M. anisopliae* strains and from *M. verrucaria* are described in subsequent sections.

Isolation of fungal biomass

The mycelial biomass of three different fungal strains was used for chitosan isolation. (i) After solid state fermentation (SSF) of *M. anisopliae*MTCC5190 for 14 days, and separation of conidia (60 g/kgrice grains) using liquid extraction method, mycelial biomass (27.11 ± 3.0 g/kgrice grains) was separated from the rice grains after repeated washings with distilled water. (ii) The mycelial biomass of *M. anisopliae*MCC1197, CDA producer (29.07± 2.42 g/l) from YPG medium was separated after 72 h and washed with distilled water.(iii)*M. verrucaria* MTCC5191 mycelial biomass (28.85 ± 2.5g/l) was separated from extracellular enzyme mixture and unutilized chitin after 7 days. In view of the requirement of either conidia or enzymes in the fields to control different pests and pathogens, the amount of mycelial biomass generated will be high. It was reported that for effective control of *Helicoverpaarmigera* in 4.2 ha field of pulses, 1.5 kg *M. anisopliae* conidia were used [14]. After harvesting conidia, 680 g mycelial biomass waste could be generated. Considering the area under pulses in India (25.23 million ha) this can be a better source for chitosan isolation. Similarly, total enzyme requirement for the control of pests and pathogens in grapes can generate almost 200 tons of mycelial biomass every year which can be used to reduce the cost of production [16].

Table 1 Amount of chitosan, degree of deacetylation and molecular weight of chitosan isolated from *Metarhiziumanisopliae* and *Myrotheciumverrucaria*

Organism	Fermentation	Chitosan (mg/10gof dry biomass)	Degree of deacetylation (%) (FT-IR)	Degree of deacetylation (%) (^{1}H-NMR)	Molecular weight (KDa)
M. anisopliae MTCC5190	SSF	124.4 ± 2.98	84.68	87.80	0.77
*M. anisopliae*MCC1197	SmF	180.8 ± 4.79	84.70	82.05	1.36
M. verrucaria MTCC 5191	SmF	193.5 ± 2.98	81.68	86.34	2.38
Marine chitosan (Commercial)	-	-	81.16	85.38	464.83

Chitosan extraction from fungal biomass

The yield of chitosan from *M. anisopliae* MTCC5190 mycelial biomass was 124.4 ± 2.98 mg/10 g dry wt. (Table 1). In other words, per kg of substrate While CDA producer, *M. anisopliae* MCC1197 yielded 180.8 ± 4.79 mg/10 g dry biomass in 72 h. From *M. verrucaria* MTCC 5191 the yield of chitosan was 193.5 ± 2.98mg/10 g of dry biomass obtained after 7 days (Table 1). In case of *L. edodes* wheat straw was used as a substrate for SSF and 6.8 g chitosan per kgof the substrate was extracted from the mycelial biomass[24]. Pochanavanich and Suntornsuk reported chitosan extraction from different fungi such as *Aspergillusniger, L. edodes, Pleurotussajo-caju, Zygosaccharomycesrouxii* and *Candida albicans*[28]. The reported yield of chitosan was in the range of 10–140 mg/g cell dry weight.

Degree of deacetylation

Pochanavanich and Suntornsuk measured degree of deacetylation by titration with NaOH which hydrolyzed the acetyl groups in chitosan which were measured. The % DDA for isolated fungal chitosans was in the range 83-90%[28]. Interestingly, for crab shell chitosan % DDA

was reported to be 97% which was high. While Mane *et al.* reported 80-86 % DDA of crab shell chitosan measured using FTIR and ^{1}H NMR[6]. Using FTIR, the DDA of the chitosans from *Metarhizium* and *Myrothecium* strains were found to be in the range 81-85% (Table 1; Fig.1).The chitosan isolated from *Metarhizium*MTCC5190 grown in SSF was 84.68% (Table 1; Fig.1).

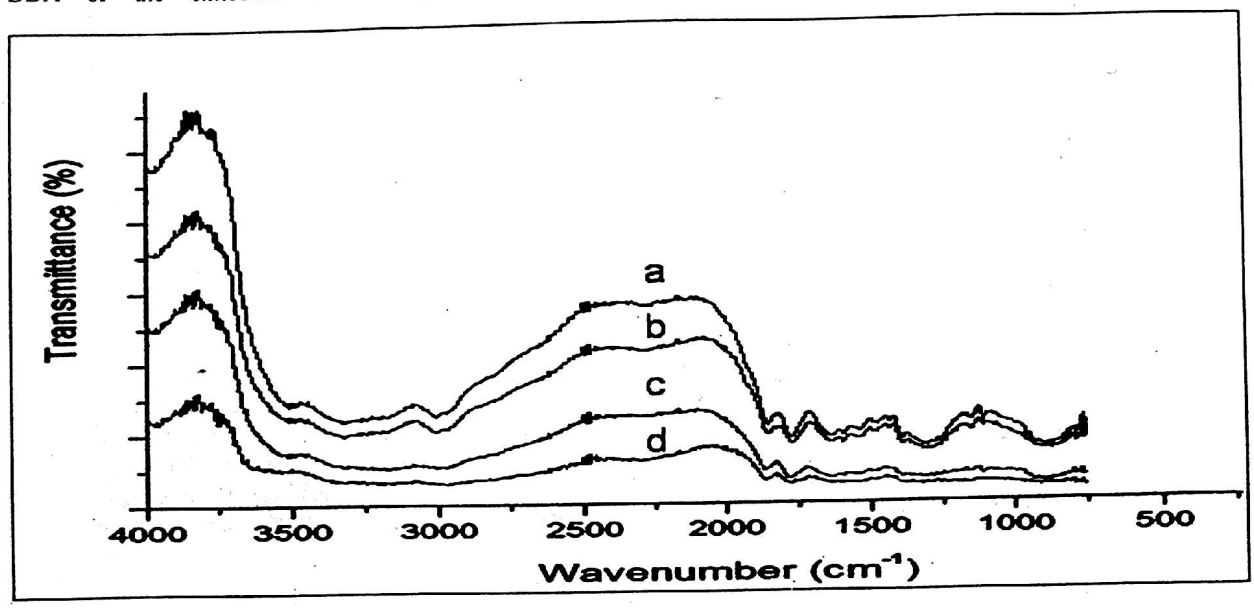

Fig. 1.FTIR profiles of chitosan from different sources. a, chitosan isolated from *M. anisopliae*MTCC5190; b, chitosan isolated from *M. verrucaria* MTCC5191; c, chitosan isolated from *M. anisopliae*MCC1197 d, commercial chitosan from marine source.

In case of hyphal form of *M. verrucaria*MTCC5191 the DDA was 81.68% (Table 1).With ^{1}H NMR the DDA of the chitosans from *Metarhzium* and *Myrothecium* strains were in the range 82-88% (Table 1; Fig.2). According to Lago*et al.* the characterization of chitosan using FTIR method is more qualitative than ^{1}H NMR analysis of degree of deacetylation[29]. In the present investigations, however, the values for % DDA were slightly higher with ^{1}H NMR. The chitosan isolated from the mycelial biomass of *Metarhizium* MTCC5190 harvested after SSF showed unusually higher (87.8%) DDA which can be attributed to the interference of other polysaccharides from the substrates in ^{1}H NMR measurements.

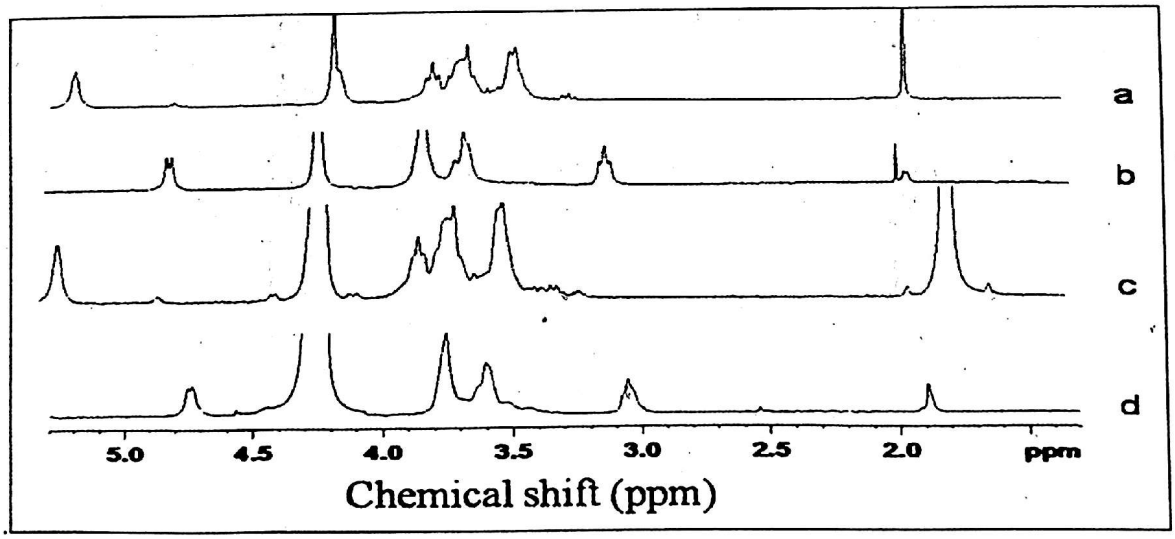

Fig.2. ^{1}H-NMR profiles of chitosan from different sources.a, chitosan isolated from *M. anisopliae*MTCC5190; b, chitosan isolated from *M. verrucaria* MTCC5191; c, chitosan isolated from *M. anisopliae*MCC1197 d, commercial chitosan from marine source.

Molecular weight

In the present investigations, chitosans isolated had low molecular weights (0.77- 2.38 KDa) (Table 1). Chitosan extracted from *A. niger* waste biomass from citric acid industry was very high, 267.97 KDa[7]. Chitosans extracted from zygomycetous fungus *Gongronellabutleri*had molecular weight in the range 30-100 KDa[30]. It was reported further that the chitosan produced from mycelial biomass developed in SSF had lower mol wt. (30 KDa) as compared to the chitosan produced from biomass developed in SmF (100 KDa). It was in accordance with our investigations for *M. anisopliae* grown in SSF and SmF. Chatterjee*et al.* reported use of *Mucorrouxii*mycelial biomass grown in different media for chitosan isolation. The molecular weights were in the range from 24-50 KDa[31]. The molecular weight of chitosan isolated from *Benjaminiellapoitrasii*was 42.82 KDa[6] while that from *A. coruleaw*as 500 KDa[8].

Antimicrobial activity

Usually low molecular weight chitosans have applications in human health care[6]. Kulikov*et al.* reported that chito-oligomers with molecular weights ranging from 2.09-19.99 KDa had high antifungal activity against several *Candida* strains [32]. While Mane *et al.* demonstrated that *B. poitrasii* chitosan with 42.82 KDa molecular weight had MIC_{90} in the range 0.025-0.8 mg/ml against human pathogens such as *A. niger*, *Candida albicans*, *Candida glabrata*, and *Cryptococcus neoformans*[6]. While chitosan from marine source with a DDA 79.6% and of low molecular weight (70 KDa) was found to be effective against plant pathogenic fungi such as *Fusariumoxysporum* and others [33]. In view of this, potential of the chitosans isolated from *Metarhizium* and *Myrothecium* strains was tested for antibacterial and antifungal activities against 2 plant pathogens (Table 2). As compared to chitosan isolated from marine source (MIC_{90} 0.8 mg/ml), the fungal chitosans showed lesser MIC_{90} against *F. oxysporum* (0.05-0.2 mg/ml). Against *X. campestris*, MIC_{90} for chitosans were in the range0.025-0.4 mg/ml.

Table 2 Antimicrobial activity of chitosan isolated from *Metarhiztumanisopliae* and *Myrotheciumverrucaria*. OD_{600} is given in parenthesis

Minimum Inhibitory Concentration (MIC_{90}) mg/ml		
	Xanthomonascampestris NCIM 5028	*Fusariumoxysporum* NCIM 1043
Control (2% acetic acid)	- (1.251)	- (1.117)
M. anisopliae MTCC1197	0.05 (0.133)	0.1(0.113)
M. anisopliae MTCC5190	0.4 (0.113)	0.2 (0.109)
M. verrucarria MTCC5191	0.025 (0.134)	0.05 (0.101)
Commercial chitosan	0.4 (0.112)	0.8 (0.119)

Further work is necessary to explore the possibility of use of these low molecular weight biopolymericchitosans for other health care applications such as drug delivery and generation of scaffolds for tissue regeneration. Low molecular weight chitosan displays less viscosity and is more amenable towards modifications for increasing water solubility. Such biopolymers hold promise for drug delivery as they can provide electrostatic interactions with cells and a stealth effect [34]. Fungal origin chitosan, with improved physico-chemical properties as compared to crustacean chitosan, improved the mechanical properties of scaffolds that displayed uniform porosity and water retention [35]. Therefore, fungal chitosan holds immense potential for applications in human health care.

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Article

Isolation and Characterization of Chitosans from Different Fungi with Special Emphasis on Zygomycetous Dimorphic Fungus *Benjaminiella poitrasii*: Evaluation of Its Chitosan Nanoparticles for the Inhibition of Human Pathogenic Fungi

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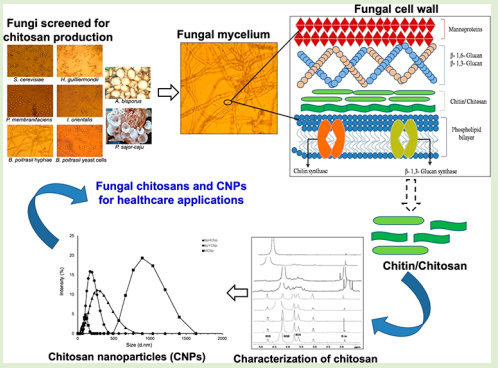
ABSTRACT: The cell wall chitosan was extracted from fungi belonging to different taxonomic classes, namely, *Benjaminiella poitrasii* (Zygomycetes, dimorphic), *Hanseniaspora guilliermondii*, *Issatchenkia orientalis*, *Pichia membranifaciens*, and *Saccharomyces cerevisiae* (Ascomycetes, yeasts), and *Agaricus bisporus* and *Pleurotus sajor-caju* (Basidiomycetes). The maximum yield of chitosan was 60.89 ± 2.30 mg/g of dry mycelial biomass of *B. poitrasii*. The degree of deacetylation (DDA) of chitosan extracted from different fungi, as observed with ^{1}H NMR, was in the range of 70−93%. *B. poitrasii* chitosan exhibited the highest DDA (92.78%). The characteristic absorption bands were observed at 3450, 1650, 1420, 1320, and 1035 cm^{-1} by FTIR. Compared to chitosan from marine sources (molecular weight, MW, 585 kDa), fungal chitosans showed lower MW (6.21−46.33 kDa). Further, to improve the efficacy of *B. poitrasii* chitosan (Bp), nanoparticles (Np) were synthesized using the ionic gelation method and characterized by dynamic light scattering (DLS). For yeast and hyphal chitosan nanoparticles (BpYCNp and BpHCNp), the average particle size was <200 nm with polydispersity index of 0.341 ± 0.03 and 0.388 ± 0.002, respectively, and the zeta potential values were 21.64 ± 0.34 and 24.48 ± 1.58 mV, respectively. The *B. poitrasii* chitosans and their nanoparticles were further evaluated for antifungal activity against human pathogenic *Candida albicans* ATCC 10231, *Candida glabrata* NCYC 388, *Candida tropicalis* ATCC 750, *Cryptococcus neoformans* ATCC 34664, and *Aspergillus niger* ATCC 10578. BpHCNps showed lower MIC$_{90}$ values (0.025−0.4 mg/mL) than the chitosan polymer against the tested human pathogens. The study suggested that nanoformulation of fungal chitosan, which has low molecular weight and high % DDA, is desirable for antifungal applications against human pathogens. Moreover, chitosans as well as their nanoparticles were found to be hemocompatible and are therefore safe for healthcare applications.



INTRODUCTION

Chitosan is known to be a natural glucosamine polymer [β-(1→4)-linked] synthesized by the deacetylation of chitin [β-(1→4)-linked *N*-acetylglucosamine] polymer. Landings of marine organisms such as crabs, lobsters, and shrimp are the primary commercial source of chitin and chitosan. Fungi by definition are organisms that contain chitin as a main structural component in their cell walls.[1] The possible fungal sources for chitin and chitosan extraction include waste fungal biomass generated from biotech industries and fermentation of zygomycetous fungi.[1] The use of fungi as a source of chitosan is advantageous due to its homogeneous, highly deacetylated nature and also availability around the year.

Indeed, thousands of tons of waste fungal biomass are produced from Mycotech industries every year and are subjected to land filling or incineration for easy disposal. Varelas et al. identified utilization of wine lees, primarily spent yeasts, to produce β-glucan, which has a wide range of applications in the food sector.[2] The same can also be used to extract chitosan. The main yeast genera of lees include *Hanseniaspora guilliermondii*, *Issatchenkia orientalis*, *Pichia membranifaciens*, and *Saccharomyces cerevisiae*.[3] As yeast lees are available in huge quantities, this can also be a potential source for chitosan isolation.

As a potential source of chitosan, zygomycetous fungi, such as *Absidia coerulea*,[4] *Benjaminiella poitrasii*,[5] *Cunninghamella elegans*,[6] *Gongronella butleri*,[7] *Mucor rouxii*,[8] *Mucor indicus*,[9] and

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Rhizopus oryzae,[10] have been studied. Chitosan extraction has also been reported from other fungi from different classes such as Ascomycetes (*Metarhizium, Myrothecium*)[11] and Basidiomycetes (*Agaricus bisporus, Lentinus edodes, Pleurotus sajor-caju*) to name a few.[12,13]

Chitosan has several applications in cosmetics, pharmaceuticals (such as, lowering of serum cholesterol), drug delivery, agriculture, and food additives.[14] Chitosan shows antifungal activity due to its positive charge, which interacts with negatively charged phospholipids of the fungal cell membrane.[15] The molecular weight and higher degree of deacetylation (DDA) contribute significantly to the antifungal activity. Ing et al.[15] reported that chitosan nanoparticles showed higher antifungal activity against *Candida albicans* and *Fusarium solani*. This inhibitory effect was attributed to the particle size and zeta potential.

In this work, we have screened fungal organisms that fall into either of the sources mentioned above, waste fungal biomass from wine and mushroom industries and fermentation of zygomycetous fungus *B. poitrasii.* Along with chitosan production and characterization, synthesis of fungal chitosan nanoparticles for the determination of their antifungal potential against human pathogenic fungi was also carried out.

EXPERIMENTAL SECTION

Fungal Strains and Growth Conditions. The fungal cultures used for chitosan production, *B. poitrasii* (NCIM 1240), *H. guilliermondii* (FJ231450), *I. orientalis* (FJ231418), *P. membranifaciens* (FJ231459), and *S. cerevisiae* (FJ231433) were maintained on 1% YPG agar slants (0.3% yeast extract, 0.5% mycological peptone, and 1% D-glucose, pH 6.5). The yeast cultures were identified by Chavan et al.[3] The mushroom *Pleurotus sajor-caju* DMRP112 was procured from ICAR-Directorate of Mushroom Research, Himachal Pradesh, Solan, and maintained on 2% potato dextrose agar (2% PDA). *Agaricus bisporus* (1 kg) was directly procured from a local market.

The human pathogens *Candida albicans* ATCC 10231, *Candida glabrata* NCYC 388, *Candida tropicalis* ATCC 750, and *Cryptococcus neoformans* ATCC 34664 were maintained on 1% YPG, while the filamentous fungus *Aspergillus niger* ATCC 10578 was maintained on 2% PDA. All the slants were stored at 4 °C.

Submerged Fermentation of Yeasts. The yeast cultures were grown in 1000 mL Erlenmeyer flasks containing 200 mL of 1% YPG medium, and the flasks were incubated at 28 °C at 180 rpm, for 48 h. Inoculum (24 h old) of *H. guilliermondii*, *I. orientalis*, *P. membranifaciens*, and *S. cerevisiae* was prepared in 1% YPG medium, and the final concentration of 10^7 cells/100 mL was adjusted based on hemocytometer counting under light microscope. The yeast biomass was obtained by centrifugation at 8000*g* for 20 min and dried at 50 °C until further use.

Biomass Production of Zygomycetous Fungus *B. poitrasii*. The media components and other incubation parameters to produce *B. poitrasii* biomass were applied as mentioned earlier.[5] For production of exclusively yeast biomass of *B. poitrasii*, spores (1.6×10^7 sporangiospores/100 mL) were inoculated into 1% YPG medium and incubated at 37 °C for 48 h. After 48 h, the yeast biomass was harvested as mentioned in above section, while the mycelial biomass was harvested by filtration.[5] The biomass was further dried at constant temperature in a hot air oven at 50 °C.

Cultivation of *P. sajor-caju*. The cultivation of *P. sajor-caju* was carried out using wheat straw as a substrate. The spawn culture was developed using wheat. The overnight soaking of wheat was carried out in water, after which it was boiled for about 30 min and drained on a plain surface to remove excess water to retain 50% moisture. Calcium carbonate (20 g/kg of wheat straw) was added to the bags, which were then autoclaved at 121 °C for 30 min. The bags were inoculated with the spawn culture and incubated at 28 °C. Further the cultivation was carried out as described by Tupatkar and Jadhao.[16]

Extraction of Chitosan from Fungal Biomass. All preparations were carried out at 0−4 °C, unless otherwise mentioned. The cells were washed and suspended in cold distilled water and disrupted in a 90s Braun homogenizer using glass beads (0.45−0.5 mm) for 4−5 cycles of 30 s each through mechanical shaking. The cell wall preparations were observed by light microscopy to confirm the absence of cytoplasmic content.[17] Further, the purified cell wall samples were lyophilized and stored at −20 °C until used. Chitosan was extracted as described by Mane et al.[5] The alkali-insoluble fraction (AIF) was separated by centrifugation at 8000*g* for 15 min and neutralized by washing with cold distilled water. The pellet obtained was treated with 2% acetic acid for 24 h at 95 °C, followed by centrifugation at 8000*g*, and the supernatant was collected. Chitosan was precipitated from the supernatant using 1 N NaOH. A white precipitate of chitosan obtained at pH 8.5−10 was washed with acetone and dried at 50 °C to a constant weight.

The commercial chitosan from shrimp shells procured from Sigma-Aldrich, USA (product no. C3646), was used as reference in the present study, unless otherwise mentioned.

Biophysical Characterization of Chitosan. *^{1}H Nuclear Magnetic Resonance.* The chitosan samples were prepared using 10 mg of chitosan in a solution containing 1.96 mL of D_2O and 0.04 mL of deuterated HCl with continuous stirring for half an hour to confirm complete dissolution of the chitosan. Samples were lyophilized and dissolved in 1 mL of D_2O; 600 μL of the sample was used for the NMR spectra. All the NMR spectra were taken on a Bruker AV400 NMR spectrophotometer. The NMR spectra were obtained at 70 °C, the temperature at which the solvent (HOD) peak does not interfere with any of the chitosan peaks. The degree of deacetylation (DDA) was calculated using the following formula:[18,19]

$$\mathrm{DDA}\ (\%) = \left(\frac{\mathrm{H1D}}{\mathrm{H1D} + \mathrm{HAc}/3}\right) \times 100$$

where H1D is the peak of proton H1 of the deacetylated monomer and HAc is the peak of the three protons of the acetyl group.

Fourier Transform Infrared (FT-IR) Spectroscopy. The samples were analyzed using IRAfinity-1S (Shimadzu, USA) FT-IR spectrophotometer.

The FT-IR spectrum of each chitosan sample was recorded in the 400−4000 cm^{-1} region. The sample was dried at 105 °C for 1 h, powdered, and mixed with KBr with appropriate control. The degree of deacetylation was determined using the absorbance ratio A_{1320}/A_{1420}.[20]

Viscosity Determination. Viscosity measurements were carried out by using an Ubbelöhde-type viscometer (Fisher, Germany) at constant temperature (25 °C). The viscometer was attached to a viscoclock (Schott viscoclock) that automatically recorded the time of solution flow via the viscometer's two markings. Every value was the average of three measurements. Chitosan samples (0.1%) were prepared in 0.5 M acetic acid and 0.5 M sodium acetate. The flow time was determined in seconds.[21]

Molecular Weight Determination of Fungal Chitosan by Gel Permeation Chromatography (GPC). A chromatography system with OHpak SB-800 HQ series columns (Agilent Technologies, USA) was applied to determine the molecular weight of fungal chitosan using gel permeation. The fungal chitosan was dissolved in 2% acetic acid at a final concentration of 2 mg/mL. Further the solution was filtered through 0.22 μm filters to remove debris. The mobile phase used in this study was 0.2 M acetic acid/0.1 M sodium acetate. The filtered chitosan solution (50 μL) was run at 0.8 mL/min flow rate and 40 °C column temperature. Pullulan 20201 Shodex standards were used for the calibration of column. The molecular weight determination was carried out as described earlier by Zielinska et al. with a refractive index detector.[22]

Fungal Chitosan Nanoparticles Synthesis and Characterization. *B. poitrasii Hyphal and Yeast Chitosan Nanoparticles Synthesis. B. poitrasii* hyphal and yeast chitosan and marine chitosan solutions at concentration of 0.5 mg/mL were prepared by dissolving in 10 mL of 2% acetic acid. The synthesis of nanoparticles was carried out by ionic gelation method through the interaction with sodium

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Table 1. Extraction of Chitosan from Different Fungi and Their Biophysical Characterization

Fungal organisms	Chitosan (mg/g of dry biomass)	DDA (%) (^{1}H NMR)	DDA (%) (FTIR)	Viscosity (cP)	Molecular weight (kDa)
		Zygomycetes			
Benjaminiella poitrasii (H)	60.89 ± 2.30	92.78	88.51	0.621	46.33
Benjaminiella poitrasii (Y)	31.00 ± 0.7	90.86	87.30	0.379	25.15
		Ascomycetes (Yeasts)			
Hanseniaspora guilliermondii	21.38 ± 3.46	73.10	85.16	0.063	6.95
Issatchenkia orientalis	15.00 ± 0.91	70.32	83.52	0.013	6.21
Pichia membranifaciens	25.64 ± 1.0	89.66	86.40	0.058	6.82
Saccharomyces cerevisiae	31.00 ± 0.70	83.59	86.68	0.073	6.55
		Basidiomycetes			
Agaricus bisporus	15.92 ± 0.85	*a*	81.72	0.012	6.48
Pleurotus sajor-caju	13.52 ± 1.22	*a*	79.33	0.003	6.72
		Commercial source			
Marine chitosan		85.66	82.64	2.67	585[b]

[a]Not determined. [b]The molecular weight was determined by using viscometry data.

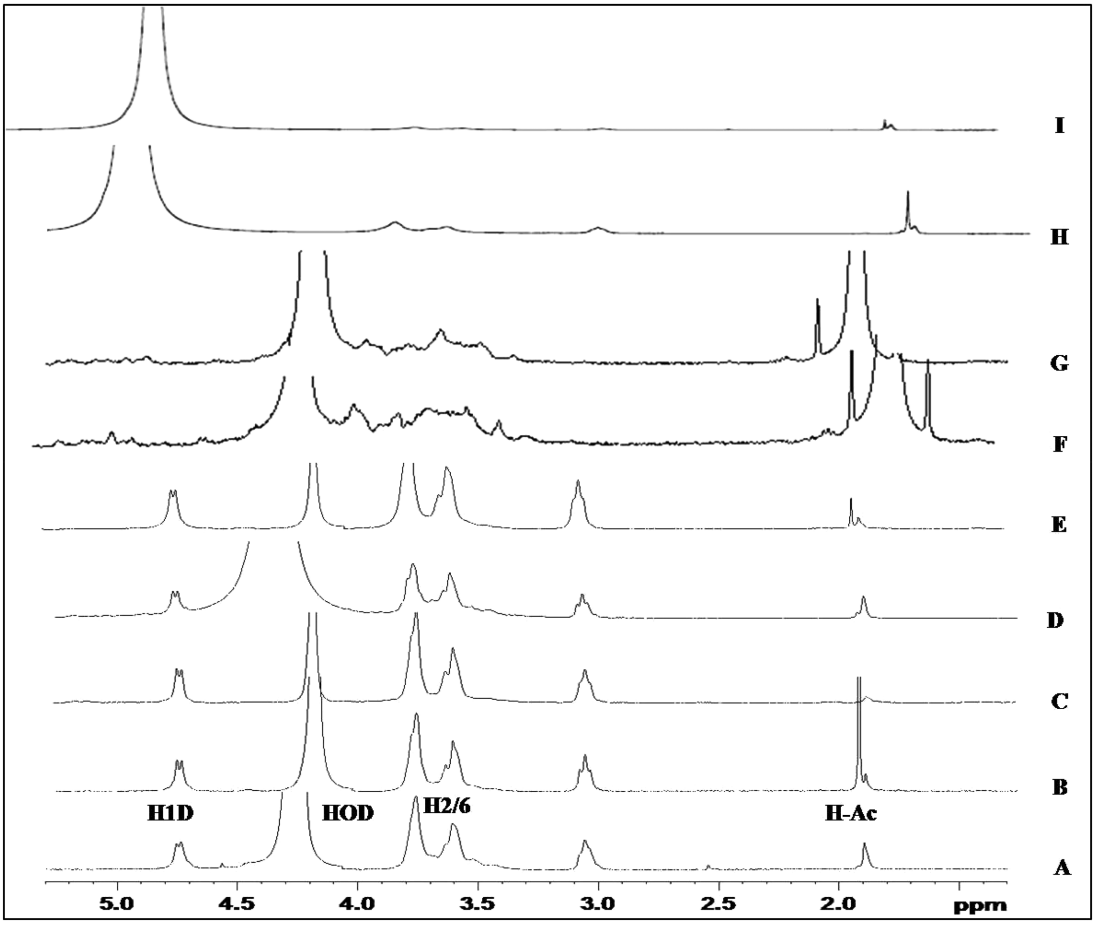

Figure 1. ^{1}H NMR profiles of chitosan isolated from fungi of different classes, Zygomycetes (B, C), Ascomycetes (D−G) and Basidiomycetes (H, I) compared with chitosan from marine source (A). Chitosan isolated from *B. poitrasii* hyphae (B), *B. poitrasii* yeast (C), *S. cerevisiae* (D), *P. membranifaciens* (E), *I. orientalis* (F), *H. guilliermondii* (G), *A. bisporus* (H), and *P. sajor-caju* (I).

tripolyphosphate (TPP). A stock of 1.6 mg/mL TPP was prepared in sterile distilled water. Under continuous magnetic stirring at 1000 rpm, 1.25 mL of TPP solution was added in dropwise manner to 10 mL of chitosan solution (1:5 molar ratio), and nanoparticles were produced by ionic gelation.[23] The particles were then kept at room temperature for 30 min. The nanoparticles were then centrifuged at 25000*g* for 30 min. The nanoparticles were redispersed in distilled water and used for characterization.[24]

Characterization of Synthesized Fungal Chitosan Nanoparticles. Mean particle size (*Z*-average), polydispersity index (PDI), and zeta potential of the *B. poitrasii* hyphal chitosan nanoparticles (BpHCNps), *B. poitrasii* yeast chitosan nanoparticles (BpYCNps), and marine chitosan nanoparticles (MCNps) were measured using Malvern Zetasizer Pro. The measurements were carried out in triplicate at 25 °C.

Antifungal Assay. Chitosan extracted from different fungi was dissolved in 2% acetic acid solution at a final concentration of 25 mg/mL. The solution was kept stirring overnight for dissolution of chitosan. NaOH (1 N) was used to adjust the final pH of the solution to 5. For the determination of antifungal activity of chitosan

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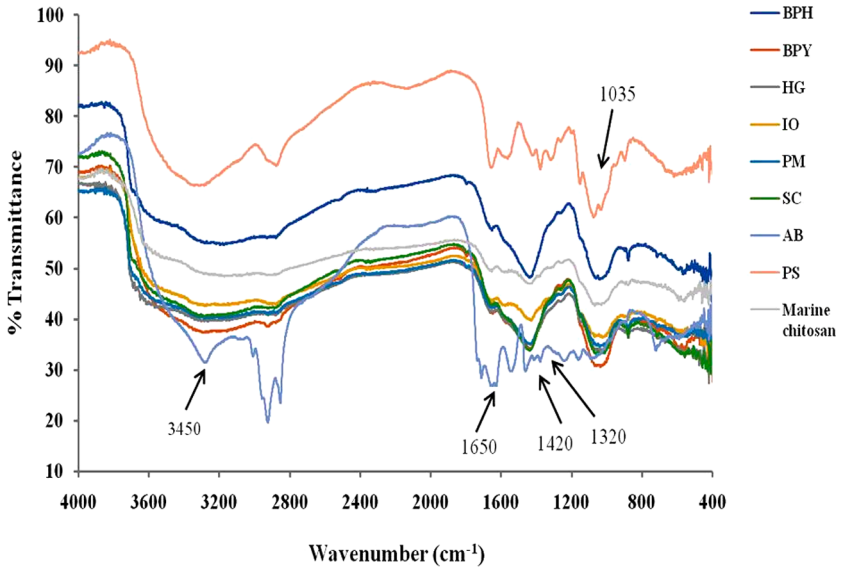

Figure 2. FTIR profiles of chitosan isolated from different fungi [*B. poitrasii* hyphae (BPH); *B. poitrasii* yeast (BPY); *H. guilliermondii* (HG); *I. orientalis* (IO); *P. membranifaciens* (PM); *S. cerevisiae* (SC); *A. bisporus* (AB); *P. sajor-caju* (PS)] and commercial chitosan (marine chitosan).

nanoparticles, particles dispersed in distilled water were used directly. The antifungal activity was checked against human pathogenic *Candida albicans* ATCC 10231, *Candida glabrata* NCYC 388, *Candida tropicalis* ATCC 750, *Cryptococcus neoformans* ATCC 34664, and *Aspergillus niger* ATCC 10578 by Clinical Laboratory Standards Institute's (CLSI) broth microdilution assay as reported earlier by Pathan et al.[25] The stock was prepared in Roswell Park Memorial Institute (RPMI) 1640 medium and added in the first row of a 96-well microtiter plate. The chitosan samples were serially diluted using RPMI 1640 medium in successive wells to get final concentrations in the range of 0.025−5.0 mg/mL. The yeast cells (~2 × 10^3 cfu/mL) were grown overnight and diluted in RPMI 1640 medium for inoculation (100 μL) into the wells of the microtiter plate. For filamentous fungi, 2 × 10^4 spores/mL were used as the inoculum. The plates were further incubated for 24−72 h. Acetic acid (2%) was used as control. After incubation, the absorbance was measured at 600 nm via a microtiter plate reader (Epoch, Biotech Instruments) to evaluate the growth. The lowest concentration exhibiting >90% inhibition of growth as compared to the control was described as the MIC_{90}.

RBC Hemolysis Assay. To study the hemocompatibility of chitosan and chitosan nanoparticles, the red blood cell (RBC) hemolysis assay was performed as described by Pathan et al.[25] Chitosan and chitosan nanoparticles were tested in the concentration range of 0.039 to 5 mg/mL. Triton X-100 (0.1% (v/v) in PBS) was used as a positive control, whereas PBS was used as a negative control. The experiment was performed in triplicate with two biological replicates, and the mean values were considered for the calculation of percent hemolytic activity of chitosan and chitosan nanoparticles.

RESULTS AND DISCUSSION

Biomass Production Using Different Class of Fungi. Under similar growth conditions, the biomass obtained from the fungi used in the study was in the range of 1.2−9.2 g/L. The ascomycetous yeasts yielded higher dry biomass (3.90−5.16 g/L) when grown in 1% YPG medium, while the zygomycetous *B. poitrasii* gave 9.17 ± 0.32 g of dry mycelial biomass and 1.78 ± 0.28 g of yeast biomass per liter of medium when grown under the modified growth conditions as described earlier.[5] It has been reviewed extensively that the available waste biomass from different industries, including wine and mushroom industries, is in huge quantities,[1] while to carry out fermentation exclusively for the extraction of chitosan from the fungal biomass is relatively expensive.[1] Nevertheless, the desired characteristics (molecular weight, DDA, and viscosity) for healthcare applications prompt researchers to go for the cultivation of fungal biomass.

Chitosan Extraction from Different Fungi. Gooday reported the common wall constituents found in each division of fungi. The chitin and chitosan contents vary from 1 to 50%.[26] In the present investigation, chitosan isolation from winery yeasts such as *H. guilliermondii*, *I. orientalis*, *P. membranifaciens*, and *S. cerevisiae* and fruit bodies of mushrooms, namely, *A. bisporus* and *P. sajor-caju* (Basidiomycetes) and from *B. poitrasii* is reported in Table 1.

The chitosan yield in ascomycetous and basidiomycetous fungi was in the range of 13−31 mg/g of dry biomass, while in case of *B. poitrasii* grown in optimized medium, the chitosan yield was 60.89 ± 2.30 mg/g of dry mycelial biomass and 31.00 ± 0.7 mg/g of dry yeast biomass (Table 1). Among the unicellular ascomycetous yeasts, *S. cerevisiae* showed highest content of chitosan (31.00 ± 0.70 mg/g of dry biomass). Earlier Ramos Berger et al.[27] isolated chitosan from Mucorales fungi, and the yield of chitosan was between 5 and 15 mg/g of dry mycelial biomass.

Biophysical Characterization of Fungal Chitosan. As reported in Table 1, the degree of deacetylation (DDA) of chitosan measured using ^{1}H NMR was in the range of 70−89% for chitosan isolated from yeast and mushrooms, while DDA measured using FTIR was in the range of 79−87%. On the other hand, DDA of chitosan isolated from *B. poitrasii* mycelial and yeast cells was >90% when measured by ^{1}H NMR (Figure 1), while by FTIR (Figure 2), it was in the range of 87−88%.

The characteristic absorption bands were observed at 3450 cm^{-1} (O−H stretching), 1650 cm^{-1} (amide II), 1420 cm^{-1} (C−H deformations), 1320 cm^{-1} (amide III, C−N stretching), and 1035 cm^{-1} (O bridge stretching) by FTIR for all chitosans. Pochanavanich and Suntornsuk, isolated chitosan from fungi of genera *Aspergillus*, *Rhizopus*, and *Lentinus*.[13] They reported % DDA measured by titration with NaOH, which hydrolyzed the acetyl groups in chitosan, in the range 86−90%. Moreover the DDA of chitosan from crab shell was 97%. In the present

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Table 2. Antifungal Activities[a] of Chitosan Isolated from Different Fungi against Human Pathogens

	Candida albicans ATCC 10231	*Candida glabrata* NCYC 388	*Candida tropicalis* ATCC 750	*Cryptococcus neoformans* ATCC 34664	*Aspergillus niger* ATCC 10578
			Zygomycetes		
B. poitrasii (H)	0.025	0.2	0.8	0.8	0.8
B. poitrasii (Y)	0.8	2.5	1.6	0.8	0.8
			Ascomycetes (Yeasts)		
H. guilliermondii	>5	>5	>5	>5	>5
I. orientalis	>5	>5	>5	>5	>5
P. membranifaciens	>5	>5	>5	>5	>5
S. cerevisiae	>5	>5	>5	>5	>5
			Basidiomycetes		
A. bisporus	>5	>5	>5	>5	>5
P. sajor-caju	>5	>5	>5	>5	>5
			Commercial Source		
Marine chitosan	5	2.5	2.5	2.5	>5
Control (2% acetic acid)	*b*	*b*	*b*	*b*	*b*

[a]MIC_{90} (minimum inhibitory concentration causing 90% inhibition), mg/mL. [b]Not detected.

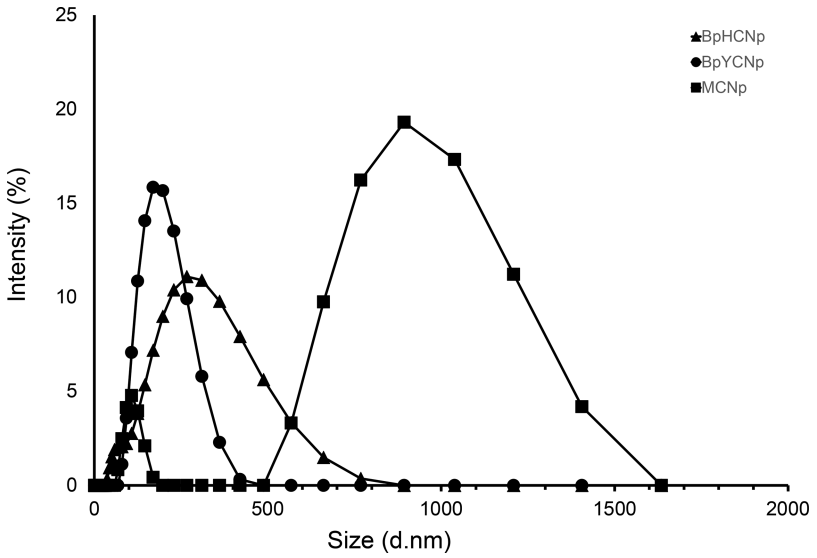

Figure 3. Size distribution of fungal chitosan TPP nanoparticles at 0.5 mg/mL concentration. BpHCNp, *B. poitrasii* hyphal chitosan nanoparticles; BpYCNp, *B. poitrasii* yeast chitosan nanoparticles; MCNp, marine chitosan nanoparticles.

study, using ^{1}H NMR and FTIR, DDA for crab shell chitosan was 80−86% (Table 1).

The viscometry analysis was carried out to determine the viscous nature of fungal chitosan. The chitosan isolated from ascomycetous yeast showed viscosity in the range of 0.013−0.073 cP. The viscosity of chitosan from mushroom was in the range of 0.003−0.012 cP. In the present study, the viscosity of chitosan isolated from zygomycetous fungus *B. poitrasii* hyphae and yeast cells was 0.621 cP and 0.379 cP, respectively. The marine chitosan (2.67 cP) was more viscous as compared to fungal chitosans (Table 1).

The molecular weights of chitosan isolated from ascomycetous yeasts were low (6.21−6.95 kDa) (Table 1). Earlier Mane et al.[11] reported low molecular weight (0.77−2.38 kDa) chitosan from 2 filamentous Ascomycetes *Metarhizium anisopliae* and *Myrothecium verrucaria*. While molecular weight of chitosan isolated from a zygomycetous *B. poitrasii* hyphae was 46.33 kDa and that of yeast form cells was 25.15 kDa. The molecular weight of chitosan isolated from Basidiomycetes fungi, *A. bisporus* and *P. sajor-caju*, was 6.48 and 6.72 kDa, respectively (Table 1, Figure S1).

Antifungal Activity of Fungal Chitosan. Chitosan has antiviral, antibacterial, and antifungal properties and can be used in different applications.[28] The antimicrobial activity of chitosan is dependent on the properties like molecular weight and % DDA, which define its solubility in water or dilute acid solutions.[15] The chitosan isolated from ascomycetous and basidiomycetous fungi did not inhibit the growth of any of the tested human pathogenic yeasts and filamentous fungal strains. This can be attributed to the lower % DDA and very low molecular weights of these chitosan samples. As compared to the commercial chitosan from marine source (585 kDa, 85% DDA; MIC_{90} 2.5−5.0 mg/mL), the *B. poitrasii* hyphal (46.33 kDa, 92.78% DDA) and yeast chitosans (25.15 kDa, 90.86% DDA) inhibited the growth of human pathogenic *C. albicans* ATCC 10231, *C. glabrata* NCYC 388, *C. tropicalis* ATCC 750, *C. neoformans* ATCC 34664 and *A. niger* ATCC 10578 at relatively lower concentrations (MIC_{90} 0.025−2.5 mg/mL; Table 2). The antifungal activity of chitosan polymer against *A.*

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niger, *C. albicans*, *Rhizopus oryzae*, *Rhizopus stolonifer*, *Alternaria alternata*, and others have been reported.[15,29,30] These findings suggested that chitosan polymer properties, namely, molecular weight, DDA, types of fungal source, concentration, and polycationic nature, determine its antifungal potential.[15,29,30]

The increased MIC values of chitosan from ascomycetous fungi and mushrooms against fungal pathogens is mostly attributed to the plasma membrane's free fatty acid (FFA) composition. Nutrient deprivation alters cell wall design, which has an impact on fungal growth. The higher contents of glucan reported in cell walls of ascomycetous and basidiomycetous fungi reduces their sensitivity toward chitosan.[31] To improve the activity of promising *B. poitrasii* chitosan, nanoparticles were prepared and tested against the human pathogenic organisms.

Characterization of Fungal Chitosan Nanoparticles. The determination of hydrodynamic diameter and zeta potential of nanoparticles is carried out by dynamic light scattering (DLS). The average particle size of BpHCNps was found to be 196 ± 2.32 nm with polydispersity index 0.388 ± 0.002. For BpYCNps, the average size was 178 ± 4.09 nm with polydispersity index 0.341 ± 0.03, while the marine chitosan nanoparticles (MCNps) showed higher average particle size (689 ± 57.82 nm) with polydispersity index 0.644 ± 0.05 (Figure 3).

The morphology of nanoparticles prepared at different chitosan concentrations was determined using field emission scanning electron microscopy (FE-SEM; Figure S2) and high-resolution transmission electron microscopy (HR-TEM; Figure S3). The morphology of chitosan nanoparticles was found to be spherical (Figure S2). The average size of BpHCNps, BpYCNps, and MCNps were found to be 63.58, 51.34, and 23.04 nm, respectively. The possibility of particle aggregation during DLS estimation could be attributed to the differences in the particle size measurement by the TEM and DLS methods.

The zeta potential of BpHCNps, BpYCNps, and MCNps was 24.48 ± 1.58, 21.64 ± 0.34 and 43.81 ± 0.22 mV, respectively (Table 3).

Table 3. Mean Particle Size, Polydispersity Index, and Zeta Potential of Chitosan Nanoparticles

	Particle size (nm)	PDI	Zeta potential (mV)
BpHCNp	196 ± 2.32	0.388 ± 0.002	24.48 ± 1.58
BpYCNp	178 ± 4.09	0.341 ± 0.03	21.64 ± 0.34
MCNp	689 ± 57.82	0.644 ± 0.05	43.81 ± 0.22

As shown in Table 3, the size and charge of MCNps differed significantly compared to size and charge of BpHCNps and BpYCNps. This difference in the sizes and charges of synthesized nanoparticles are mainly due to the variations in molecular weight and % DDA of chitosan samples used for synthesis of nanoparticles. The other parameters, namely, initial concentration of chitosan, chitosan to TPP ratio used, agitation speed, incubation temperature, choice of solvent, and presence or absence of different salts, which affect the physicochemical properties of chitosan nanoparticles, were reported to contribute to their size and charge variations.[24]

Antifungal Activity of Fungal Chitosan Nanoparticles. Compared to the *B. poitrasii* hyphal chitosan, its nanoparticles showed improved antifungal activity at lower concentrations against human pathogenic *C. albicans* ATCC 10231 (MIC_{90} 0.025 mg/mL), *C. glabrata* NCYC 388 (MIC_{90} 0.1 mg/mL), *C. tropicalis* ATCC 750 (MIC_{90} 0.4 mg/mL), *C. neoformans* ATCC 34664 (MIC_{90} 0.4 mg/mL), and *A. niger* ATCC 10578 (MIC_{90} 0.2 mg/mL), while the MIC_{90} for *B. poitrasii* yeast chitosan nanoparticles were in the range of 0.4−0.8 mg/mL against the tested organisms. However, the marine chitosan nanoparticles showed higher MIC_{90} (0.8 to >1.6 mg/mL) than the fungal chitosan nanoparticles (Table 4).

The chitosan nanoparticles (CNPs) showed improved antifungal activity against *Candida albicans, Fusarium solani*, and *Aspergillus niger* than the chitosan polymer.[15] This improved antifungal activity of CNPs was attributed to the size and zeta potential of chitosan nanoparticles.[15] The nanoformulation of chitosan and chitosan−silver nanocomposites also effectively inhibited the growth of plant pathogen *Fusarium oxysporum*.[32] In general, chitosan nanoparticles showed higher antifungal potential than the chitosan polymer.[15,33] Qi et al.[34] attributed this improved antimicrobial potential of chitosan nanoparticles to their properties such as small size, compact shape, and higher surface charge (zeta potential). The small size and low molecular weight of CNPs reduces the viscosity and thereby improves their solubility in water or dilute acid solutions. This led to protonation of more amino groups on CNPs allowing more efficient interaction between polycationic CNPs with the negatively charged host plasma membrane.[15] Singh et al.[35] also suggested that polycations primarily target the negatively charged plasma membrane. In this context, polycationic chitosan nanoparticles are more likely to interact with the fungal cells than the chitosan polymer. BpHCNps were more effective against the tested fungi compared to the BpYCNps. This could be attributed to the increased cationic charges on the nanoparticle surfaces that may contribute to enhanced interaction with the negatively charged cell membrane and its disruption. In case of MCNps, the increased aggregation could contribute to lower antifungal efficacy.

Hemocompatibility of Chitosan and Chitosan Nanoparticles. RBC hemolysis is an important parameter for *in vivo* applications of any pharmaceutical agents as it can lead to anemia, jaundice, and other pathological conditions. Therefore, in the present study RBC hemolytic activities of chitosan samples and their nanoparticles were tested (Figure S4). The *B. poitrasii* yeast and hyphal chitosans, as well as their

Table 4. Antifungal Activity[a] of *B. poitrasii* Chitosan Nanoparticles against Human Pathogens

	Candida albicans ATCC 10231	*Candida glabrata* NCYC 388	*Candida tropicalis* ATCC 750	*Cryptococcus neoformans* ATCC 34664	*Aspergillus niger* ATCC 10578
BpHCNp	0.025	0.1	0.4	0.4	0.2
BpYCNp	0.4	0.8	0.8	0.4	0.4
MCNp	0.8	1.6	1.6	0.8	0.8

[a]MIC_{90} (mg/mL).

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nanoparticles, were found to be hemocompatible as they showed <5% hemolysis at the highest tested concentration (5 mg/mL). The commercial chitosan from marine source however showed 6.53% hemolysis at 5 mg/mL, higher than the chitosan isolated from *B. poitrasii* hyphae (3.18% hemolysis) and yeast cells (3.06% hemolysis) under similar sets of conditions. The CNPs from marine source, *B. poitrasii* hyphae, and yeast cells showed 3.30%, 2.96%, and 1.49% hemolysis, respectively, at the highest tested concentration (Figure S4). These observations agree with reports where chitosan and CNPs did not show any cytotoxic effect against human corneal epithelial (HCEC) and hepatoma (HepG2) cell lines.[36−38] The study suggested that fungal chitosans and their nanoformulations are hemocompatible and therefore are safe for healthcare applications.

CONCLUSIONS

The chitosan content in zygomycetous fungal cell walls is relatively higher than fungi from other classes, however the availability of waste biomass for other fungi from biotech industries is much more significant. In view of this, isolation of chitosan from different industrially important fungi was studied. The chitosan from the ascomycetous yeast, as well as basidiomycetous mushrooms, showed low molecular weight compared to the chitosan from *B. poitrasii*. However, they showed poor antifungal potential, which was attributed to their very low molecular weights and low polycationic nature (i.e., low % DDA) as compared to the chitosan from *B. poitrasii*. The present investigations suggest that % DDA is more important for the antifungal activity of chitosan polymers and their nanoparticles than the molecular weight. Although the raw waste biomass available for ascomycetous and basidiomycetous fungi is higher, the isolated chitosan cannot be used for healthcare applications due to their low cationic nature (i.e., low % DDA). On the other hand, chitosan isolated from the zygomycetous fungus *B. poitrasii* was found to be a promising (DDA 92.78%, molecular weight 46.33 kDa) source for healthcare applications, *per se*. Furthermore, the nanoparticles synthesized using *B. poitrasii* chitosan effectively inhibited the growth of human fungal pathogens at lower MIC_{90} than the chitosan polymer. In the light of these results, it is suggested that chitosan nanoparticles prepared from *B. poitrasii* chitosan with high % DDA have the potential of becoming a powerful and safe natural antifungal agent.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.1c01248.

Gel permeation chromatography profiles of isolated fungal chitosans for molecular weight determination, field emission scanning electron micrographs to analyze the morphologies of chitosan nanoparticles and methodology followed, high-resolution transmission electron microscopy (HR-TEM) of chitosan nanoparticles to determine their sizes and methodology used for sample preparation and analysis, and red blood cells (RBC) hemolysis assay to check the hemocompatibility of chitosan polymers and their nanoparticles (PDF)

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Notes

The authors declare no competing financial interest.

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Taguchi design of the experimental approach to increase the biomass and cell wall chitosan contents of zygomycetous dimorphic fungus *Benjaminiella poitrasii*

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Abstract

A dimorphic fungus *Benjaminiella poitrasii* contains high chitin/chitosan (35% of the cell wall) in the mycelial (M) form than its yeast (Y) form (20% of the cell wall). However, the relative proportion of chitosan is more in yeast form cells (chitosan: chitin ratio, 6:1) than mycelial cells (chitosan: chitin ratio, 3:1). Using the Taguchi design of experimental (DOE) approach, interactions among eight different parameters showed that carbon source (starch, 10 g/L), incubation time (48 h), inoculum (M and Y mixed 10%), yeast extract (6 g/L) and peptone (10 g/L), were optimum for maximum biomass production. Under these conditions, the chitosan yield from the mycelia was 60.89±2.30 mg/g of dry biomass, while that from the yeast cells was 28.29±2.7 mg/g. The molecular weights of chitosan isolated from M and Y cells were 41.28 kDa and 21.72 kDa, respectively as measured by gel permeation chromatography. The degree of deacetylation of chitosans as measured by FTIR was lower (87.3-88.5% DDA) than measured using ^{1}H-NMR (88.17-90.72% DDA). Furthermore, chitosans from M and Y cells inhibited the growth of plant pathogenic *Fusarium oxysporum* (MIC_{90} 0.1 and 0.4 mg/mL) and *Ustilago maydis* (MIC_{90}0.4 and 0.8 mg/mL) at lower concentrations as compared to chitosan isolated from marine sources (MIC_{90} 0.8 and >1.6 mg/mL).

Keywords: Antifungal activity, degree of deacetylation, fungal chitosan, molecular weight, Taguchi design of experiment

1. Introduction

Chitosan is a natural β-(1→4)-linked glucosamine polymer produced by deacetylation of chitin, a (β-1→4)-linked *N*-acetylglucosamine polymer. The main commercial source of chitin/chitosan is landings of marine organisms such as, crabs, lobsters, and shrimps. However, the supply of marine waste is seasonal and limited, resulting in variability in the source material. The fungi containing chitin/chitosan in their cell walls can be an alternative commercially viable source. Ghormade *et al* (2017) extensively reviewed the possible fungal sources for chitin and chitosan production [1]. The fermentation of zygomycetous fungi *viz., Absidia coerulea, Benjaminiella poitrasii, Cunninghamella elegans, Gongronella butleri, Mucor rouxii* and *Rhizopus oryzae*, was preferred for chitosan production due to a higher percentage of chitosan in their cell wall [1]. The attempts were also made to optimize the fermentation conditions to increase biomass production, ultimately increasing the chitosan yield. Previously, we have optimized the mycelial biomass production in *B. poitrasii* by using a one variable at time (OVAT) approach [2]. Xu *et al.,* 2003 also used a OVAT and orthogonal matrix method to optimize submerged conditions for *Paecilomyces tenuipes* C240 strain [3]. However, the optimization of fermentation parameters by OVAT approach becomes tedious and time-consuming. Alternatively, statistical methods can be used to study the interaction of variables in generating the process response. Taguchi design of experiments (DOE) developed by Genichi Taguchi is one such tool. It involves studying the system by a set of independent variables (factors) over a specific region of interest (levels). Taguchi's parameter design concept is related to finding the appropriate design factor levels to make the system insensitive to variations in noise (uncontrollable factors). Kim *et al.*, 2005 used this statistical approach to optimize submerged culture conditions to produce mycelial biomass and exopolysaccharides (EPS) by *Agrocybe cylindracea*[4] Under the optimal culture condition, the maximum EPS concentration achieved bout three times higher than the basal medium[4].

In the present study, fermentation conditions were optimized to increase the biomass and extractable cell wall chitosan contents of *B. poitrasii* using the Taguchi DOE approach. The effect of interactions between different fermentation parameters on growth and chitosan contents of *B. poitrasii* were also studied. Further, biophysical properties of chitosan isolated from *B. poitrasii* yeast and mycelia grown under optimized fermentation conditions were studied. Furthermore, the antifungal potential of *B. poitrasii* yeast and hyphal chitosan was also evaluated against plant pathogenic fungi.

2. Material and Methods

2.1 Fungal strains and growth conditions

The parent strain of *B. poitrasii* (NCIM 1240) isolated at CSIR-National Chemical Laboratory, Pune, was maintained on YPG agar (g/L: yeast extract, 3; mycological peptone, 5; glucose, 10; agar, 20, pH adjusted to 6.5) slant. The plant pathogens *Fusarium oxysporum* CMI113138 and *Ustilago maydis* PRL 1549 were maintained on 2% potato dextrose agar (PDA). All the slants were stored at 4°C and routinely sub-cultured every week.

2.2 Optimization of fungal biomass using Taguchi design of the experimental approach

The optimization of media components and other incubation conditions for *B. poitrasii* biomass was done using the Taguchi design of experimental approach (DOE). Eight different control factors were identified to evaluate their role in the objective function, *i.e.*, optimizing medium for maximum biomass production. The factors selected were starch, yeast extract (YE), peptone, pH, temperature, the ratio $MgSO_4$: KH_2PO_4, inoculum size, and incubation time. The temperature factor has been assigned with only two levels, namely 25°C and 28°C, whereas three were selected for the remaining seven factors. Based on the number of factors and levels, L18 ($2^1X\ 3^7$) orthogonal array was used for the design. In the present study, all 8 columns were assigned with different factors (Table 1 depicts the details).

Table 1 Design of experiment to optimize fermentation conditions to increase *B. poitrasii* biomass and chitosan contents using L18 orthogonal array

	Control factors							
Expt. No.	Temp (°C)	Starch (%)	pH	YE (%)	Peptone (%)	Inoculum Size (%)	Incubation Time (h)	$MgSO_4$: KH_2PO_4
1	25	0.5	5.5	0.3	0.5	8	24	1:2
2	25	0.5	6.5	0.6	1	10	36	1:2.5
3	25	0.5	7.5	0.9	1.5	12	48	1:3
4	25	1	5.5	0.3	1	10	48	1:3
5	25	1	6.5	0.6	1.5	12	24	1:2
6	25	1	7.5	0.9	0.5	8	36	1:2.5

7	25	1.5	5.5	0.6	0.5	12	36	1:3
8	25	1.5	6.5	0.9	1	8	48	1:2
9	25	1.5	7.5	0.3	1.5	10	24	1:2.5
10	28	0.5	5.5	0.9	1.5	10	36	1:2
11	28	0.5	6.5	0.3	0.5	12	48	1:2.5
12	28	0.5	7.5	0.6	1	8	24	1:3
13	28	1	5.5	0.6	1.5	8	48	1:2.5
14	28	1	6.5	0.9	0.5	10	24	1:3
15	28	1	7.5	0.3	1	12	36	1:2
16	28	1.5	5.5	0.9	1	12	24	1:2.5
17	28	1.5	6.5	0.3	1.5	8	36	1:3
18	28	1.5	7.5	0.6	0.5	10	48	1:2

The growth experiments were carried out in a 1000 mL Erlenmeyer flask containing 200 mL of optimized medium. The different inoculums were prepared in the respective medium using sporangiospores (1.6 x 10^7/100 mL), incubated for 24 h under shaking conditions and used for inoculations. All flasks were incubated at 28 °C for 48 h on a rotary shaker (180 rpm). The mycelial biomass was harvested by filtration through Whatman filter paper No.1 and washed with distilled water (3-4 times). The above filtrate solution was centrifuged at 7100rpm () for 20 min to harvest the yeast cells and washed with distilled water several times to remove the starch. The removal of starch was confirmed with the iodine test. For instance, 1 g of wet biomass (yeast or mycelia) was taken into a test tube, and a few drops of iodine solution were added.No change in color indicated the absence of starch in biomass. The dried mycelial and yeast biomass was further used for the isolation of chitosan.

2.3 Effect of different inoculum types on the growth of fungal biomass

B. poitrasii, *viz.*, sporangiospores, mycelia, yeast cells, alone and in combination, were used as an inoculum to obtain biomass. *B. poitrasii* growth from slant was gently scraped to obtain

sporangiospores, free from yeast, mycelium, and zygospores, as determined by light microscopy. The yeast inoculum was obtained by inoculating 1.6 x 10^7 sporangiospores in 100 mL YPG medium under shaking conditions (180 rpm) at 37°C for 24h. To prepare mycelial inoculum, sporangiospores were inoculated in 100 mL YP medium and incubated at 28°C, 180 rpm for 24h. The mix inoculum was prepared by growing the sporangiospores in the optimized medium at 28°C, 180 rpm for 24h. All the experiments were carried out in a 1000 mL Erlenmeyer flask containing 200 mL of optimized medium, inoculated with different inoculum types and incubated at 28 °C, 180 rpm for 48h. The biomass was harvested and dried until constant weight.

2.4 Extraction of chitosan from *B. poitrasii* biomass

The biomass was washed and suspended in cold distilled water and broken by mechanical shaking with glass beads (0.45-0.5mm) in a Braun homogenizer. The absence of cytoplasmic material was confirmed by light microscopy as described earlier by Khale and Deshpande 1992[5]. The purified cell wall samples were lyophilized and stored at -20 °C. Chitosan was extracted as described by Mane *et al* 2017[2]. The alkali-insoluble fraction (AIF) was separated by centrifugation at 7100 rpm for 15 min and neutralized by washing with cold distilled water. The pellet obtained was treated with 2% acetic acid at 95°C for 24 h, followed by centrifugation at 7100 rpm for 30 min and the supernatant was collected. Chitosan was precipitated from the supernatant using 1N NaOH. A white precipitate of chitosan obtained at pH 8.5-10 was washed with acetone and dried at 50 °C to a constant weight.

2.5 Biophysical characterization of chitosan

2.5.1 Fourier Transform Infrared (FTIR) spectroscopy

The samples were analyzed using FTIR spectrophotometer (IRAfinity-1S, Shimadzu,Japan). The FT-IR spectrum of chitosan was recorded in the 400-4000 cm^{-1} region. The sample dried at 105°C for 1h was powdered and then mixed with KBr with appropriate control. The degree of deacetylation was determined using the absorbance ratio A_{1320}/A_{1420} [6].

2.5.2 Gel permeation chromatography

The gel permeation chromatography to determine the molecular weight of chitosan samples was carried out using OHpak SB-800 HQ series columns (Agilent Technologies, USA). For instance, chitosan samples were dissolved in acetic acid (2%) solution to get the final concentration of 2 mg/mL and filtered to remove the debris, if any. The 0.2 M acetic acid/0.1 M sodium acetate was used as mobile phase. The 50 µL of chitosan sample was run through the column at 0.8 ml/min

flow rate at 40 °C. The column was calibrated using Pullulan 20201 Shodex standards and molecular weight of chitosan samples were determined [7].

2.5.3 ^{1}H-Nuclear Magnetic Resonance

Chitosan sample (10 mg) was dissolved in a solution containing 1.96 mL of D_2O and 0.04 mL of deuteriated HCl. The samples were kept on magnetic stirrer for half an hour to confirm complete dissolution of the chitosan. Further the samples were lyophilized and dissolved in 1mL of D_2O, 600 µL of the sample was used to record the NMR spectra. All the NMR spectra were taken on Bruker AV400 NMR spectrophotometer. The Degree of Deacetylation (DDA) was calculated using formula as mentioned in Lavertu *et al.*[8]

2.6 Antifungal assay

Chitosan extracted from *B. poitrasii* was dissolved in 2% acetic acid solution to make 10 mg/mL stock. The solution was kept for stirring overnight for dissolution of chitosan. The final pH of the solution was adjusted to 5 using 1 N NaOH. The antifungal activity was tested against two plant pathogenic fungi, *F. oxysporum* CMI 113138 and *U. maydis* PRL 1549 by Clinical Laboratory Standards Institute's (CLSI) broth micro-dilution assay (CLSI document M27-A3 and CLSI M38-A2) as described earlier by Pulya *et al* 2016 [9]. The stock was diluted in RPMI 1640 medium and added to the first row of a 96-well microtitre plate. The chitosan samples were serially diluted using RPMI 1640 medium in successive wells to get the final concentration in the range of0.0125-1.6 mg/mL. *F. oxysporum* and *U. maydis* spores ($2x10^4$/mL) were suspended in the medium and inoculated (100 µL) in the microtitre plate wells. Acetic acid (2%) served as a control. All plates were incubated for 48h. After incubation, the growth was assessed visibly and recorded. The minimum inhibitory concentration (MIC) was defined as the concentration required to inhibit>90% of the growthcompared to the control.

3. Results

3.1 Multiple variable approaches for optimization of biomass production by *B. poitrasii*

The experimental data were processed using excel sheets prepared by Dr. P. R. Apte, TIFR, Mumbai, India (http://www.tifr.res.in/~apte) for DOE using the Taguchi approach. The individual influence of the factors at the assigned levels, severity indices for different interactions between factors and ANOVA were calculated. The biomass (dry weight, unless otherwise mentioned) after 48 h of incubation was in the range of 2-6.6 g/L and 0.1-2.83 g/L for mycelium and yeast, respectively (Table 2).

175 **Table 2**Effect of interactions between different fermentation parameters on growth and chitosan
176 contents of *B. poitrasii* studied using L18 orthogonal array

Expt. No.	Final pH	Mycelia		Yeast cells	
		Biomass (g/L)	Chitosan* (mg/g)	Biomass (g/L)	Chitosan* (mg/g)
1	7.07	2.16 ± 0.33	29.33 ± 0.48	0.63±0.30	8.88±0.32
2	8.25	4.93 ± 0.43	30.28 ± 0.40	0.9±0.10	10.48±0.36
3	8.31	4.76 ± 0.15	31.46 ± 0.45	0.26±0.05	9.15±0.29
4	7.37	5.94 ± 0.48	33.98 ± 0.37	1.7±0.10	12.79±0.21
5	7.90	3.80 ± 0.55	30.71 ± 0.51	1.46±0.15	11.43±0.34
6	7.90	5.32 ± 0.44	33.71 ± 0.53	0.97±0.15	8.28±0.21
7	7.46	4.81 ± 0.21	30.44 ± 0.39	2.53±0.87	14.48±0.36
8	8.20	6.62 ± 0.44	38.49 ± 0.35	0.96±0.15	8.42±0.10
9	8.21	4.11 ± 0.21	28.95 ± 0.44	1.3±0.20	12.44±0.25
10	7.77	4.74 ± 0.39	30.16 ± 0.38	1.56±0.25	13.87±0.83
11	7.03	2.58 ± 0.50	28.88 ± 0.31	2.83±0.12	15.33±0.26
12	7.56	2.50 ± 0.52	31.18 ± 0.38	0.14±0.04	6.41±0.19
13	7.39	5.57 ± 0.15	35.30 ± 0.42	0.5±0.17	7.56±0.37
14	6.81	3.44 ± 0.37	32.65 ± 0.47	0.7±0.26	9.36±0.12
15	7.47	3.86 ± 0.27	33.21 ± 0.25	0.16±0.05	4.49±0.32
16	6.67	3.03 ± 0.94	35.85 ± 0.32	0.26±0.25	6.58±0.23
17	7.90	4.23 ± 0.15	30.34 ± 0.27	0.11±0.01	5.52±0.13
18	7.09	5.08 ± 0.27	29.83 ± 0.28	0.12±0.005	5.79±0.18

*The mycelia and yeast cells were homogenized in liquid nitrogen and chitosan was isolated as mentioned under Materials and Methods.

The maximum biomass of *B. poitrasii* hyphae (6.50-7.00 g/L) was obtained in experiment no. 8 (Table 2). The factors *viz.*, incubation temperature of 25°C, starch (1.5 %), yeast extract (0.9%), peptone (1%), $MgSO_4$ and KH_2PO_4 at 1:2 proportion in the growth medium of pH 8, and incubation time of 48 h, were found to be optimum for production of maximum mycelial biomass (6.62 ± 0.44 g/L) and chitosan (38.49 ± 0.35 mg/g of dry biomass) (Table 2). The optimum conditions for yeast biomass production were found to be as of experiment no. 7 (Table 2).

3.2 Effect of different types of inoculums on the growth of *B. poitrasii* biomass

The yeast inoculum produced higher proportions of yeast cells (3.15±0.05 g/L) and fewer mycelia ((1.37±0.21 g/L). The least biomass was obtained with mycelial inoculum (2.60±0.22 g/L of M and 1.88±0.10 g/L of Y) (Table 3).

Table 3 Effect of different types of inoculums on the growth and chitosan contents of *B. poitrasii*

Inoculum Type	**Biomass (g/L)**		**Chitosan dry wt. (mg/g of dry biomass)**	
	M	Y	M	Y
Spores	4.27±0.16	1.80±0.18	57.20±1.1	26.23±2.7
Mycelia	2.60±0.22	1.88±0.10	38.10±0.5	27.41±2.4
Yeast	1.37±0.21	3.15±0.05	36.30±0.8	25.63±1.6
Mix (M+Y)	4.71±0.19	2.70±0.10	58.72±1.0	23.64±1.2

M, mycelia; Y,yeast cells.

The mixed inoculum (Y+M) produced the highest biomass (4.71±0.19 g/L of M and 2.70±0.10 g/L of Y) followed by spore inoculum (4.27±0.16 g/L of M and 1.80±0.18 g/L of Y) in the optimized media. Therefore, the mixed inoculum containing both the yeast and mycelium cells

was used in further experiments.

3.3 Validation of optimized conditions to produce*B. poitrasii* biomass and cell wall chitosan

The software automatically calculated optimized process conditions on pooled ANOVA: (g/L): yeast extract 6; peptone 15; KH_2PO_4, 4; $MgSO_4$ 2; starch, 10; and 1 ml trace metal solution containing [(mg/mL): $FeSO_4.7H_2O$, 5; $MnSO_4$, 1.5; $ZnSO_4$, 3.34; $CoCl_2$, 2.0], pH 6.5, inoculum 10%, incubation temperature, 28 °C, incubation time, 48 h and agitation 180 rpm. The validation experiment was carried out under the above-optimized process conditions (Table 4).

Table 4 Yield of *B. poitrasii* chitosan from One Variable at Time (OVAT) and Validationexperiment using Taguchi Design of experimental approach (DOE)

Method	Biomass Dry wt. (g/L)		Chitosan(mg/g dry wt.)		% DDA (FTIR)		Molecular weight (kDa)	
	M	Y	M	Y	M	Y	M	Y
OVAT method	10.00±0.50	ND	51.00±0.52	ND	92.89	ND	42.82	ND
Taguchi Method	9.17±0.32	1.78±0.28	60.89±2.30	28.29±2.7	88.51	87.30	41.68	19.49

M, mycelia; Y,yeast cells; ND, not determined. From 1 L optimized medium, 10.95±0.3 g biomass and 608±2.5 mg of chitosan was obtained using Taguchi DOE approach.

It produced seventimes more biomass (9.17±0.32 g/L of mycelial biomass and 1.78±0.28 g/L yeast biomass) than the biomass obtained in basal medium (1.28± g/L)[2]. The Taguchi optimized produced substantial amount of both mycelial and yeast biomass which increased the final yield of chitosan as compared to the OVAT approach (Table 4).

3.4 Degree of deacetylationof chitosan isolated from *B. poitrasii*(M and Y)

The degree of deacetylation (DDA) is one of the most important factors responsible for chitosan's physical and chemical properties. The solubility of chitosan is mainly dependent on the DDA. The fungal chitosan showed characteristic peaks for O-H stretching at 3450 cm^{-1}, for amide II at 1650 cm^{-1}, at 1420 cm^{-1} for C-H deformations, for amide III, C-N stretching at 1320 cm^{-1} and at 1035 cm^{-1} for O bridge stretching in FTIR spectroscopy. These peaks are comparable with the FTIR spectra obtained for commercial chitosan from marine source (Fig. 1).

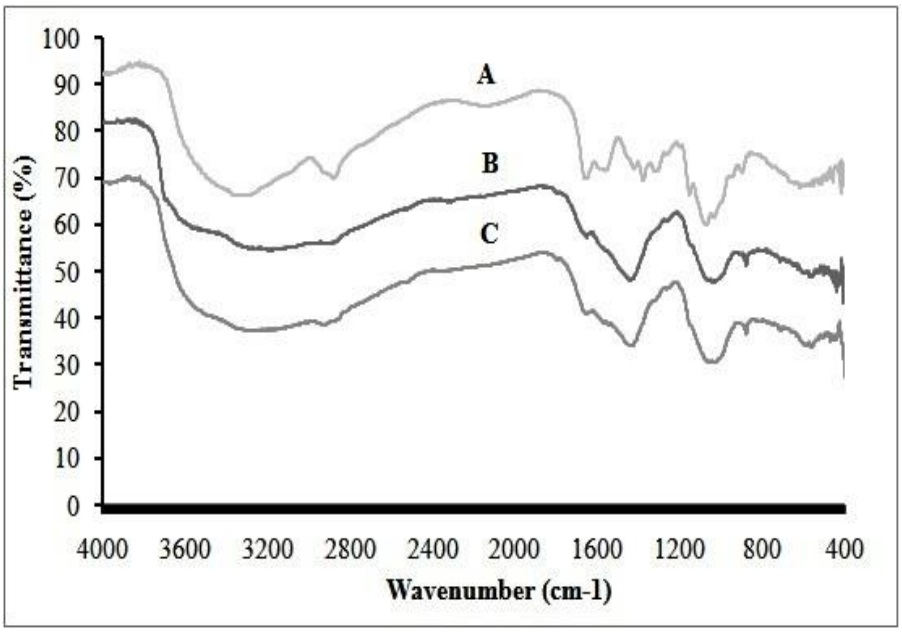

Fig. 1. FT-IR profiles of *Benjaminiella poitrasii* mycelium (B) and yeast(C) chitosans comparedwith chitosan obtained from marine source (A).

In validation experiment, % DDA of chitosan isolated from mycelia and yeast cells were 88.51% and 87.30%, respectively. The chitosan from marine source showed 79.26 % of DDA, lower as compared to the fungal chitosan.

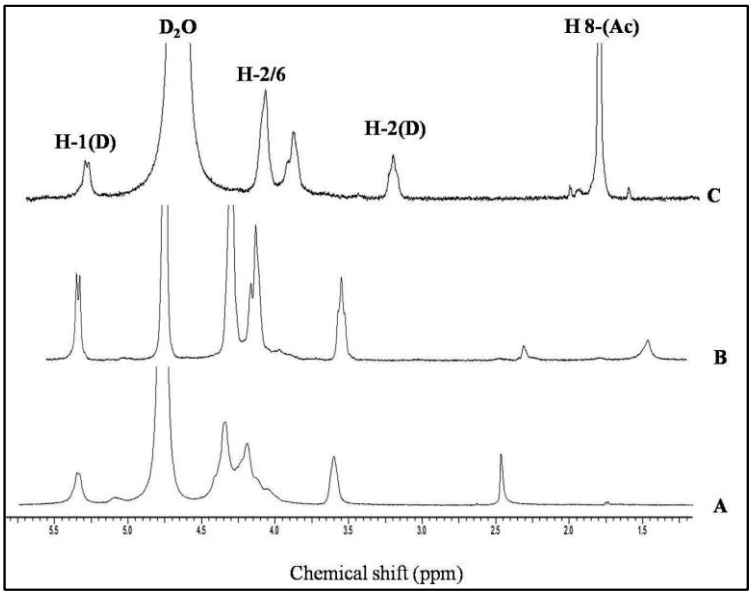

Fig. 2.[1]H-NMR profiles of chitosan from marine source (A), *B. poitrasii* mycelium(B) and *B. poitrasii* yeast (C).

Further, chitosan samples from validation experiments were also analyzed by ^{1}H-NMR spectroscopy. The characteristic peak of H1 proton (a deacetylated monomer, H1D) and peak of three protons of acetyl group (H-Ac) for chitosan were observed. These peaks observed in fungal chitosan samples were comparable with the commercial chitosan from marine source (Fig. 2). The % DDA as calculated from ^{1}H-NMR of marine chitosan was 86.51 whereas for *B. poitrasii* hyphal and yeast chitosan were 90.72 and 88.17, respectively.

3.5 Molecular weight of chitosan isolated from *B. poitrasii* mycelia and yeast cells by gel permeation chromatography

The molecular weight of chitosan samples from validation experiment was determined by gel permeation chromatography (GPC). The molecular weight of mycelial and yeast chitosan samples by GPC were 41.28 kDa and 21.72 kDa, respectively (Fig. 3). These values were found to be comparable with the values obtained with viscometry method. The molecular weight of commercial chitosan from marine sources as determined by GPC was 251 kDa (Fig. 3).

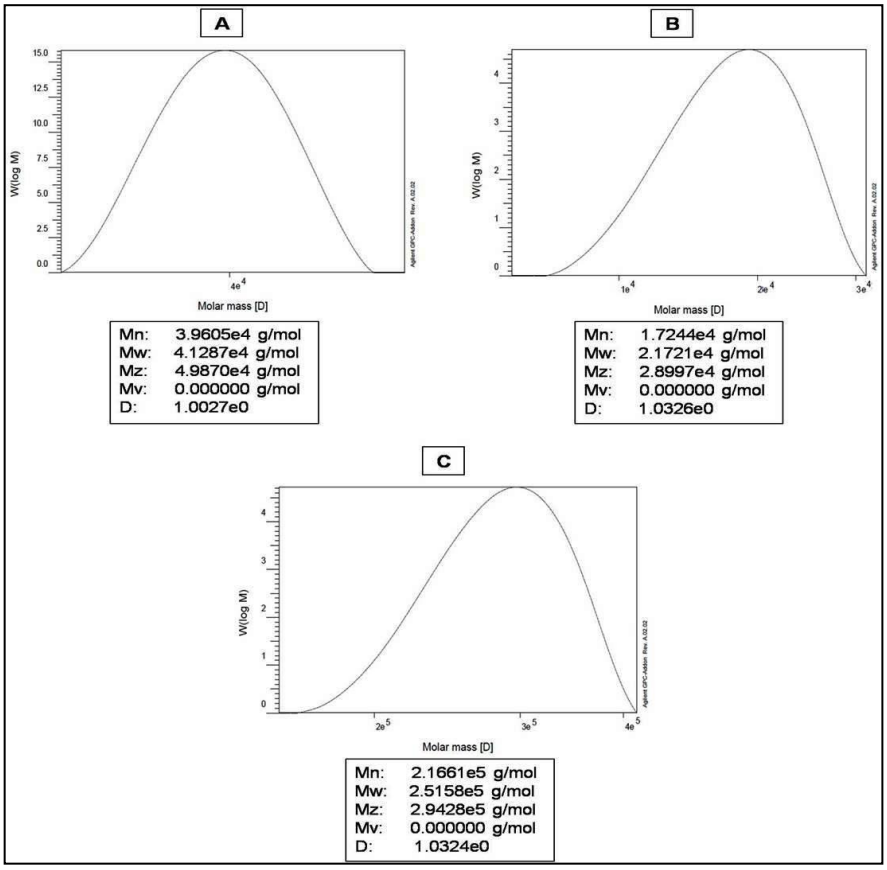

Fig. 3. Gel permeation chromatography to determine the molecular weight of chitosan samples isolated from (A) *B. poitrasii* mycelia, (B) *B. poitrasii* yeast and (C) Marine sources.

3.6 Antifungal activity of *B. poitrasii* chitosan against plant pathogens

The chitosan extracted from *B. poitrasii* yeast and mycelia were tested against plant pathogenic fungi *viz.*, *F. oxysporum* and *U. maydis*. The mycelial chitosan inhibited the growth of *F. oxysporum* (MIC_{90} 0.1 mg/mL) and *U. maydis* (MIC_{90} 0.4 mg/mL) more effectively than the yeast chitosan (MIC_{90} 0.4 and 0.8 mg/mL, respectively). The marine chitosan showed least effectiveness under studied conditions (MIC_{90} 0.8and >1.6 mg/mL)(Table 5).

Table 5 Antifungal activity of chitosan polymers against plant pathogenic fungi

Chitosan source	**MIC_{90} (mg/mL)**	
	Fusarium oxysporum	*Ustilago maydis*
B. poitrasii (M)	0.1	0.4
B. poitrasii (Y)	0.4	0.8
Marine chitosan	0.8	>1.6
Control	ND	ND

ND, not detected;2% acetic acid (used to dissolve chitosan polymer) was served as control; The antifungal assay was carried out using CLSI micro-broth dilution method; All the assays were carried out in triplicates and average values are presented.

4. Discussion

Media optimization is a crucial step in increasing the production of fungal biomass that ultimately increases the yield of extractable chitosan. Conventional optimization procedures involve altering one parameter at a time, keeping the rest of the parameters constant, enables understanding the impact of only those parameters on the process.Further, optimization by the conventional approach becomes erroneous. On the contrary, statistical optimization methods consider the interaction of variables in generating the process response. Chenthamarakshan *et al.*[10]described the optimization of extracellular production of laccase from *Marasmielluspalmivorus*LA1 byTaguchi method using L8 orthogonal array.The optimization improved the laccase yield by 17.6-fold. The process parameters for the growth of *Aspergillus sp.*to remove the copper and nickel were also optimized using the Taguchi method [11]. In the

present study, optimized conditions gave 7-fold higher biomass than the biomass obtained in the basalmedium[2]. Previously, it was reported that peptone's source might affect the % germ tube formation in *B. poitrasii* [12]. Asimilar effect was observed during the two optimization methods. Earlier, the concentration of peptone was less (10 g/L), resulting in more mycelial biomass (10 g/L). However, in the case of Taguchi media optimization, the higher peptone concentration (15 g/L) gave at-par mycelial biomass (9.17 g/L) with a substantial amount of yeast cells (1.78 g/L). So, ultimately the total yield of biomass was increased (10.95 g/L).The DDA is a crucial biophysical characteristicin deciding the quality and solubility of chitosan polymer[1].Thechitosan extracted from yeast and mycelia of *B. poitrasii* showed ~90% DDA. The chitosan isolated from another zygomycetous fungus *Mucor rouxii*, grownin three different media, showed variation (82.2-89.8%) in % DDA [13]. The chitosan's molecular weight varies depending on the source of isolation. Chitosans from crabs and shrimps havehigh molecular weights as compared to fungal chitosans[1]. The molecular weight of chitosan isolated from *Cunninghamella elegans* was 27.2 kDa[14]. Munoz *et al*. reported thechitosan extraction from *A.niger*(molecular weight 190 kDa) to synthesizehydrogels for biomedical applications[15].The chitosan also has antiviral, antibacterial, and antifungal properties thatcan be used fordifferent applications. Previously, Mane *et al*.showedthat *B. poitrasii* mycelial chitosan (molecular weight 42.82 kDa)inhibited the growth of *A. niger*, *Candida albicans*, *Candida glabrata*, and *Cryptococcus neoformans* (MIC_{90} 0.025-0.8 mg/mL)[2]. In the present study,*B. poitrasii*mycelial and yeast chitosan (molecular weights 41.68 and 19.49 kDa, respectively) were compared for their antifungal potential against dreadful plant pathogenic fungi*F. oxysporum* and *U. maydis*.The high molecular weight chitosan from the marine source was the least effective among tested chitosans against plant pathogens. It suggested that the molecular weight of chitosan along with % DDAare important factors in defining its antifungal potential.

5. Conclusions

TheTaguchi DOEapproach used in the present study for media optimization considersthe interaction between multiple factors affecting the growth and cell wall chitosan content of zygomycetous fungus *B. poitrasii*.The biomass (yeast and mycelia) obtained under optimized growth conditions gave maximum extractable chitosan, which has low molecular weight and high % DDA, the properties that define its antifungal potential.

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Conflict of interest

No conflict of interest was declared.

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