Synthesis and Characterization of Biopolymers Chitosan

Abhijeet Deshmukh, Research Scholar, Department of Pharmacy, SunRise University Alwar, Raj.
Bharat Tekade, Associate Professor, Department of Pharmacy, SunRise University, Alwar, Rajasthan, India.

Abstract

Chitosan was first discovered in 1811 by Henri Braconnot, director of the botanical garden in Nancy, France. Braconnot observed that a certain substance (chitin) found inmushrooms did not dissolve in sulphuric acid. Over the last 200 years, the exploration of chitosan has taken on many different forms. Several other researchers continue to build on the original finding of Braconnot, discovering new uses for chitin as they find different forms of it in nature. As seen in above Figure, both chitin and chitosan have similar chemical structure. Chitin is made up of a linear chain of acetylglucosamine groups while chitosan is obtained by removing enough acetyl groups (CH_3 - CO) for the molecule to be soluble in most diluted acids and the only difference between chitosan and cellulose is the amine (- NH_2) group in the C-2 position of chitosan instead of the hydroxyl (-OH) group. However, unlike plant fiber, chitosan possesses positive ionic charges, which give it the ability to chemically bind with negatively charged fats, lipids, cholesterol, metal ions, proteins and macromolecules Chitosan is a cationic biopolymer consisting of (1,4)-linked 2-amino-deoxy-β-d- glucan is derived from chitin, a homopolymer of ß-(1-4)-linked *N*-acetyl-D-glucosamine.

Keywords: Chitosan, biopolymer, characterization, antimicrobial activity

INTRODUCTION

Biopolymers based polysaccharides are versatile bio-products with various applications in many industries. Worldwide every year approximately 140 million tons of synthetic polymers are produced that are stable with limited degradation cycle in the biosphere. This compels the need for the natural biodegradable polymers from renewable resources which will fit into the ecological cycle. Biosynthesized microbial polysaccharides produced through fermentation can be further improved easily by genetic engineering of the strains used, alteration in the design and working principle of the fermentor and efficient downstream processing. Exopolysaccharides based polymer production by fermentation is more advantageous because it is extruded into the environment and makes isolation of the polymer from broth easier[3].

MATERIALS AND METHODS

- **Maintenance Media:** All the strains obtained from National collection of Industrial Microorganisms, National Chemical Laboratory, Pune, India. The cultures were maintained on a Potato Dextrose Agar slant. This culture was transferred once a month to a fresh slant.
- **Identification of fungi by microscopy: Morphology of Fungi:** This is a rapid technique for preparing a temporary microscopicmount of a fungus without disturbing the arrangement of conidia and conidia bearing hyphae, the conidiophores.

Requirements: Fungus colony on agar plate, Strip of clear cello tape 10 cm,Lactophenol cotton blue, Microscopic slide.

- **Procedure:**

1. A clean slide was taken and a drop of lactophenol cotton blue wasplaced in the centre of the slide.
2. The Tape was held the tape with sticky side down, between thumb andforefinger of each hand and pressed firmly.
3. The centre of the sticky side of the tape was pressed firmly on to thesurface of the fungus colony, where sporulation was visible.
4. The tape was gently pulled away from the colony and placed on thedrop of lactophenol cotton blue.

- The extended ends of the tape were folded over the ends of the slide.**Inoculum Preparation:** Whenever required, all cultures were subcultured on Potato Dextrose Agar plates, incubated at room temperature (25°C) and exposed to black light to stimulate sporulation. The cultures were allowed to grow for 3-5 days for spores formation. Spores were harvested by flooding the culture plates with 5% Tween 80

in sterile distilled water. A final spore suspension(1.8 x $l0^6$ spores/ml) was prepared for each fungal species and used to inoculate the fermentation broth.**Fermentation Medium** : The fermentation medium contains 20g/L of glucose, 10 g/L ofpeptone, 1g/L of yeast extract, 5 g/l of ammonium sulphate, 1 g/L of a K_2HPO_4, 1 g/L of $MgSo_4 .7H_2O$, 0.1 g/L calcium chloride and sodiumchloride 1g/L. After inoculation the fungi was grown in the fermentation broth for an additional two days in a shaking incubator set at 28˚ C with agitation of 200 rpm, the pH of the fermentation medium was maintained between 3-4, throughout the fermentation . At the end of the desired incubation period the mycelia was harvested by filtration.**Chitosan Isolation**: The biomass was recovered from the fermentation medium by filtration (no. 1 Whatman) and washed with distilled water to get clear filtrate. The mycelium was then treated with 1 M sodium hydroxide (1:30g/ v) and the mixture was autoclaved at 121˚ C for 15 minutes. The mixture was subsequently centrifuged at 12000g for 5 minutes to sediment the alkaliinsoluble materials (AIM) and washed with distilled water and ethanol. Thewashed material was further extracted with 10% acetic acid solution (1:40g/ml) reflexed at 60˚C for 6 hours. The resulting slurry was then isolated by centrifugation at 12,000 g for 15 minutes yielding an acid soluble supernatant (containing chitosan) and an acid insoluble precipitate (containing chitin). The pH of the supernatant was adjusted to 10 with 4 Msodium hydroxide, thereby precipitating out the chitosan. The chitosan was finally washed with distilled water, 95% ethanol (1: 20 w/v), acetone (1:20 w/v) subsequently, and air dried. (Chen MH et al., 2001, Pochanavanich P.et al., 2002, Thayaza et al., 2007)Determination of Growth Curve, Extractable Chitin and Chitosan (Shimahara K., et al., 1998): The growth curves, extractable chitosan and chitin of *R. oryzae*, *M.hiemalis* and *A.niger* were determined by culturing each fungus in the fermentation medium. This was done by inoculating 30 ml of spore inoculum in 270 ml of fermentation medium. The mycelial dry weight was determined by filtration and drying the biomass at 65^0C extractable chitosanand chitin after 24, 48, 72, 96, 120, 144, 168 and 192 hours of growth were determined as described above. Three replicate cultures were prepared for each incubation period.Medium Optimization (Chen MH., et al., 2001; Chen MH., et al.,2002): In the media optimization for the three strains two variations in the experimental procedure was adopted.

Carbon Source Optimization: The nitrogen source i.e. peptone and mineral sources used earlier was kept as a constant and various carbon sources, namely dextrose, maltose, sucrose and corn starch were used.

Nitrogen Source Optimization: The carbon source i.e. glucose and mineral sources used earlier was kept as a constant and various nitrogen sources, namely soybean meal, corn steep liquor, arginine and urea were used.

CHITOSAN CHARACTERIZATION

Viscosity (Vilai Rungsardhong et al., 2005): The viscosity of 0.1% chitosan in 0.5% acetic acid solution was determined by using Brook Field viscometer (Version 5.1, Spindle No 62, rpm 100) at 25^0C.

Infrared spectroscopy (Deacetylation degree-DDA %): The degree of deacetylation is determined by using the absorbanceratio A1655/A3450 and calculated according to the following equation:

DDA % = 118.883-[40.1647 X (A1655/A3450)]

Two milligrams of fungal chitosan which had been dried overnightat 60˚C was thoroughly blended with 100mg of KBr, to produce 0.5 mm thick discs. Spectrum was recorded using JASCO FTIR-410

^{1}HNMR (Lavertu M., et al., 2003): ^{1}HNMR measurement was carried in Bruker NMR spectrometer under static magnetic field of 300 Mhz. chitosan preparations was dissolved in D_20 and poured into 5-mm inner diameter NMR tubesAntioxidant Activity by DPPH Method

Principle: DPPH is a free radical, stable at room temperature, which produces aviolet coloration in ethanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncolored ethanol solutions. The use of DPPH provides an easy and rapid way to evaluate antioxidants. Procedure: Each chitosan sample (0.1- 10mg/ml) in 2g/l acetic acid solution (2.5ml) was mixed with 1ml of ethanolic solution containing DPPH (Sigma) radicals, resulting in a final concentration of 10mmol/l DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance is then measured at 518 nm against a blank.

The absorbance value was converted into the % of antioxidant activity (AA) using the following formula.

$$AA\ (\%) = 100 - \frac{[\text{Absorbance of Sample} - \text{Absorbance of Blank}]\ X\ 100}{\textbf{[Absorbance of Control]}}$$

Ethanol (1.0 ml) plus sample solution was used as blank. DPPH solution (1.0 ml; 10 mM) plus ethanol (2.5 ml) was used as negativecontrol. The positive controls were those using the standard solutions.

EC_{50} value (mg/ml) is the effective concentration at which DPPH radicals is scavenged by 50% and it was obtained by interpolation from linear regression analysis. Ascorbic acid was used for comparison

Sterilization: 20-25ml of Mueller Hinton Agar was transferred to test tubes and sealed with non-absorbent cotton. It was then autoclaved at a pressure of 15psi (121^{o}c) for not less than15 minutes.

Organisms Used: *Staphylococcus aureus* NCIM 2079, *Pseudomonas aeruginosa*NCIM 2036, *Escherichia coli* NCIM 2118 (Clinical isolate of *E.coli*, obtained from Sri Ramakrishna hospital, Coimbatore-44.) *Bacillus subtilis* NCIM 2063, *Micrococcus luteus* NCIM 2704, *Aspergillus niger* NCIM 545and *Candida albicans* NCIM 3100 were procured from National Chemical Laboratory, Pune and stored in the Pharmaceutical Biotechnology Laboratory, College of Pharmacy, SRIPMS, Coimbatore-44. The strains were confirmed for their purity and identity by Gram's staining method andtheir characteristic biochemical reactions. The selected strains were preserved by sub culturing them periodically on nutrient agar slants and storing them under frozen conditions. For the study, fresh 24 hr brothcultures were used after standardization of the culture.

Working Conditions: The entire work was done using vertical laminar flow hood so as to provide aseptic conditions. Before commencement of the work air samplingwas carried out using a sterile nutrient agar plate and exposing it to the environment inside the hood. After incubation it was checked for the growth of microorganism and absence of growth confirmed aseptic working conditions.

Preparation of Inoculum: The inoculum for the experiment was prepared fresh in MuellerHinton broth for bacteria and potato dextrose broth for fungi from preserved frozen slants. It was incubated at 37^{o}C for 18-24 hrs and used after standardization.

RESULTS AND DISCUSSION

Strain identification: All the three procured strains were confirmed, its macro scopic and microscopic morphology and further work was carried out. Chitosan Identification in Fungi: The chitosan containing fungi were identified under light microscopeby novel staining method using Ponceau S and Stains all. All the three procured strains were stained by Ponceau S and Stains all. It confirmed thepresence of chitosan.

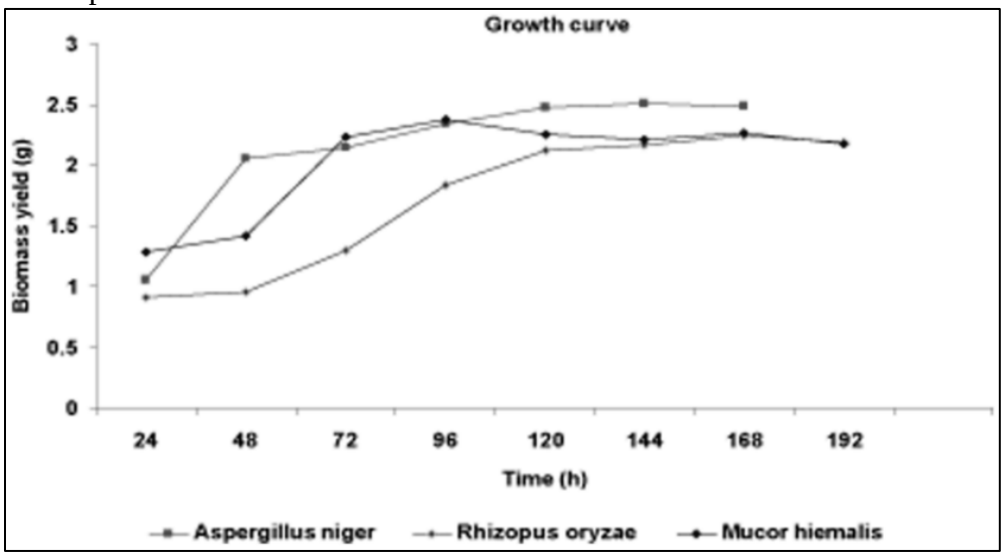

Fig. 1: Growth curve of *Aspergillus niger, Rhizopus oryzae and M.hiemalis*

Table 1: Weight of Biomass, Extractable Chitosan, Chitin and Degree of Deacetylation of *Aspergillus niger* NCIM 545

Sr. No	Time (h)	Weight of Biomass(g)	Weight of Chitosan (g)	Weight of Chitin (g)	Degree of Deacetylation
01	24	1.06±0.04	0.02±0.09	0.82±0.02	91.66±1.4
02	48	2.06±0.02	0.03±0.005	0.95±0.04	87.33±1.20
03	72	2.14±0.03	0.03±0.05	1.01±0.02	89±2.51
04	96	2.34±0.03	0.03±0.03	1.17±0.08	85.67±3.2
05	120	2.47±0.12	0.06±0.05	1.27±0.2	86.33±2.33
06	144	2.31±0.01	0.06±0.12	1.19±0.01	83.67±2.40
07	168	2.28±0.07	0.05±0.17	1.15±0.26	84±2.51

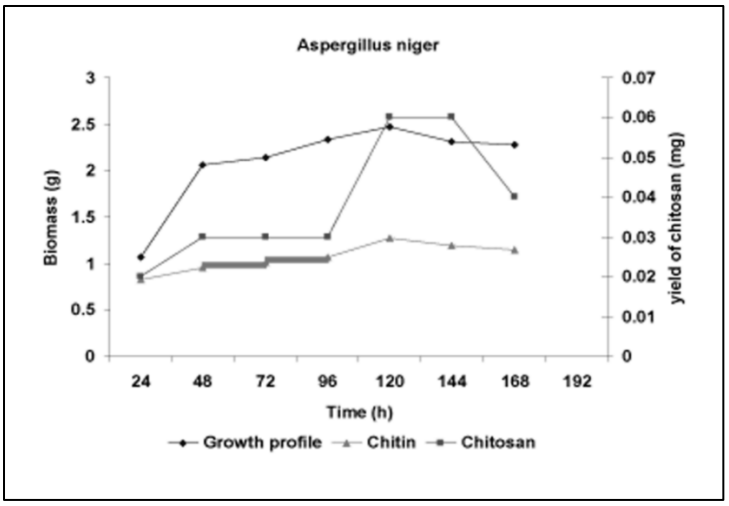

Fig 2: Weight of Biomass, Extractable Chitosan, and Chitin from *Aspergillus niger* NCIM 545 with Incubation Time

Table 2: Weight of Biomass, Extractable Chitosan, Chitin and Degree of Deacetylation of *Mucor hiemalis* NCIM 873

Sr. No.	Time (h)	Weight of Biomass (g)	Weight of Chitosan (g)	Weight of Chitin (g)	Degree of Deacetylation
01	24	1.29±0.03	0.03±0.13	0.91±0.2	91.33±2.02
02	48	1.42±0.04	0.03±0.02	0.96±0.01	84.67±1.86
03	72	2.23±0.05	0.04±0.1	1.10±0.9	90.33±1.20
04	96	2.37±0.02	0.07±0.05	1.14±0.9	90.21±2.72
05	120	2.26±0.07	0.05±0.06	1.48±0.04	84.67±1.85
06	144	2.21±0.02	0.04±0.6	1.57±0.01	89.61±2.92
07	168	2.26±0.1	0.04±0.3	1.77±0.1	88.67±0.88
08	192	2.18±0.6	0.02±0.003	1.68±0.02	84.01±2.54

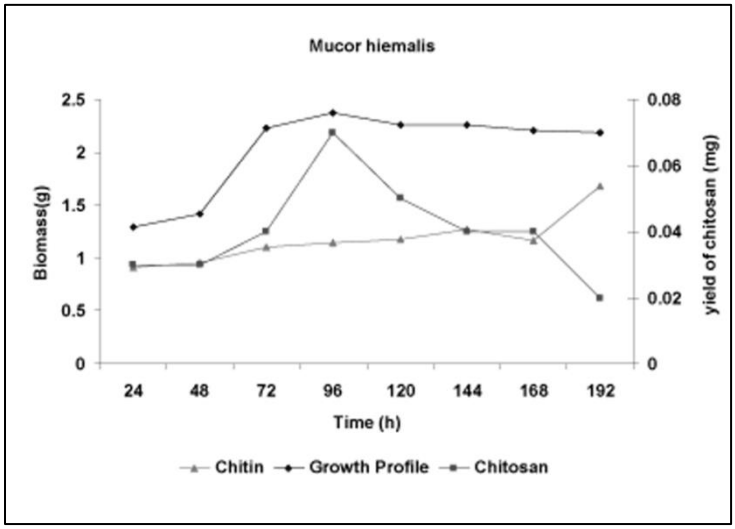

Fig.3 Weight of Biomass, Extractable Chitosan and Chitin from ***Mucor hiemalis* NCIM 873 with Incubation Time**

Table 3: Weight of Biomass, Extractable Chitosan, Chitin and Degree of Deacetylation *Rhizopus oryzae* NCIM 879

Sr. No.	Time (h)	Weight of Biomass (g)	Weight of Chitosan (g)	Weight of Chitin (g)	Degree of Deacetylation
01	24	0.91±0.04	0.01±0.003	0.63±0.03	90.33±1.85
02	48	0.96±0.1	0.02±0.01	0.74±0.07	90.3±2.37
03	72	1.3±0.11	0.04±0.3	1.12±0.21	84.33±2.84
04	96	1.84±0.3	0.04±0.3	1.37±0.05	92±1.15
05	120	2.34±0.5	0.06±0.03	1.50±0.9	91.64±6.36
06	144	2.24±0.08	0.04±0.02	1.75±0.5	87.01±1.21
07	168	2.19±0.04	0.04±0.03	1.78±0.03	82.31±3.28
08	192	2.17±0.05	0.04±0.12	1.72±0.4	88.33±0.81

Values are mean ± S.E.M (n=3)

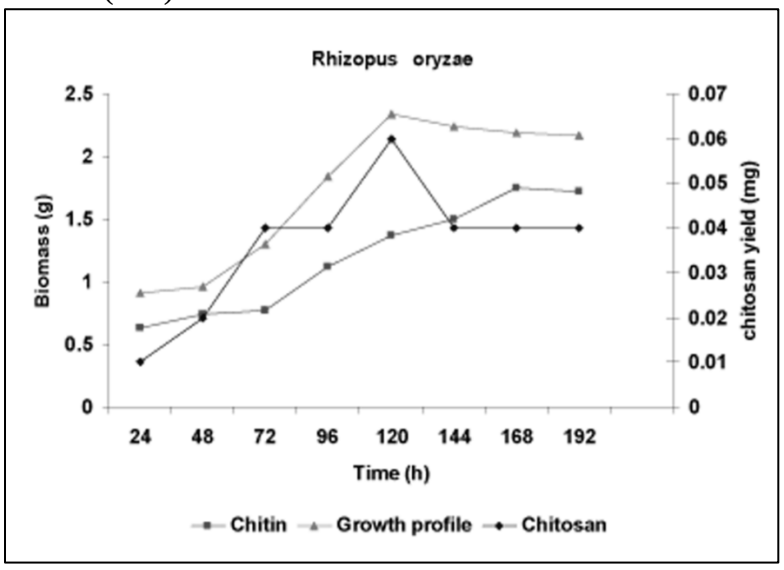

Figure 4 : Weight of Biomass, Extractable Chitosan and Chitin from
***Rhizopus oryzae* NCIM 879 with Incubation Time**

A. niger NCIM 545 had the highest growth rate with a maximal mycelial dry weight of 2.47gm with 0.06 g of chitosan after 8 d of cultivation, while *M.hiemalis* NCIM 873 and *R. oryzae* NCIM 879 grew equally with a maximal biomass of 2.37g with 0.07g of chitosan and 2.34gm with 0.06g of chitosan after 6d and 8d of cultivation respectively.

The dry weight of mycelia (biomass) and extractable chitosan of the three genus increased with time. The fungal biomass increased rapidlyduring the first 74 hours of incubation and continued to increase until 120 hours after which the growth slowed down and the fungus appeared to enter the stationary phase.

The decline of the extractable chitosan seen in the time-culture curvemight be due to physiological changes in the fungal cell wall (McGahren *et al.*, 1984). Chitosan is produced in the fungal cell wall by deacetylating its precursor, nascent chitin. During the exponential phase, the amount of extractable chitosan is relatively high, due to the active growth. Once the culture enters the stationary growth phase, more of the chitosan is anchored to the cell wall of the fungi by binding to chitin and other polysaccharides and extraction becomes more difficult. Therefore, although the fungal biomass was highest during the stationary growth phase, less chitosan is obtained. The results in Figure 13, 14, 15 indicate that the late exponential growth phase of the fungus would give the best yield for chitosan.

Therefore, all fungi should be harvested at their late exponential growth phase and the content of extractable chitosan was determined. Thisis because different fungi have different growth rates, and the time needed for them to reach their late exponential growth phase will also different. If mycelia were harvested at a fixed incubation time, the amount of extractable chitosan might not be maximum. In light of the above results,the late exponential growth phase was determined for all genera of fungi used in this study. The late exponential phase of *Mucor hiemalis* was at 96 hour, and that of *Aspergillus niger*, *Rhizopus oryzae were* at 120 hours.

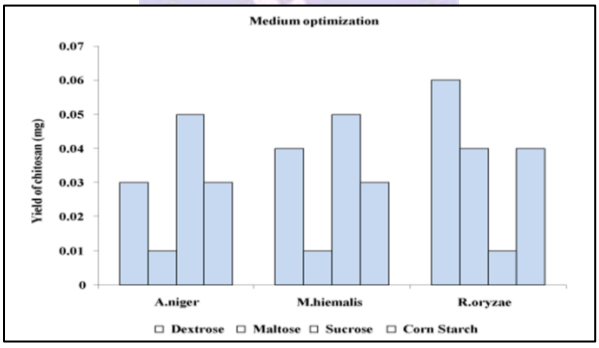

Figure 5: Comparison of Yield of Chitosan for Different Carbon Sources

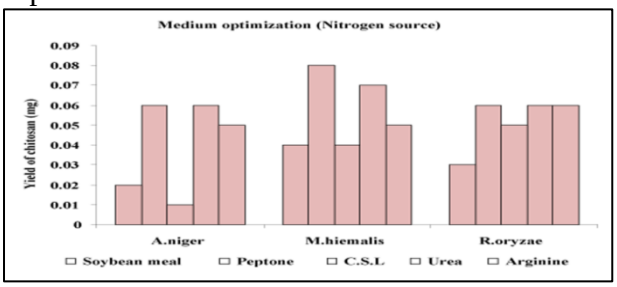

Figure 6: Comparison of Yield of Chitosan for Different Nitrogen Sources

The inclusion of sucrose and glucose as a carbon source and urea asa nitrogen source led to the highest yield of chitosan and chitin in *Aspergillus niger*. The inclusion of glucose and urea led to the highest yield of chitosanand chitin in *Mucor hiemalis*.

The inclusion of dextrose as a carbon source and urea as a nitrogen source led to the highest yield of chitosan and chitin in *Rhizopus oryzae*. The results confirmed that the chitin and chitosan

content of thefungi depends on the fungal strains, mycelial age and the composition of thegrowth medium. (Chatterjee S., et al., 2005)

Table 4: Antioxidant Activity of Isolated Chitosan

Sr. No.	Conc. of Sample (mg/ml)	Percentage of Antioxidant activity			
		Ascorbic Acid	*Aspergillus niger*	*Mucor hiemalis*	*Rhizopus oryzae*
01	0.1	33.42±0.14	15.45 ± 0.03	15.3±1.08	16.50±0.02
02	0.5	35.82±1.10	21.88 ± 0.1	26.79±0.22	22.66±0.78
03	1.0	37.14±0.15	31.43 ±0.03	28.34±0.23	28.36±0.27
04	2	38.89±0.21	44.70 ±0.16	40.4±0.20	37.30±0.58
05	4	41.14±0.09	54.33 ±0.07	47.99±0.45	49.55±0.38
06	6	45.01±0.11	58.57 ±0.23	50.41±0.83	53.06±0.67
07	8	46.78±0.20	65.42 ±0.26	59.07±0.39	57.52±0.12
08	10	47.44±0.13	78.52 ± 0.03	66.14±0.28	61.55±0.47
EC_{50} value	-	-	3.1mg/ml	5.3mg/ml	4.1mg/ml

ANTIOXIDANT ACTIVITY

The results of antioxidant activity shown in (Table no. 10) the isolated chitosan possess good antioxidant properties in scavenging ability on hydroxyl radicals. The EC_{50} value of the isolated chitosan was in the range of 3.1-5.3.

All the three isolated fungal chitosan shown good scavenging ability on DPPH free radical it may be due to the higher degree of deacetylation.

CHARACTERIZATION OF CHITOSAN BY VISCOMETRY, FT-IR AND ^{1}HNMR SPECTROSCOPY

➢ **Viscometry:** The viscosity of the isolated fungal chitosan was in the range of 13-18 cP whereas commercial chitosan having the viscosity of 123 cP. The viscosity of fungal chitosan was considerably lower than commercial chitosan highly viscous solutions are not desirable for industrial handling. Alow viscosity chitosan from fungal mycelia as obtained in this work may facilitate easy handling in industries.

Table 5: Viscosity of Isolated and Commercial Chitosan

Sr. No.	Samples	Viscosity (cP)
01	Commercial Chitosan	123
02	*Aspergillus niger*	13.8
03	*Mucor hiemalis*	15.94
04	*Rhizopus oryzae*	17.8

➢ FT- IR spectroscopy: To prove that the acid extractable material contains chitosan, its FT-IR spectra were measured in comparison with IR spectrum ofcommercial chitosan from Sigma. All the three isolated chitosan shownsimilar FT- IR spectrum to that of the commercial chitosan. The resultindicated that acid extractable material contains chitosan. The degree of deacetylation was also calculated. The degree of deacetylation value of the fungal isolated chitosan was in the range of 80-99 % but the commercial chitosan shows only 64.96%. from *Mucor hiemalis* NCIM 873 from *Rhizopus oryzae* NCIM 879NMR Spectroscopy: The ^{1}H NMR spectra was measured in comparison to commercial chitosan and it proved that the isolated fraction contains chitosan. The isolated fraction showed less intense peak at 2.0 ppm (acetyl group) as compared to commercial chitosan confirming that t he isolatosan had a higher degree of deacylatioin.

The degree of deacetylation of the isolated chitosan was 80-99%it is relatively higher than that of the commercial chitosan. The degree of deacetylation is an important parameter affecting the physicochemical properties of chitosan. Chitosan with high degree of deacetylation has high positive charges and is more suitable for food applications as a coagulating or chelating agent, a clarifying agent or an antimicrobial agent.

❖ ANTIMICROBIAL STUDIES

➢ **Antibacterial Studies:** The isolated fungal chitosan and commercial chitosan were tested fortheir anti-bacterial activity against Gram positive and Gram negative organisms by disc diffusion method using Ciprofloxacin (5µg/disc) as standard.

➢ **Gram Positive Organisms:** Both *Staphylococcus aureus* and *Bacillus subtilis was* found to be sensitive to all the isolated fungal chitosan and commercial chitosan. The chitosan did not show sensitivity against *Micrococcus luteus* except chitosan isolated from *M.hiemalis.*

➢ **Gram Negative Organisms:** Both *E.coli* and clinical isolate of *E.coli* was found to be highly sensitive to all the isolated fungal chitosan and commercial chitosan. *Pseudomonas aeruginosa* was found to be moderately sensitive towards all the isolated chitosan and commercial chitosan. The growth inhibitory effect of the isolated chitosan against bacteria is due to free-NH_3^+ groups, which are responsible for the binding of negative charges on the bacterial cell surface to bring about antibacterial activity (Chen C., et al., 1998).

ANTI FUNGAL ACTIVITY

All the isolated fungal chitosan and commercial chitosan were tested for their activity against *Candida albicans* and *Aspergillus niger* by agar diffusion method using Fluconazole (10 µg/disc) as the standard.All the isolated fungal chitosan and commercial chitosan were found to be inactive against both *Aspergillus niger* and *Candida albicans*.

As per earlier reports, the fungal chitosan showed good antifungal activity to certain genus and the derivatives of the chitosan showed better growth inhibitory activity than compared to normal chitosan. (Liu et al., 2004)

Antibacterial Activity of Isolated and Commercial Chitosan

Samples Tested	**Diameter of Zone of Inhibition (mm) (n=2)**					
	Gram Negative Bacteria			**Gram Positive Bacteria**		
	E.Coli	**Clinical Isolate *(E.coli)***	***P. aeruginosa***	***S. aureus***	***B. subtilis***	***M. luteus***
Commercial Chitosan	30±0.5	30±0.2	10.33±0.3	16.33±0.7	27	-
A.niger	31±0.5	33±0.5	15.32±0.6	19±0.4	27.67±0.2	12.32±0.5
M.hiemalis	34.66±0.3	28.33±0.3	14.66±0.33	21.67±0.2	21.33±0.3	-
R.oryzae	33.33±0.3	22±0.5	13.3±0.6	19	20.33±0.3	-
Ciprofloxacin	37	34	39	35	30	31

CONCLUSION:

It may be concluded that the mycelium of*Mucor hiemalis, Rhizopus oryzae and A.niger* could be used as good alternative sources for commercial chitosan production. The achievement ofdesired physicochemical properties such as higher degree of deacetylation and lower viscosity of the isolated fungal chitosan than the commercial chitosan indicates the superiority for using it for various pharmaceutical and medicinal applications. Fungal chitosan with alleged antioxidant properties may be used as a source of antioxidant in the pharmaceutical industry. The antibacterial activity of fungal chitosan against, *S.aureus*, *E.coli including*a and clinical isolate of *E.coli*

revealed that it can be used for therapeutic purposes.

References

1. Luis MDS, Faustino S, Gullieremo RC. A spectrophotometric method for thequantitative measurement of pullulan. J Microbiol Methods. 1992;16:253-8.
2. Kafetzopoulos D, Martinou A, Bouriotis V. Bioconversion of chitin to chitosan:purification and characterization of chitin deacetylase from *Mucor rouxii*. Proc Natl Acad Sci. 1993;90(7):2564-8.
3. Wood PJ, Fulcher RG. Specific interaction of aniline blue with (13)-β-D-glucan. Carbohydr Polym. 1984;4 (1):49-72.
4. Amemura A, Hisamatsu M, Harada T. Spontaneous mutation of polysaccharideproduction in *Alcaligenes faecalis var. myxogenes* 10C3. Appl Environ Microbiol. 1977;34(6):617-20.
5. Nakanishi I, Kimura K, Kusui S, Yamazaki E. Complex formation of gel- forming bacterial (1,3)-β-D-glucans (curdlan type polysaccharides) with dyes inaqueous solution. Carbohydr Res. 1974;32:47-52.
6. Vijayendra SVN, Bansal D, Prasad MS. Jaggery: A novel substrate for pullulanproduction by *Aureobasidium pullulans* CFR-77. Process Biochem. 2001;37:359-64.
7. Shengjun WU, Zhengyu J, Moon KJ. Downstream processing of pullulan fromfermentation broth. Carbohydr Polym. 2009;77:750-3.
8. Rho D, Mulchandani A, Luong JHT. Oxygen requirement in pullulan fermentation. Appl Microbiol Biotechnol. 1988;28:361-6.
9. Roukas T. Pre-treatment of beet molasses to increase pullulan production.Process Biochem. 1998;33:805-10.
10. Seviour RJ, Stasinopouslos SJ, Auer DPF, Gibbs PA. Production of pullulan and other exopolysaccharides by filamentous fungi. Crit Rev Biotechnol. 1992;12:279-98.
11. Harada T, Misaki A. Curdlan: A bacterial gel forming (1□3) β-glucan. ArchBiochem Biophy. 1968;124:292-8.
12. Chalikwar SS, Belgamwar VS, Talele VR, Surana SJ, Patil MU. Formulationand evaluation of Nimodipine-loaded solid lipid nanoparticles delivered *via* lymphatic transport system. Colloids Surf B Biointerfaces. 2012;97:109-6.
13. Reddy LH, Vivek K, Bakshi N, Murthy RSR. Tamoxifen citrate loaded solid lipid nanoparticles (SLN): preparation, characterization, *in vitro* drug releaseand pharmacokinetic evaluation. Pharm Dev Technol. 2006;11:167-77.
14. Helbok A, Decristoforo C, Dobrozemsky G, Rangger C, Diederen E, Stark B,Prassl R, von Guggenberg E. Radio labeling of lipid-based nanoparticles for diagnostics and therapeutic applications: A comparison using different radio metals. J Liposome Res. 2010;20:219-27.
15. Bourne DW. Pharmacokinetics. In: Banker GS, Rhodes CT, Editors. ModernPharmaceutics, 4th ed. New York: Marcel Dekker Inc; 2002.
16. Thomas PW, Strohfus BA. Pullulan degrading enzyme activity of *Aureobasidium pullulans*. J Basic Microbiol. 1996;36:377-80.
17. Goksungur Y, Ucan A, Guvenc U. Production of pullulan from beet molasses and synthetic medium by *Aureobasidium pullulans*. Turk J Biol. 2004;28:23-30.
18. Seviour RJ, Kristiansen B, Harvey L. Morphology of *Aureobasidium pullulans* during polysaccharide elaboration. T Brit Mycol Soc. 1984;2:350-6.
19. Campbell BS, Siddique AB, McDougall BM, Seviour RJ. Which morphologicalforms of the fungus *Aureobasidium pullulans* are responsible for pullulan production? FEMS Microbiol Lett. 2004;232: 225-8.
20. Li BX, Zhang N, Peng Q, Yin T, Guan FF, Wang GL, Li Y. Production of pigment-free pullulan by swollen cell in *Aureobasidium pullulans* NG which cell differentiation was affected by pH and nutrition. Appl Microbiol Biotechnol. 2009;84:293-300.