ISOLATION OF A NATURAL POLYSACCHARIDE FROM TWO DIMORPHIC FUNGI AND ITS BIOLOGICAL ACTIVITIES

Dissertation submitted to

The Tamil Nadu Dr. M. G. R. Medical University, Chennai

in partial fulfillment of the award of degree of

MASTER OF PHARMACY

(PHARMACEUTICAL BIOTECHNOLOGY)

Submitted by

ARUN RAJ T.R

Under the guidance of

Mrs. RM.AKILA, M. Pharm., (Ph.D.)

Assistant Professor

Department of Pharmaceutical Biotechnology

MARCH – 2009

COLLEGE OF PHARMACY

SRI RAMAKRISHNA INSTITUTE OF PARAMEDICAL SCIENCES

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Mrs. RM. AKILA, M.Pharm., (Ph. D.)
Assistant Professor,
Department of Pharmaceutical Biotechnology,
College of Pharmacy,
SRIPMS,
Coimbatore – 44

Place: Coimbatore
Date:

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Dr. T. K. Ravi, M. Pharm., Ph. D., FAGE.,
Principal,
College of pharmacy, SRIPMS
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Prof.S.Krishnan, M.Pharm., (Ph. D.),
Head, Dept. of Pharmaceutical Biotechnology,
College of Pharmacy,
SRIPMS,
Coimbatore – 44

Place: Coimbatore
Date:

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Arun Raj T.R

CONTENTS

III. INTRODUCTION

Polysaccharide-based biomaterials are an emerging class in several biomedical fields such as tissue regeneration, particularly for cartilage, drug delivery devices and gel entrapment systems for the immobilization of cells. Important properties of the polysaccharides include controllable biological activity, biodegradability, and their ability to form hydrogels. Most of the polysaccharides used derive from natural sources; particularly, alginate and chitin, two polysaccharides which have an extensive history of use in medicine, pharmacy and basic sciences, and can be easily extracted from marine plants (algae kelp) and crab shells, respectively. The recent rediscovery of poly-saccharide based materials is also attributable to new synthetic routes for their chemical modification, with the aim of promoting new biological activities or to modify the final properties of the biomaterials for specific purposes. These synthetic strategies also involve the combination of polysaccharides with other polymers.

3.1 Polysaccharides

The majority of carbohydrate materials in nature occur in the form of polysaccharides. By our definition, polysaccharides include not only those substances composed only of glycosidically linked sugar residues, but also molecules that contain polymeric saccharide structures linked via covalent bonds to amino acids, peptides, proteins, lipids and other structures.

Polysaccharides, also called glycans, consist of monosaccharides and their derivatives. If a polysaccharide contains only one kind of monosaccharide molecule, it is known as a homopolysaccharide, or homoglycan, whereas those containing more than one kind of monosaccharide are heteropolysaccharides. The most common constituent of polysaccharides is D-glucose, but D-fructose, D-galactose, L-galactose, D-mannose, L-

arabinose, and D-xylose are also frequent. Some monosaccharide derivatives found in polysaccharides include the amino sugars (D-glucosamine and D-galactosamine) as well as their derivatives (*N*-acetylneuraminic acid and *N*-acetylmuramic acid), and simple sugar acids (glucuronic and iduronic acids). Homopoly- saccharides are often named for the sugar unit they contain, so glucose homopolysaccharides are called glucans, while mannose homopolysaccharides are mannans. Polysaccharides differ not only in the nature of their component monosaccharides but also in the length of their chains and in the amount of chain branching that occurs. Although a given sugar residue has only one anomeric carbon and thus can form only one glycosidic linkage with hydroxyl groups on other molecules, each sugar residue carries several hydroxyls, one or more of which may be an acceptor of glycosyl substituents. This ability to form branched structures distinguishes polysaccharides from proteins and nucleic acids, which occur only as linear polymers.

The main functions played by polysaccharides in nature are either storage or structural functions. By far the most common storage polysaccharide in plants is starch, which exists in two forms: α-amylose and amylopectin. Structural polysaccharides exhibit properties that are dramatically different from those of the storage polysaccharides, even though the compositions of these two classes are similar. The structural polysaccharide cellulose is the most abundant natural polymer in the world. Found in the cell walls of nearly all plants, included marine algae, cellulose is one of the principal components, providing physical structure and strength. (Cima *et al,.* 1996)

Exopolysaccharides (EPSs) are high molecular weight carbohydrate polymers that make up a substantial component of the extracellular polymers surrounding most microbial cells in the marine environment. In recent years, there has been a growing interest in isolating new EPS-producing bacteria, particularly from various extreme marine environments (Nichols

et al. 2005). Many new microbial EPSs with novel chemical compositions, properties and structures have been found to have potential applications in fields such as adhesives, textiles, pharmaceuticals and medicine for anti-cancer, food additives, etc. (Decho AW *et al*.,1990)

Referring to polysaccharides performing structural functions in marine environment, although all of them are abundant in nature, chitin and its derivative chitosan, are important not only as abundant resources, but mainly for their attracting biological properties and potential in the biomedical field.

Chitin is the second most abundant organic compound in nature after cellulose. (Ehrlich *et al.* 2007)Chitin is widely distributed in marine invertebrates, insects, fungi, and yeast. However, chitin is not present in higher plants and higher animals.

3.2 Dimorphism in fungi

Dimorphism is a phenomenon exhibited by a variety of fungal species where fungi exist in two different morphological forms – yeast and mycelium – depending upon the nutritional and environmental conditions. The transition between the two forms is reversible. (Gooday *et al*., 1995)

Fungi from different taxonomic groups display yeast-mycelium transition, which include saprophytes such as *Mucor*, *Mycotypha*, human pathogens *Candida*, *Histoplasma* and plant pathogens *Taphrina*, *Ceratocystis.*

The dimorphism has also been viewed as a useful model eukaryotic system to study the basis of morphogenesis. Fungal dimorphism is also relevant to industrial production of single-cell protein, since the capacity to form pellicles or mats by yeast used in biomass production may alleviate difficulties inherent in harvesting individual cells from large quantities of culture fluid (Garcia and Nickerson *et al*1962)

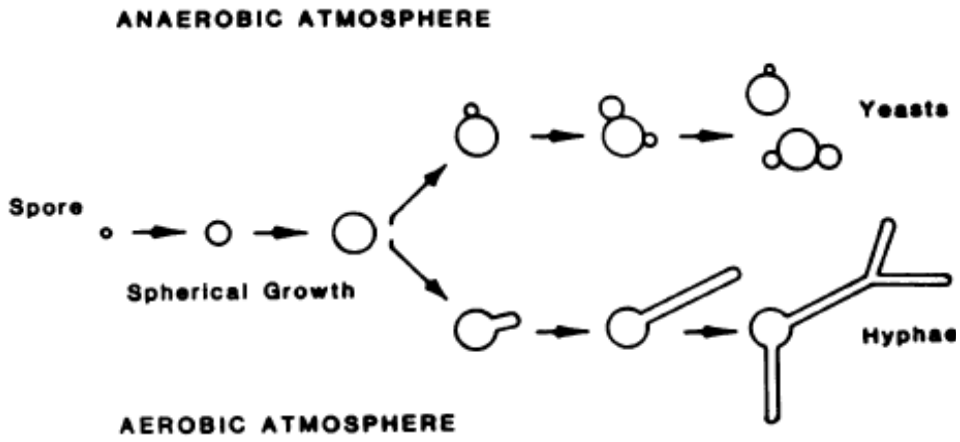


Fig. 1: Alternative morphogenetic fates of sporangiospores from dimorphic fungi

3.3 Factors influencing dimorphic transition

A large number of factors influence yeast -mycelium form transition. These include: a) environmental factors such as temperature, pH, oxygen concentration or anaerobiosis with CO_2 or N_2, b) nutritional conditions (C:N ratio, presence of metal ion etc). In most of the dimorphic fungi, temperature change is the common factor which triggers yeast to mycelium transition. For instance, in case of *B.dermatitidis, B. poitrasii, H. capsulatum*, and *P. brasiliensis* incubation at 37^{0}C favours yeast-form growth whereas in *C. albicans* yeast formation is favoured at lower temperatures (25^{0}C). In *C. albicans*, *N*-acetylglucosamine and serum stimulated hyphal growth. Elevated temperatures, neutral pH and poor growth medium stimulated the growth of hyphal cells. In *Y. lipolytica* mycelium formation is favoured by anaerobiosis. In *Ophiostoma ulmi*, dimorphic transition is regulated by controlled supply of nitrogen in the growth medium. Ammonium ions, yeast extract and certain amino acids promote hyphal development (Orlowski,*et al* 1991).

In case of *Mycotypha africana*, pH of the growth medium affected the morphological outcome. The fungus predominantly grows as yeast between pH 5.8-6.5, while on the either side (pH below 4.5 or above 7.4) it grows as mycelium.

In *Mucor*, anaerobiosis and hexoses favor yeast form and even very low concentrations of O_2 promoted mycelial development. Hexose sugars were found to be required for the formation of yeast cells. There was a close relationship between pH and filamentation. At pH 1.9, no growth occurred; only yeast like cells from the inoculum were seen. At pH 2.4, the scant growth obtained consisted of swollen distorted cells with an ellipsoidal shape and fragile walls. At pH 3.0, growth was appreciable and composed of single cells with rudimentary branching. Some cells possessed a pyriform shape, possibly indicative of a preliminary stage in the formation of filaments. At pH 3.5, distinct filaments were seen, some with arthrosporal structures. As the pH of the medium was increased, the number and length of filaments increased accordingly. Above pH 4.5, Practically all cells of the inoculum developed into filaments. (Orlowski M,*et al* 1991).

Fungal morphogenesis is a cyclical process with no beginning or end. However, it is usually convenient to consider the spore as the point of reference.

3.4 Fungal cell wall: Components, organisation and chemistry

The fungal cell wall is the outermost part of the cell and therefore provides the interface between the organism and its environment. The cell wall performs a diversity of functions such as determination of the shape and rigidity, protection from osmotic changes, maintenance of the intracellular concentrations of ions and solutes and the extracellular secretion of large molecules. The cell wall can also serve as a store of carbon reserves. Lastly, in certain parasitic and symbiotic fungi, cell wall plays an important role in establishing interaction with the host.

3.4.1 Cell wall composition, structure and organisation

For the complete characterization of cell walls, it is important to know the chemical

nature of individual wall components, their relative abundance, the spatial organisation of these components within the cell wall and the resultant architecture

The cell wall of the fungi is composed primarily of polysaccharides – both homo- and hetero-polymers. In some fungi proteins are also the significant components of the cell wall and frequently are associated with the polysaccharide components (Gooday *et al* 1995). Lipids and melanins are the minor cell wall components

Table 1: Maincomponents of the fungal cell wall

Component	Remarks
1. Aminopolysaccharides (polymers of amino sugars) • Polymers of acetyl hexosamine ○ Chitin (polymer of β-1,4 linked N-acetyl D-glucosamine • Polymerss of hexosamines ○ Chitosan (polymer of β-1,4 linked D-glucosamine) ○ Polymer of galactosamine ○ Other polymers of glucosamine	Skeletal component. Crystalline. Occurs in a complex with R- glucan. Skeletal component. Binds polyphosphates. Could link proteins and polysaccharides.
2. Non amino polysaccharides (Polymers of neutral sugars) • Glucans (Polymers composed of glucose but distinct from cellulose) ○ β-Glucans : R-glucan (β-1,3 glucan homopolymer comprised of β-1,3- and β-1,6-linked D-glucose) ○ α-Glucans:S-glucan (α-1,3 homopolymerof D-glucose) and Nigeran (α-1,3-and α-1,4- linked glucan) • Mannans (polymers of mannose)	Skeletal/ matrix element. Varying degrees of branching/ crstallinity. Some covalently linked to mannoproteins, can link with chitin. Major component of outer matrix of the wall. Linear molecule. Microcrystalline.

• Other neutral polysaccharides (polymers of hexoses such as galactose,methyl pentoses such as fructose and rhamnose, and pentoses such as Xylose) • Polyyuronids (polymers of uronic acid	Matrix component exists as mannan-protein complex Matrix component. Exist as complexes with proteins/ mannoproteins
3. Proteins	Probably exist as glycoproteins
4 Other components • Lipids • Melanins • Inorganic constituents especially phosphates • Nucleic acid deriivatives	Dark brown to black pigments in wall distinct layers of spore walls.

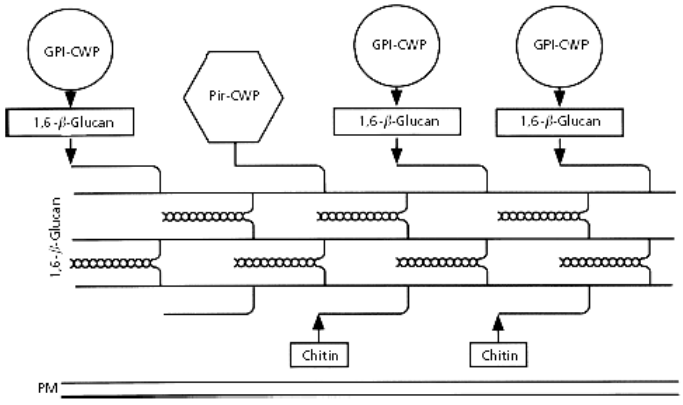


Fig. 2: Molecular architecture of the fungal cell wall

The constituents of cell walls are synthesized in the cytoplasm, linked to the walls of the hyphal tip, and polymerized and cross-linked in the wall matrix. Chitin (structure of the monomer shown in above figure 2) and the glucans are synthesized at the plasma membrane by enzymes embedded in the membrane. Nucleotide sugar precursors are accepted from the cytoplasm, linked and passed to the wall. Wall glycoproteins are synthesised in the endoplasmic reticulum, carried through the golgi to the plasma membrane, where vesicles release the glycoprotein to the wall. Enzymes cross-linking fibrils in the wall are released through the plasma membrane.

3.5 Biosynthesis of chitosan in the fungal cell wall

Chitin is synthesized by chitin synthase which catalyses glycosidic bond formation from nucleotide sugar substrate, uridine diphospho-*N*-acetyl glucosamine. Glucosamine-6-

phosphate synthase is the key enzyme of this pathway. This is the enzyme that connects the carbon and nitrogen metabolic pathways in the fungal cell. Three other enzymes are involved in chitin metabolism: chitinase, *N*-acetylglucosaminidase and *N*-acetylglucosoamine kinase. These three enzymes together provide a pathway for recycling of chitin or the digestion and utilization of exogenous chitin. After synthesis, chitin chains of the growing hyphal cells undergo important changes (like hydrogen boding between individual chains, formation of covalent links with glucans and modification to chitosan), the occurrence and extent of which differ in different fungi, giving different properties to the walls. The modification of chitin to chitosan is especially prominent in members of zygomycetes, which involves progressive deacetylation with chitin deacetylase to give a β(1-4)-linked polymer of glucosamine.

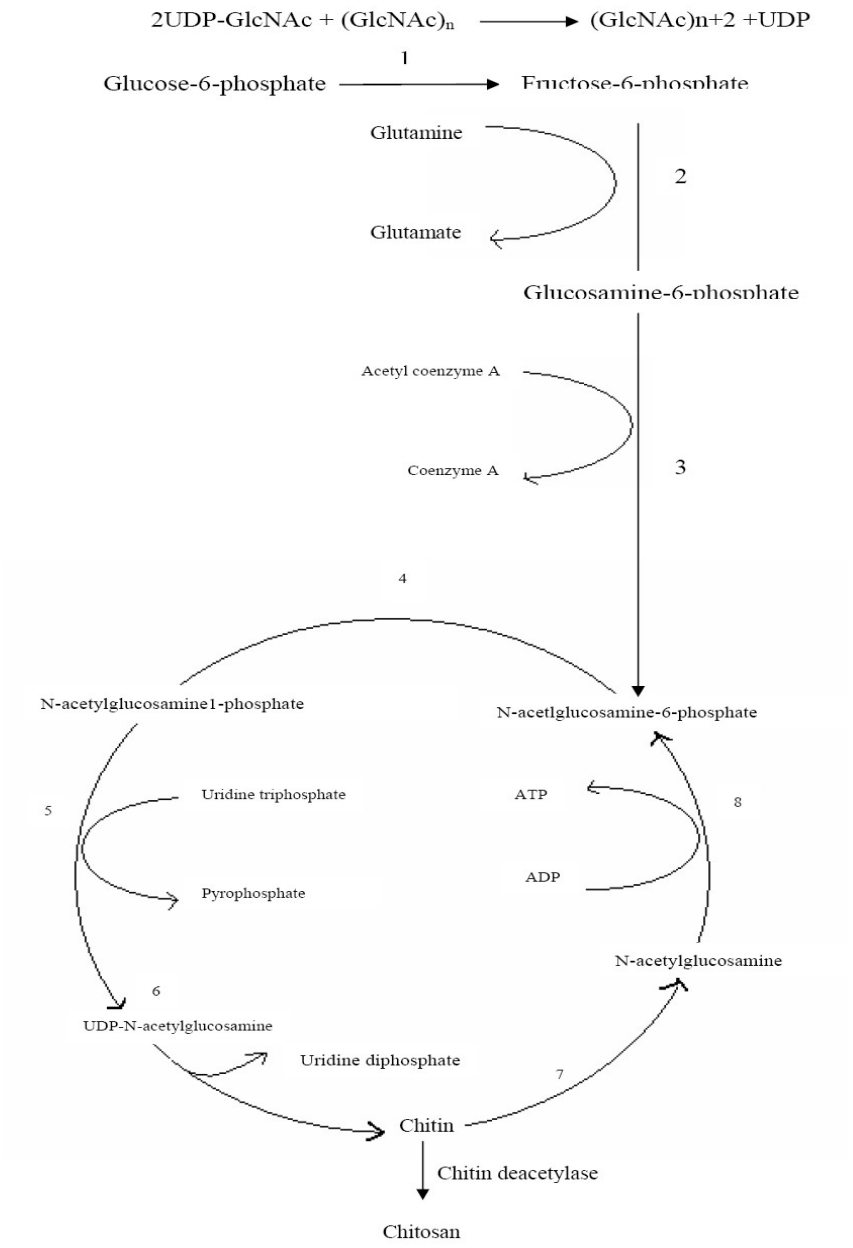
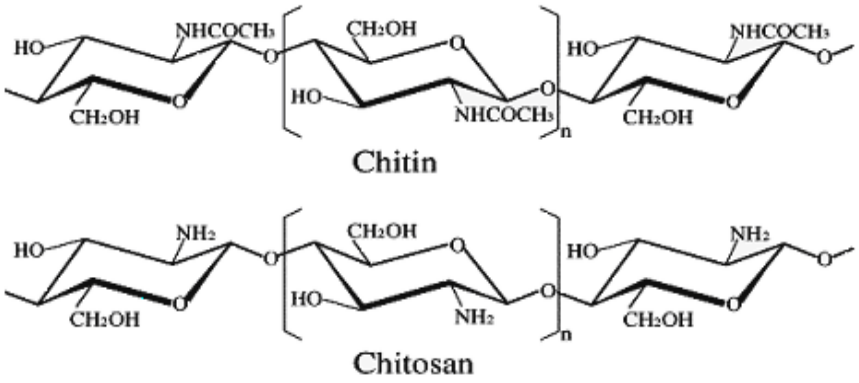


Fig. 3: Pathway of chitosan synthesis (→) and N- acetylglucosamine cycle(→) Enzymes as follows :1,Phosphoglucoisomerase:2,Glucosamine 6-phosphate synthase; 3,Glucosamine-phosphate-acetyltransferase; 4, N-acetylglucosamine phosphomutase; 5, UDP-N-acetylglucosamine pyro-phosphorylase; 6, Chitin synthase; 7, Chitinase and N- acetylglucosaminidase: 8, N-acetylglucosamine kinase. (Carlile MJ *et al*. 2001).

3.6 Chitosan

Chitin is the second most abundant polysaccaharide in the nature. 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 in mushrooms. The X-ray diffraction studies of chitinous material indicated that three different types, viz., α, β and γ-chitin are present in nature. (Jang *et al*. 2004).

Fig. 4: Structure of Chitin and Chitosan

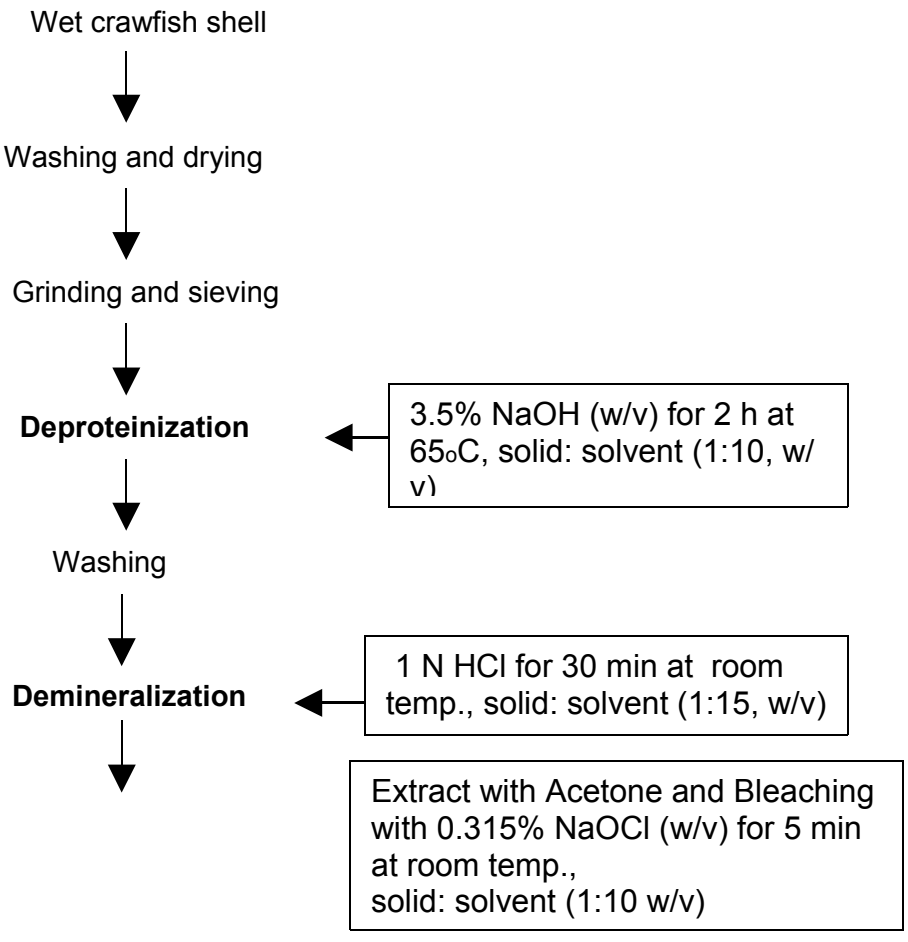


Chitosan is a cationic polysaccharide and fibre like substance consisting of (1,4)-linked 2-amino-deoxy-β-D-glucan is derived from chitin, a homopolysaccharide of β-(1-4)-linked *N*-acetyl-D-glucosamine. Chitin is made up of a linear chain of acetylglucosamine groups, while chitosan is obtained by removing enough acetyl groups (CH_3-CO) for the molecule to be soluble in most dilute acids. (NO, HK *et al.,* 1992). Chitosan is a non toxic, biodegradable polysaccharide of high molecular weight, and is very much similar to cellulose, a plant fiber. The only difference between chitosan and cellulose is the amine (-NH2) group in the position C-2 of chitosan instead of the hydroxyl (-OH) group found in cellulose. 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 macro- molecules due to which it has attained increasing commercial interest as suitable resource material due to its excellent properties like biocompatibility, biodegradability, adsorption, ability to form

films and to chelate metal ions (Li, Q., *et al* 1992).

Chitin is widely available from a variety of sources among which, the principal sources are shellfish and crustacean waste materials. Industrially, Chitosan is derived from the chemical deacetylation of chitin prepared from crab or shrimp shell. However, this process fails to produce chitosan of uniform quality. The process of deacetylation involves the removal of acetyl groups from the molecular chain of chitin, leaving behind a compound (chitosan) with a high degree chemical reactive amino group ($-NH_2$). This makes the degree of deacetylation (DDA) is an important property in chitosan production as it affects the physicochemical properties and this value also differ based on the crustacean species and preparation methods used. Hence the degree of deacetylation determines its appropriate applications. Deacetylation also affects the biodegradability and immunological activity of chitosan. (Arcidiacono S., *et al*., 1992: Jin woo lee *et al*., 2003; Davidson G.R., *et al*., 2003).

Chitosan production by traditional method



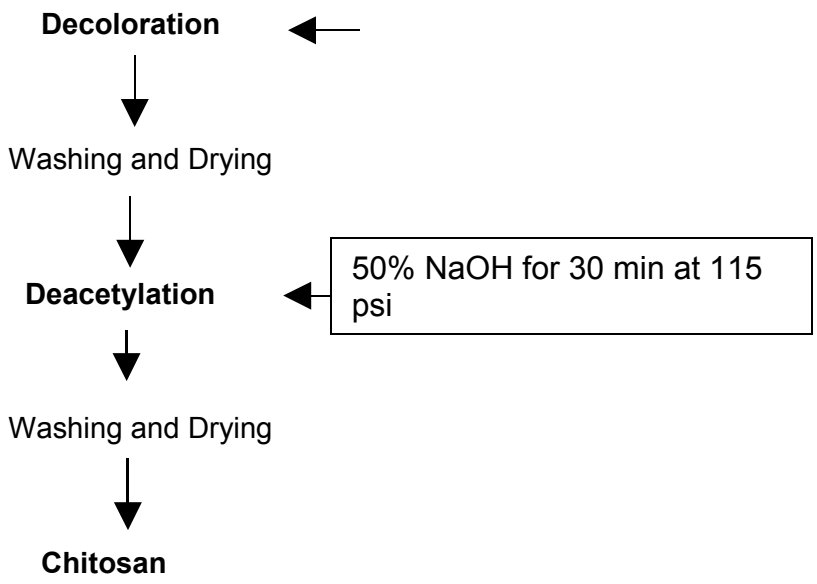


Fig. 5: Flow scheme of traditional chitosan production
(Jin woo lee *et al*., 2003)

Traditionally chitosan is derived from the chemical deacetylation of chitin, using strong alkali. Depending on the severity of these treatments such as temperature, duration, concentration of the chemicals, concentration and size of the crushed shells, the physico-chemical characteristics of the extracted chitin will vary. For instance, the three most important characteristics of the chitin i.e., degree of polymerization, acetylation and purity, will be affected. Shell also contains lipids and pigments. Thus the traditional industrial production of chitin from shellfish wastes such as shrimp, crab, and lobster processing involves problems with seasonal and limited supply, confined production locations, product variability, and high processing costs associated with the chemical conversion of chitin to chitosan appear to have limited the potential industrial acceptance of this biopolysaccharide.

3.7 FUNGAL PRODUCTION OF CHITOSAN

An alternative to solve the above described problems related to traditional chitosan production is the fungal production of chitosan, which contain chitin and chitosan as cell wall

component. The amount of these polysaccharides depends on the fungi species and culture conditions. The main advantage of fungal production of chitosan is that it is eco friendly, independent of seasonal factor, possibility of wide scale production, simultaneous extraction of chitin and chitosan, easy handling, harvesting, absence of protein contamination and controlling the production of high quality of chitosan. The physicochemical properties and yield of chitosan isolated directly from a fungus may be optimized by controlling the fermentation and processing parameters. Fungal biomass can be produced by solid state/substrate fermentation (SSF) and submerged fermentation (SmF). The fungal mycelial wastes of the either antibiotic or citric acid industry can also serve as a rich alternative source for chitosan production. (New N *et al,* 2007), (White SA *et al* 1979).

3.8 Physico chemical properties

3.8.1. Physical properties

It is off white amorphous translucent flake or powder with pearly color.

3.8.2. Solubility

Chitin is insoluble in most organic solvents, chitosan is readily soluble in dilute acidic solutions below pH 6.0. Organic acids such as acetic, formic and lactic acids are used for dissolving chitosan. The most commonly used is 1% acetic acid solution at about pH 4.0 as a reference. (Zamani A *et al* 2007)

3.8.3. Degree of Deacetylation (DDA) (Tanverr A., *et al*., 2002)

The degree of deacetylation can be employed to differentiate between chitin and chitosan because it determines the content of free amino groups in the polysaccharides. The degree of deacetylation is an important parameter associated with the physical and chemical properties of chitosan, because it is directly linked to the chitosan cationic properties.

The degree of deacetylation of chitosan ranges from 56% to 99% with an average of 80%, depending on the crustacean species and the preparation methods. Various methods have been reported for the determination of the degree of deacetylation of chitosan. These included Ninhydrin test, linear potentiometric titration, near-infrared spectroscopy nuclear magnetic resonance spectroscopy, hydrogen bromide titrimetry, infrared spectroscopy, and first derivative UV-spectrophotometry.(Chan H.Y., *et al*., 2005).

3.8.4.Determination of degree of deacetylation by Infrared spectroscopy (Muzzareli *et al*., 1997, Brugnerato J., *et al*., 2001)

The IR spectroscopy method, which was first proposed by Moore and Roberts (1980), is commonly used for the estimation of chitosan Degree of Deacetylation. This method has a number of advantages and disadvantages,

- First, it is relatively fast and unlike other spectroscopic methods, does not require purity of the sample to be tested nor require dissolution of the chitosan sample in an aqueous solvent.
- However, the IR method utilizing baseline for DDA calculation, and as such there may be possible argument for employment of different baseline which would inevitably contribute to variation in the DDA values.
- The sample preparation, type of instrument used and conditions may influence the sample analysis. Since chitosan is hygroscopic in nature and samples with lower DDA may absorb more moisture than those with higher DDA, it is essential that the samples under analysis be completely dry.

The following are some baselines proposed for the determination of the degree of deacetylation of chitosan

i. Domszy and Roberts (1985), DDA = 100-[(A1655/ A3450) x 100/1.33]

ii. Sabnis and Block (1997), DDA = 97.67-[26.486X (A1655 / A3450)]

iii. Baxter et al (1992), DDA = 100 – [(A1655 / A3450) X 115]

iv. Rout (2001), DDA = 118.883-[40.1647 X (A1655/A3450)]

3.8.5 Molecular Weight

Chitosan is a biopolymer of high molecular weight. Like its composition, the molecular weight of chitosan varies with the raw material source and method of preparation. Molecular weight of native chitin is usually larger than one million Daltons while commercial chitosan products have the molecular weight range of 100,000 – 1,200,000 Daltons, depending on the process and grades of the product.

3.8.6 Purity

The purity of the product is vital particularly for high-value product (biomedical or cosmetic area) purity is quantified as the remaining ashes, proteins, insolubles and also in the Bio-burden (microbes, yeasts and moulds, endotoxins). Even in the lower value chitosan such as that used for the waste water treatment, the purity is a factor because the remaining ashes or proteins tend to block active sites, the amine grouping. Application of chitosan can be classified mainly in three categories according to the requirement on the purity of chitosan:

- Technical grade for agriculture and water treatment
- Pure grade for the food and cosmetics industries
- Ultra-pure grade for biopharmaceutical uses

3.9. APPLICATIONS OF CHITOSAN: (Podolski J.S.*et al.,*1999 ; Jin Woo Lee *et al*., 2003; Davidson G.R. *et al*., 2003).

3.9.1 Medical

- Chitosan is widely used for improving immunity, activating cells, preventing cancer, adjusting blood sugar and fighting against ageing.
- Treating Athlete's foot, haemostatic material, exhibits hypocholesterolemic activity.
- Bone disease treatment, tumor inhibition, blood cholesterol control, skin burns/ artificial skin, bandages, sponges, artificial blood vessels,
- Reduces blood levels of uric acid
- May treat and prevent irritable bowel syndrome.
- Promotes healing of ulcers/lesions.
- Helps to control blood pressure.
- Acts as an antacid

3.9.2 Ophthalmology

- Research suggests that chitin and chitosan can make lenses more permeable to oxygen than other lens material.
- Chitosan lenses accelerate the healing of the cornea.
- Eye humor fluid

3.9.3 Orthopedics

- chitosan as an agent that helps bones to heal.

3.9.4 Surgical Suture

- chitosan based sutures remain in tissue long enough to permit healing to occur ,they slowly degrade and need not be removed.

3.9.5 Drug Delivery System

- Chitin or chitosan can be used for controlled release of drugs over specific times or locations.
- Diluents in direct compression of tablets.
- Binder in wet granulation
- Slow-release of drugs from tablets and granules

- Drug carrier in microparticle systems
- Films controlling drug release
- Preparation of hydrogels, agent for increasing viscosity in solutions.
- Wetting agent, and improvement of dissolution of poorly soluble drug substances
- Disintegrant
- Bioadhesive polymer
- Site-specific drug delivery (e.g. to the stomach or colon)
- Absorption enhancer (e.g. for nasal or oral drug delivery)
- Biodegradable polymer (implants, microparticles)
- Carrier in relation to vaccine delivery or gene therapy.

3.9.6 Biotechnology

- Enzyme immobilization, cell immobilization, cell recovery, protein separation, chromatography and glucose electrode

3.9.7 Cosmetics and Toiletries

- Make-up powder, nail polish, moisturizers, fixtures and bath lotion.
- Face, hand and body creams, toothpaste and foam enhancing agent.
- Maintain skin moisture, treat acne, tone skin.
- Protect the epidermis, reduce static electricity in hair, fight dandruff and improve suppleness of hair
- Chitosan can be provided as hair washes, coloring shampoos, hair conditioners, agents for permanent hair deformation, and as cosmetic agents for the care, protection, or cleaning of skin.

3.9.8 Water Treatment

- Removal of metal ions
- Flocculant/coagulant
- Proteins, dyes, amino acids, filtration

3.9.9 Food

- removal of dyes, solids, acids
- preservatives and color stabilization
- animal feed additive
- thickening and stabilizing agent.
- chitosan as a flocculating agent , it is used to clarify beverages.
- It also having the antioxidant activity.

3.9.10 Agriculture

- Seed coating
- Leaf coating
- Hydroponics/ fertilizer
- Controlled agrochemical release
- Frost protection
- Bloom and fruit-setting stimulation
- Stimulates growth and induces certain enzymes (synthesis of phytoalexins, chitinases, pectinases, glucanases, and lignin)
- Timed release of product into the soil (fertilizers ,organic control agents, nutrients)
- Protective coating for fruits and vegetables.

3.9.11 Pulp and Paper

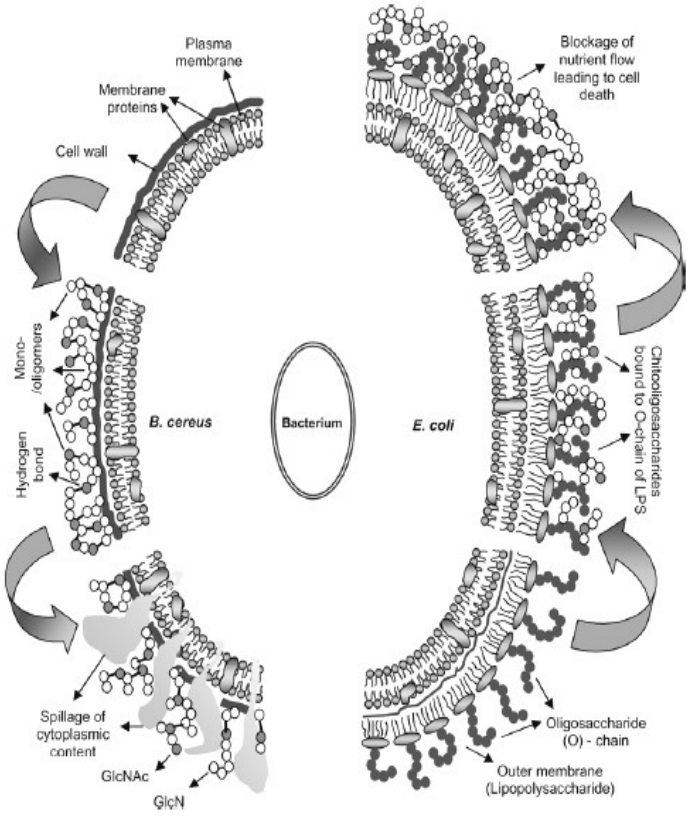
- Surface treatment
- Photographic paper
- Carbonless copy paper

3.9.12 Membranes

- Reverse osmosis
- Permeability control
- Solvent separation

3.10. ANTIBACTERIAL PROPERTIES OF CHITOSAN

Chitosan is antimicrobial against a wide range of target organisms. Activity varies considerable with the type of chitosan, the target organism and the environment in which it is applied. There are several factors, both intrinsic and extrinsic, that affect the antimicrobial activity of chitosan. It has been demonstrated that lower molecular weight chitosans (of less



than 10kDa) have greater antimicrobial activity than native chitosans. However, a degree of polymerisation of at least seven is required; lower molecular weight fractions have little or no activity (Ralston GB *et al*., 1964). Highly deacetylated chitosans are more antimicrobial than those with a higher proportion of acetylated amino groups, due to increased solubility and higher charge density (Sekiguchi S *et al*.1994), (Chen C., *et al*., 1998)

Fig.6: Mechanism of antimicrobial activity of chitosan

Mechanism of bactericidal action: The mode of action of cationic antibacterial agents is widely believed to be due to interacting with and disrupting the wall membrane structure.

3.11 Antioxidants

Antioxidants are substances whose presence in relatively low concentration significantly inhibit the rate of OXIDATION of the major targets of oxidative activity viz., cell membranes & components, proteins and other cellular constitutes.

The antioxidants that are therapeutically are conveniently divided as;

1. Natural antioxidants (physiological)- normally present in the body
2. Pharmacological antioxidants (synthetic)

Within each group three categories of antioxidants have been recognized viz.,

- Antioxidants enzymes catalyzing the breakdown of free radicals
- Preventative antioxidants (sequestration of metal ions)- those which prevent the participation of transition metal ions in free radical generation.
- Those which are Free radical SCAVENGERS (chain breaking) Antioxidants.

Natural Antioxidants:

a) Enzyme Antioxidants

- Super oxide Dismutase (SOD) – intracellular removes superoxide radical by speeding their dismutation.
- Catalase – intracellular removes H_2O_2
- Glutathione Peroxidase – Selenium containing enzyme removes H_2O_2 and other peroxides , regenerates ascorbate from dehydroascorbate and detoxification of xenobiotics.

b) Natural Preventive Antioxidants

- Transferrin & lactoferrin
- Caeruloplasmin – Cu containing has ferroxidase activity, prevents Fe^{++} from reacting with H_2O_2.
- Albumin = SH- groups contribute to chain breaking antioxidants

c) Natural Chain Breaking antioxidants

- Ascorbic acid – potent inhibitor of lipid peroxidation (25-100 µmol/L) regenerates tocopherol.
- Uric acid, bilirubin, thiols etc.
- Tocopherol (20-30 µmol/L) – Major chain breaking antioxidant and oxides to un reactive tocopherol radicals.
- Beta carotene – Lipid soluble – Retinol (vitamin A) precursor synergises with tocopherol in lipid peroxidase.
- Ubiquinol -10 - Reduced from Coenzyme Q_{10}
- Flavanoids – fruit, vegetables & wing plays a contributory role.
- Estrogens – some activity has been detected in vitro but not in vivo

Pharmacological antioxidants:

a) Augmenting antioxidant enzymes

- SOD – Generated by recombinant synthesis in pharmacological doses, SOD may reduce adherence neutrophils to endothelium.
- Catalase – Liposome encapsulated or PEG conjugated

b) Preventive Antioxidants

- Desferoxamine – Iron chelator – useful in Thalassemia

c) Scavenging (chain breaking antioxidants)

- Probucol – retards atherosclerosis, regression of Lipid deposits (xanthlasma)
- Salicylates – NSAIDs have antioxidant and radical scavenging effects.
- Lazaroids – (21 aminosteroids) – Inhibits iron dependent Lipid peroxidation.
- Mannitol, Dimethyl sulfoxide, Dimethyl thiourea – Hydroxy radical scavengers.
- Drugs: Captopril, Beta Blockers, Calcium antagaonists, Cinnarizine, Amiodarone, NSAIDs, Methylprednisolone etc.,

d) Xanthine Oxidase Inhibitors

- Allopurinol and oxypurinol

  Neutrophil & macrophage inhibitors (NADPH oxidase inhibitors) eg; adenosine NSAIDs ,Ca antagonists

  Antineutrophil serum – depletes circulating neutrophils
- Antiadhesion agents – Monoclonal antibodies- CD 11/CD18

Sources of Antioxidants

- Allium sulphur compounds: onion and garlic
- Anthocyanins: grapes and berries
- β carotene: pumpkin, mangoes, apricots, carrots and spinach
- Flavonoids: green tea, citrus fruits, red wine, onion and apples
- Isoflavonoids: soyabeans, lentils, peas and milk; Lutein: spinach
- Polyphenols: thyme;Selenium – seafood, lean meat and whole grains
- Vitamin C: oranges, mangoes, spinach and strawberries

Therapeutic Applications of Antioxidants

Antioxidants scavenge the free radicals from the body cells and prevent or reduce the damage caused by oxidation. Diets high in antioxidants may also be associated with a decrease risk of breast cancer.

Studies have shown that antioxidant therapy improves responses to therapeutic drugs and stop fibrinogenesis in people with hepatitis of various cases. Antioxidants have been shown to raise CD 4^{+} cells. This is considered an important goal for the management of HIV.

In heart disease and stroke, it is possible that higher levels of antioxidants slow or prevent the development of arterial blockages, a complicated process involving the oxidation of cholesterol.

Antioxidants are effective scavenges of super oxide and other oxygen reactive species and they prevent endothelial dysfunctions caused by hyperocysteinemia. Vitamin E potentially provides additional risk reduction in retinopathy and nephropathy in addition to those achievable through insulin therapy. Therefore, antioxidants can be considered as potential prophylactic and therapeutic agents for a number of disease conditions.

3.12 ORGANISM PROFILE

3.12.1 ORGANISM : I

Microorganism : *Mucor rouxii* MTCC 386

Taxonomic classification

Kingdom : Fungi

Phylum : Zygomycota

Class : Zygomycetes

Order : Mucorales

Family : Mucoraceae

Genus : *Mucor*

Species : *rouxii*

Fig.7: Colonies on potato dextrose agar

Description and Natural Habitats

Filamentous fungus found in soil, plants, decaying fruits and vegetables. Ubiquitous in nature and a common laboratory contaminant.

Mucor species may cause infections in man, amphibians, cattle and swine.

Macroscopic Features

Colonies of *mucor* grow rapidly at 25-30^{o}C and quickly cover the surface of the agar. Its fluffy appearance with a height of several cm resembles cotton candy. From the front, the color is white initially and becomes grayish brown in time. From the reverse, it is white.

Microscopic Features

Nonseptate or sparsely septate, broad (6-15µm) hyphae, sporangiophores, sporangia, and spores are visualized. Intercalary or terminal arthrospores (oidia) located through or at the end of the hyphae and few chlamydospores.

(a) (b)

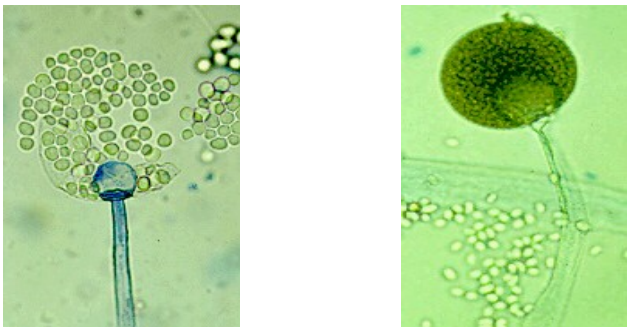


Fig. 8: (a) Sporangiospores,(b)Sporangia

Laboratory Precautions

No special precautions other than general laboratory precautions are required.

Pathogenicity and Clinical Significance

Mucor species are among the fungi causing the group of infections referred as zygomycosis. Zygomycosis includes muco- cutaneous and rhinocerebral infections, as well as septic arthritis, dialysis-associated peritonitis, renal infections, gastritis and pulmonary infections.

Susceptibility

Amphotericin B, Ketoconazole, Itraconazole, and Voriconazole

3.12.2 ORGANISM: II

Microorganism : *Mycotypha africana* NCIM 1230

Taxonomic classification

Kingdom : Fungi

Phylum : Zygomycota

Class : Zygomycetes

Order : Mucorales

Family : Mycotyphaceae

Genus : *Mycotypha*

Species : *africana*

Fig. 9: Colonies on potato dextrose agar

Description and Natural Habitats

Filamentous fungus found in dung and soil

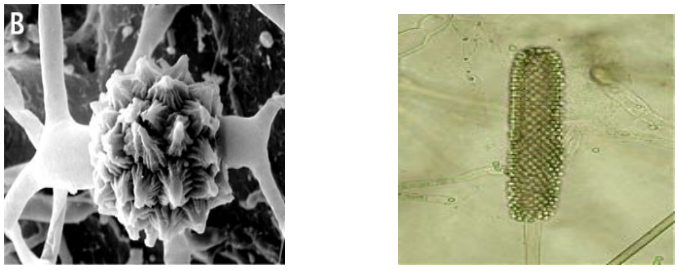
Macroscopic Features

Mycotypha species form yeast cells when grown on nutrient rich media such as Meye (Malt extract – Yeast extract agar). Colonies of *mycotypha* grow rapidly at 25-35^{o}C and initially white, becoming pale violet as sporulation occurs; short nap. Heliotropic (fructifications incline toward light source); formed in darkness.

Microscopic Features

Species of *mycotypha* produce cylindrical vesicles at the apex of sporangiophores that usually are initially simple but that can become branched in age. The vesicles bears two distinct types of dentricles: 1) one that is short and that bears a globose, unispored sporangium, and 2) a longer one that give rise to an obovoid or cylindrical unispored sporangium. Zygospores are formed between opposed suspensors, and an ornamented and

pigmented zygosporangium is produced. (Benny *et al*., 1985). *M.africana* do not form a columella in their unispored sporangia. (Fenner *et al*., 1932)



(a) (b)

Fig. 10: (a) Zygosporangium, (b) cylindrical vesicle

Laboratory Precautions

No special precautions other than general laboratory precautions are required.

Pathogenicity and Clinical Significance

No literature reports of human or animal disease

I. SCOPE AND PLAN OF WORK

The aim of the present work was to isolate and characterize chitosan from two dimorphic fungi and explore some of its applications. This work was taken up for the following purposes.

1. To produce a natural polysaccharide which is pharmaceutically very important.
2. To overcome the disadvantages of the traditional processes of chitosan production using crustacean shells.

1. To produce a natural polysaccharide

Approximately 140 million tonnes of synthetic polys- accharides are extremely stable, their degradation cycle in the biosphere is limited. This problem necessitates the need for natural biodegradable polysaccharides from the renewable sources which fit into the ecological cycle. Nowadays, a variety of natural polysaccharides have become available for use in many applications that are biocompatible and eco-friendly. Among this chitin and its derivative chitosan have been chosen for this study because of their versatile applications in pharmaceutical, biomedical, food and agricultural fields.

2. To overcome the disadvantages of the traditional processes of chitosan production using crustacean shells.

Currently, commercial chitosan is mostly produced from the crustacean shells. This source contains a high percentage of $CaCO_3$ which releases CO_2 into the environment which cannot be considered to be an eco-friendly process. The seasonal supply of crustacean shells and lengthy extraction process contributes to high production costs that limit the actual industrial demand of this polysaccharides.

Perhaps, the most sustainable alternative source in the long run is fungal mycelial

wastes as it contains chitin and chitosan in their cell wall components. The non-animal origin of the fungal chitosan avoids the risk of allergy, reduces the extraction time and cost by growing on simple medium. It also controls the molecular weight and degree of deacetylation of chitosan.

In the current study, *Mycotypha africana* NCIM 1230 was used as test organism and *Mucor rouxii* MTCC 386 was used as a reference, for the production of natural polysaccharide, **Chitosan**.

We intended to isolate, optimize, characterize and study the uses of the chitosan from *Mucor rouxii* MTCC 386 and *Mycotypha africana* NCIM 1230

Thus the research was focused to attain the following goals.

1. To screen dimorphic fungal strains producing chitosan.

2. To evaluate the optimal harvesting time of fungi for maximal chitosan production.

3. To find out the suitable carbon source, nitrogen source and optimum pH for the maximal production of chitosan.
4. To characterize isolated chitosan by FT-IR, ^{1}H NMR spectroscopy, X-ray diffraction studies, Viscometry, Ash content and Moisture content.

5. To evaluate fungal chitosan for its free radical scavenging properties and anti-microbial activities.

IV. REVIEW OF LITERATURE

- Xiao-Fang Li., *et al*., (2008) studied the antifungal activities of chitosan with different molecular weights and concentrations against *Aspergillus niger in vitro*. The results showed that the antifungal activity of chitosan against *Aspergillus niger* is molecular weight and concentration dependent. The smaller the molecular weight, the stronger would be the antifungal activity. Chitosan with higher molecular weight and concentration has no antifungal but promotion activity towards *Aspergillus niger*. The effects of chitosan on *Aspergillus niger* and hyphal ultrastructure were examined to gain more information on its mode of action. The ultrastructure morphology investigated by transmission electron microscopy results indicated that chitosan acts on *Aspergillus niger* by inhibiting the growth of sporules. The fluorescein isothiocyanate labelled chitosan observation has elucidated the antifungal activity of chitosan to be caused mainly by inhibiting the DNA to RNA transcription.

- Maghsoodi, V., *et al.*(2008) studied the effect of different nitrogen source substrates on the amount of chitosan production by *A. niger* PTCC 5012 The organism was grown on soy bean, corn seed and canola residues at 30∞C for specified cultivation days under sterilized conditions. Chitosan was extracted from the fungal mycelia using hot alkaline and acid treatment. The results were shown that soy bean residue at moisture of 37% and 8.4 ± 0.26% of nitrogen content produced the highest amount of chitosan (17.053 ± 0.95 g/kg of dry substrate), after 12 days of incubation. Corn seed residue produced very low amount of chitosan (1.9 ± 0.4% of nitrogen content). The chitosan was analyzed by FTIR spectroscopy and its spectrum was recorded.

- Dina Raafat *et al*., (2008) investigated the antimicrobial mode of action of chitosan using a combination of approaches, including in vitro assays, killing kinetics, cellular leakage measurements, membrane potential estimations, and electron microscopy, in addition to transcriptional response analysis. Chitosan, whose antimicrobial activity was influenced by several factors, exhibited a dose-dependent growth-inhibitory effect. Chitosan treatment of *Staphylococcus simulans* 22 cells did not give rise to cell wall lysis; the cell membrane also remained intact. Analysis of transcriptional response data revealed that chitosan treatment leads to multiple changes in the expression profiles of *Staphylococcus aureus*

SG511 genes involved in the regulation of stress and autolysis, as well as genes associated with energy metabolism. Finally, a possible mechanism for chitosan's activity is postulated. Binding of chitosan to teichoic acids, coupled with a potential extraction of membrane lipids (predominantly lipoteichoic acid) results in a sequence of events, ultimately leading to bacterial death.

- Stamford. *et al*., (2007) studied the microbiological processes for chitin and chitosan productions by *Cunninghamella elegans* (UCP542) grown in a new economic culture medium. The assay was carried out to evaluate the growth of *Celegans* using yam bean medium, in different times of growth (24, 48, 72 and 96hrs), incubated at 28∫C in an orbital shaker at 150 rpm. The lyophilized biomass was determined by gravimetry. The polysaccharides were extracted by alkali-acid treatment, and characterized by infrared spectroscopy, titration and viscosity. *C.elegans* grown in the yambean medium and produced higher yields of biomass (24.3g/ mL) in 96 hrs. The high level was chitosan (66mg/g), and chitin (440 mg/g) were produced at 48 and 72 hrs of growth, respectively. The polysaccharides showed degree of de-acetylation and viscosimetric molecular weight as: 6.2% and $3.25x10^4$ g/mol for chitin, and 85% and 2.72×10^4 g/mol for chitosan, respectively..

- Yen *et al*., (2007) reported the antioxidant properties of fungal chitosan from shiitake stipes. Fungal chitosan B or C was prepared by alkaline N-deacetylation of crude chitin B or C for 60, 90 and 120 min, which was obtained from air-dried shiitake stipes. Chitosan showed antioxidant activities of 61.6–82.4% at 1 mg/ml and showed reducing powers of 0.42–0.57 at 10 mg/ml. At 10 mg/ml, scavenging abilities of chitosan B60 and C60 on 1,1-diphenyl-2- picrylhydrazyl (DPPH) radicals were 28.4–31.3% whereas those of chitosan B90, B120, C90 and C120 were 44.5–53.5%. With regard to the scavenging ability on hydroxyl radicals at 0.1 mg/ml, chitosan B60 and C60 were 61.9% and 63.6%, chitosan B90 and C90 were 68.3% and 69.9% and chitosan B120 and C120 were 77.7% and 77.2%, respectively. At 1.0 mg/ml, chelating abilities of chitosan B60 and C60 on ferrous ions were 88.7–90.3% whereas those for the other chitosan were 97.8–103%. EC_{50} values of antioxidant activity were below 1 mg/ml whereas reducing power and scavenging abilities on DPPH radicals were 7.69–16.3 mg/ml. EC_{50} values of scavenging abilities on hydroxyl radicals were below 0.1 mg/ml whereas chelating abilities of ferrous ions were 0.58–0.69 mg/ml.

- Zamani *et al*., (2007) reported extraction and precipitation of chitosan from cell wall of zygomycetes fungi using dilute sulphuric acid. Chitin is insoluble in neither cold nor hot dilute sulphuric acid. Similarly chitosan is not soluble at room temperature but is dissolved in 1% H_2SO_4 at 121^0 C within 20 min. The new method was developed to measure the chitosan content of the biomass and cell wall of fungus *Rhizomucor pusillus* and results indicated 8% of the biomass as chitosan.

- Di Mario *et al*., (2007) reported isolation of chitin and chitosan isolated from the mycelium of seven species of Basidiomycetes and evaluated the possibility of using fungal biomass as a source of chitin and chitosan. The material was characterized for its purity and degree of acetylation. Chitin yields ranged between 8.5 and 19.6% dry weight and the chitosan yield was approximately 1%. The characteristics of the fungal chitins were similar to those of commercial chitin.

- Nwe *et al*., (2007) reported the decomposition of mycelial matrix and extraction of chitosan from *Gongronella butleri* USDB 0201 and *Absidia coerulea* ATCC 14076. Free chitosan, 2g/100g mycelia from *Gongronella butleri* and 6.5g/100g mycelia from *Absidia coerulea* were isolated by 1M NaOH at 45^oC for 13hrs and 0.35M acetic acid at 95^oC for 5hrs. According to these results, *G. butleri* has higher amount of complexed chitosan and *A. coerulea* has higher amount in free chitosan.

- Kalaivani Nadarajah *et al*., (2006) reported that chitosan was extracted from 60-hour old biomass of *Absidia* sp. dr was subjected to different alkaline and acid treatment in the extraction protocol. In both alkaline and acid treatment, the temperature, incubation period and concentration of acid or alkaline significantly affects the production and quality of the chitosan produced ($P<0.05$). Quality analysis was performed to determine the degree of deacetylation, molecular weight and colour of chitosan. Usage of hydrochloric acid in the extraction of chitosan gave a significantly higher degree of

deacetylation (DD) compared to acetic acid and formic acid. The average molecular weight of selected chitosan samples obtained ranged from 6.765 × 10^4 Da to 2.757×10^5 Da. Employment of strong acid, high acid concentration and high temperature produced darker coloured chitosan whereas milder treatments gave lighter coloured chitosan.

- Amorim *et al*., (2006) investigated inexpensive carbon sources from sugar cane process for fungal growth and chitosan production from a promising fungus *Cunninghamella bertholletiae* IFM 46.114 in submerged culture and and to analyze its quality. In the traditional medium for the growth of mucoralean strains, constituted of yeast extract, peptone, and D-glucose as carbon source, the highest chitosan yield found was 55 mg/g of dry of dry mycelia in a 72 hours submerged culture. Sugar cane juice and molasses, which were supplemented with 0.35% yeast extract, were used as economic substrates to produce chitosan. The optimal production of chitosan was found in sugar cane juice medium, yielding 128 mg/g of dry mycelia in batch flasks at 28^{0}c. This condition did not need high concentration of sugar cane and gave a good yield of chitosan produced within 48 hr (580 mg per L of medium). Molasses did not show to be a good carbon source for chitosan production.

- Chatterjee *et al*., (2005) reported the production and physico-chemical characterization of chitosan from *Mucor rouxii*: Fungal culture media and fermentation condition can be manipulated to provide chitosan of more consistent physico-chemical properties compared to that derived chemically from chitin using three different media, viz., molasses salt medium, Potato Dextrose Broth and yeast extract peptone glucose medium under submerged condition. Their physico-chemical properties such as ash, moisture, protein contents and specific rotation did not show much difference.

- Seh *et al*., (2005) studied the effect of fungal chitosan on the proliferation of murine lymphocytes in comparison with crustacean chitosan. Fungal chitosan (FC) was extracted from the mycelia of *Absidia butleri* DR whereas the crustacean chitosan (CC)

is of shrimp origin. Intraperitoneal administrations of FC at 50 mg/kg or 250mg/kg body weight for 1 week or 3 weeks showed no significant effects on the proliferative responses of lymphocytes to Con A or LPS stimulation. In contrast, administration of CC at 50 mg/kg and 250 mg/kg for 1 week and 50 mg/ kg for 3 weeks significantly enhanced proliferation of lymphocytes stimulated with LPS. Neither FC nor CC was found to affect proliferation of lymphocytes *in vitro*. The proliferative response was suppressed when 100 µg/ml FC was co incubated with Con A but not with LPS. Coincubation of Con A with CC at 50 µg/ml and 100 µg/ml resulted in significant suppression of lymphoproliferation. CC at 100 µg/ml was also found to exhibit significant suppression in the proliferative response to LPS.

- Salwa A.Khalaf., (2004) developed a method for the laboratory-scale production, isolation, and characterization of chitosan by solid-state fermentation (SSF) from different fungal strains. The maximum chitosan yield was 5.63g/kg of fermentation medium. It was attained by growing fungal strain *Rhizopus oryzae* on rice straw under SSF conditions for 12 days. Fungal chitosan, had a degree of deacetylation of 73-90% with a viscosity of 2.7 - 6.8 centipoises (cP). *R. oryzae* chitosan produced at concentration of 60 mg/L exhibited the maximum antibacterial activity, compared with crab shell chitosan.

- Franco *et al*., (2004) extracted chitin and chitosan from mycelial biomass of *Cunninghamella elegans* and the performance for copper, lead and iron biosorption in aqueous solution was evaluated. The growth curve of *C. elegans* was accomplished by determination of biomass, pH, glucose and nitrogen consumption. Chitin and chitosan were extracted by alkali-acid treatment and the yields were 23.8 and 7.8%, respectively. For the adsorption analysis, the process of heavy uptake metal sorption was evaluated using polysaccharides solutions (1% w/v). The rate of metallic biosorption was dependent upon the concentration and pH of metal solutions, and the best results were observed with pH 4.0. Chitosan showed the highest affinity for copper and chitin for iron adsorption. The results suggest

that *C. elegans* (IFM 46109) is an attractive source of production of chitin and chitosan, with a great potential of heavy metals bioremediation in polluted environments

- New and Stevens. (2004) reported the effect of urea on fungal chitosan production in solid substrate fermentation. The fungus *Gongronella butleri* USDB 0201 was grown on sweet potato pieces supplied with different amounts of urea at 26^0C for 7 days. Crude chitosan was extracted and treated with amylolytic enzyme to remove bound glucan. The distribution of the molecular weight of the chitosan was studied by gel exclusion chromatography. The conditions for optimal production of fungal chitosan by solid substrate fermentation were investigated. The initial pH and the amount of urea influence the yield of fungal mycelia and chitosan.

- Lavertu *et al*., (2003) reported a method for the determination of the degree of deacetylation (DDA) of chitosan by ^{1}H NMR spectroscopy has been formally validated. Chitosans with DDA ranging from 48 to 100% have been used for the validation. The method is found to be simple, rapid and more precise than other known techniques like IR or titration for %DDA measurements. The precision, ruggedness, robustness, specificity, stability and accuracy of the technique are discussed in this paper.

- Pochanavanich and Suntornsuk. (2002) studied the production and characterization of fungal chitosan. Chitosan production by several species of fungi, including two yeast strains namely *Zygosaccharomyces rouxii* and *Candida albicans*, were investigated for their ability to produce chitosan in complex media. Fungal chitosan had a degree of deacetylation of 84-90% and a molecular weight of 2.7×10^4-1.9×10^5 Da with a viscosity of 3.1-6.2 centipoises (cP). *Rhizopus oryzae* TISTR3189 was found to be the maximum producer of chitosan.

V. MATERIALS AND METHODS

5.1. APPARATUS

Assay Petri dishes	-	Anumbra
Centrifuge tubes	-	Borosil
Glass pipettes	-	Borosil
Micropipettes	-	Vari Pipettes
Sterile discs	-	Hi Media
Sterile swab	-	Hi Media
Standard flask	-	Borosil
Test tubes	-	Borosil

Ingredients	**Manufacturers**
Acetic acid	Qualigens, Mumbai
Acetone	Qualigens, Mumbai
Agar-Agar	Himedia labs ltd, Mumbai
Ammonium sulphate	S.d fine chemicals ltd, Mumbai
Arginine	Loba chemie pvt. Ltd
Calcium chloride	S.d fine chemicals ltd, Mumbai
Corn starch	Himedia labs ltd, Mumbai
Corn steep liquor	Sigma-Aldrich
Dextrose	Loba chemicals
DPPH	Sigma-Aldrich, Germany
Ethanol	Qualigens, Mumbai
Glucose	S.d fine chemicals ltd,mumbai
Magnesium sulphate 7 H_2O	S.d fine chemicals ltd,Mumbai
Maltose	Nice chemicals, Cochin
Maltose	Loba chemie pvt. Ltd
Methanol	Qualigens, Mumbai
Non absorbent cotton	Ramaraju surgical cotton ltd
Peptone	Himedia Labs ltd,Mumbai
Ponceau S	Loba Chemie pvt. Ltd
Potassium bromide	S.d Fine Chemicals Ltd,Mumbai
Potassium chloride	S.d Fine Chemicals Ltd,Mumbai
Potassium phosphate dibasic	Loba Chemie Pvt. Ltd
Sodium chloride	S.d Fine Chemicals Ltd,Mumbai
Sodium hydroxide	Hi-Pure Fine Chem. Ltd Chennai
Soy bean meal	Himedia Labs Ltd,Mumbai
Stains -all	Sigma-Aldrich, Ugraine
Sucrose	Commercial
Tween 20	Loba Chemie Pvt. Ltd
Tween 80	Loba Chemie Pvt. Ltd
Yeast extract powder	Himedia Labs Ltd,Mumbai

5.2. INSTRUMENTS

Instruments	Company
Hot air oven	Technico
Incubator	Technico
Autoclave	Universal autoclave
Conical flask	Borosil
Centrifuge	Eppendorf
Compound microscope	Motic
Digital balance	Shimadzo
Heating mantle	Guna enterprises
vertical laminar flow hood	Technico
Digital Orbital shaker	Remi motors
Micro pipettes	Vari pipettes (Hi –Tech lab)
Micro tips	Tarsons

5.3. CULTIVATION MEDIA AND CONDITIONS

Maintenance media

All the strains obtained from National collection of Industrial Microorganisms, National Chemical Laboratory, Pune, except *Mucor rouxii* MTCC 386 which was obtained from Microbial Type Culture Collection, Genebank, Chandigarh, India. The cultures were maintained on a Potato Dextrose Agar slant. This culture was transferred once a month to a fresh slant.

5.4 Identification of fungi by microscopy: (Aneja K.R., et al.,2001)

Scotch tape preparation for studying morphology of fungi

This is a rapid technique for preparing a temporary microscopic mount of a fungus without disturbing the arrangement of conidia and conidia bearing hyphae, the conidiophores.

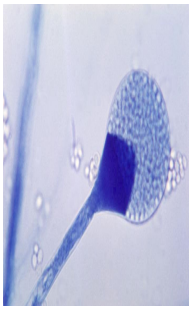
Requirements

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 was placed in the centre of the slide.
2. The transparent adhesive tape was held with sticky side down, between thumb and forefinger of each hand and pressed firmly.
3. The centre of the sticky side of the tape was pressed firmly on to the surface of the fungus colony, where sporulation was visible.
4. The tape was gently pulled away from the colony and placed on the drop of lactophenol cotton blue.
5. The extended ends of the tape were folded over the ends of the slide.

Observations:



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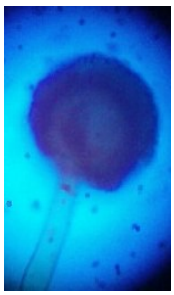

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Fig.11: Lacto phenol cotton blue staining (40x)

1. ***Mucor rouxii MTCC 386***
2. ***Mycotypha africana NCIM 1230***

5.5 Identification of chitosan containing fungi (My lien Dao. 2007)

This method is a novel method of staining for the detection of chitosan containing fungi and protozoa under a light microscope.

Requirements

This staining method comprises five reagents

1. 0.1% Ponceau S in 5% acetic acid in water.
2. 0.2% stains all in methanol (stock solution), which is diluted 1:10 in a solution of acid Methanol (solution 4) before use.
3. 0.25% sodium dodecyl sulphate in a phosphate buffered saline.
4. Acid methanol (50:10:40 deionized water: Acetic acid: Methanol)
5. Solution of PBS containing 0.05% Tween 20 and microscope.

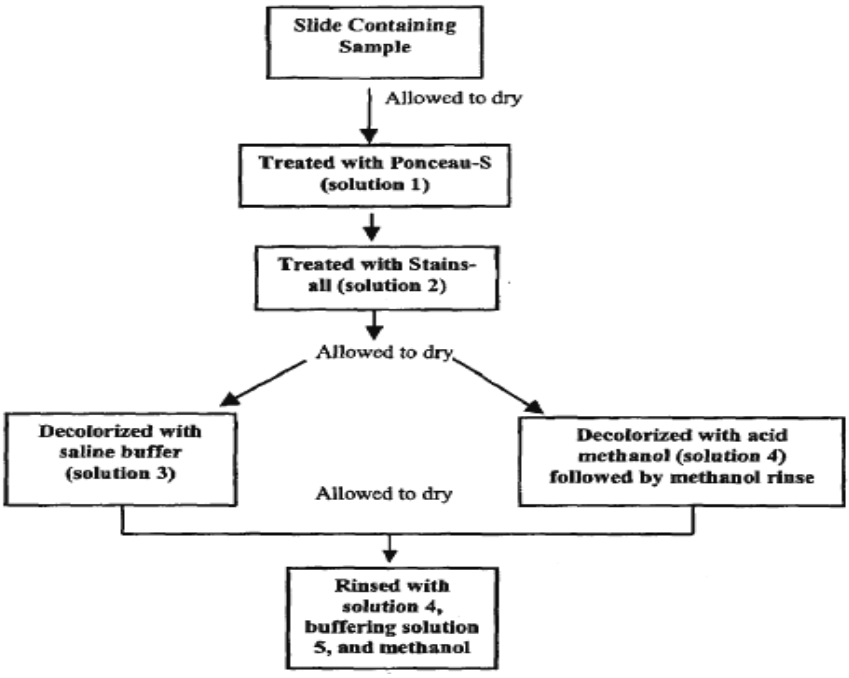


Fig. 12: Flow diagram demonstrating method of staining

Observation

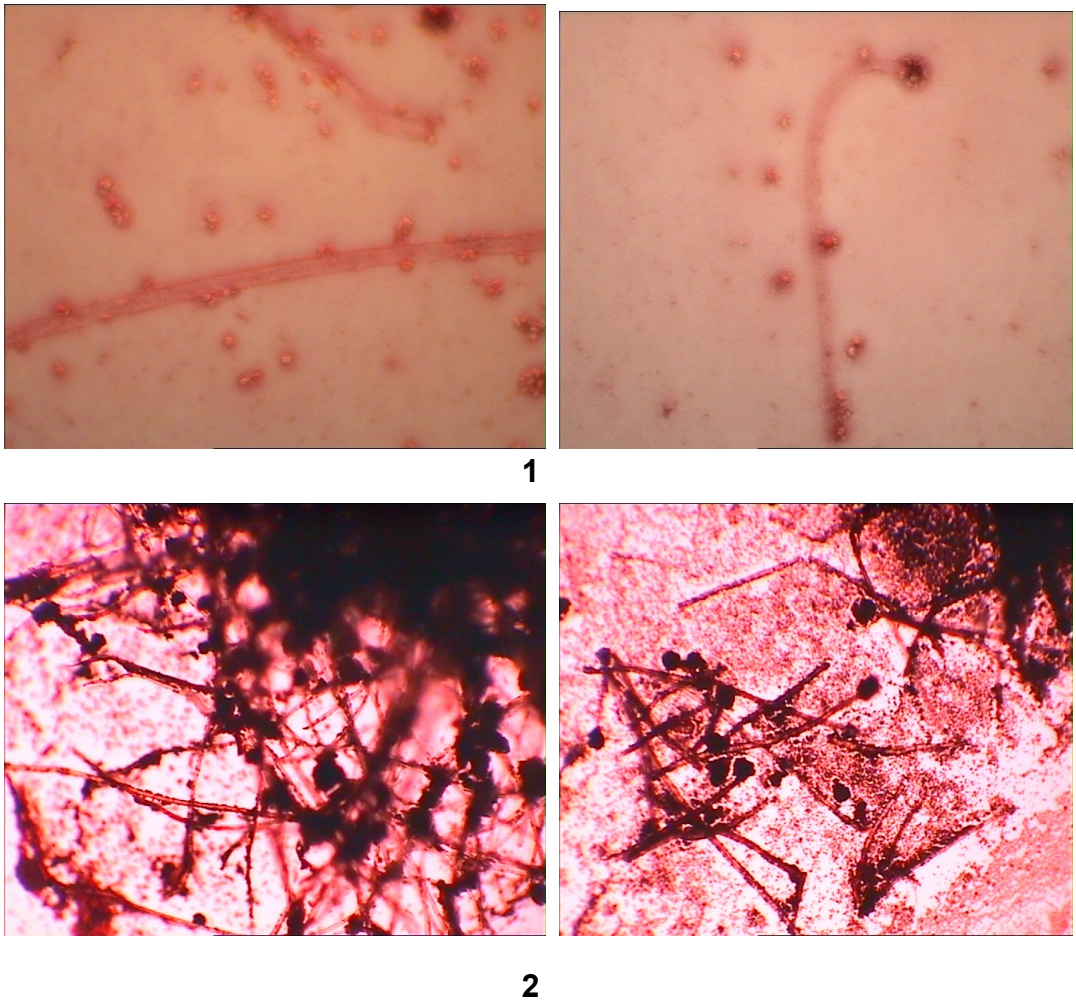


Fig. 13 . Photographic slides of the chitosan staining in fungi (40x)

1. *Mucor rouxii* MTCC 386
2. *Mycotypha africana* NCIM 1230

5.6 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 ($1.8xl0^6$ spores/ml) was prepared for each fungal species and used to inoculate the fermentation broth.

5.7. Fermentation medium (Chen M.H., *et al*., 2001)

The fermentation medium contains 20g/L of glucose, 10g/L of peptone, 1g/L of yeast extract, 5 g/l of ammonium sulphate, 1g/L of a K_2HPO_4, 1g/L of $MgSo_4.7H_2O$, 0.1g/L calcium chloride and sodium chloride 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.

5.8. 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 1M sodium hydroxide (1:30g/v) and the mixture was autoclaved at 121˚ C for 15 minutes. The mixture was subsequently filtered (no.1Whatman) to sediment the alkali insoluble materials (AIM) and washed with distilled water and ethanol. The washed 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 filtration (no.1Whatman) 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 M sodium 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).

5.9. Determination of growth curve, extractable chitin and chitosan (Shimahara K*., et al* 1998)

The growth curves, extractable chitosan and chitin of *Mucor rouxii* and *M.africana* 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 chitosan and chitin after 48, 72, 96, 120, 144 and 168 hours of growth were determined as described above. Three replicate cultures were prepared for each incubation period.

5.10. Medium optimization (Chen MH., *et al*., 2001; Chen MH., *et al*., 2002)

In the media optimization for the two strains two variations in the experimental procedure was adopted.

5.10.1. 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.

5.10.2. 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, and urea were used.

5.10.3 pH optimization (Elayaraja, *et al*., 2005)

The optimum pH for the fermentation process of *Mucor rouxii* MTCC386 and *Mycotypha africana* NCIM 1230 for the production of fungal chitosan was determined by carrying out the various pH ranges from 3 to 7. The fermentation medium was prepared as mentioned above and adjusted to a final pH such as 3,5 and 7 by using 1N HCl and 1N NaOH.

5.11. Chitosan characterization

5.11.1. Infrared spectroscopy (Deacetylation degree-DDA %) (Stamford MCT, et al.,2007)

The degree of deacetylation is determined by using the absorbance ratio 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 overnight at 60˚C was thoroughly blended with 100mg of KBr, to produce 0.5 mm thick discs. Spectrum was recorded using JASCO FTIR-410 in the Pharmaceutical analysis laboratory, College of Pharmacy, SRIPMS, Coimbatore-44.

5.11.2 ^{1}HNMR (Lavertu M., *et al*., 2003)

^{1}H NMR 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 tubes. A spectrum was recorded on Bruker at Central Electrochemical Research Institute, Karaikudi.

5.11.3 X-ray diffraction (Chatterjee *et al* 2005)

The X-ray powder diffractometry is the most powerful and established technique for material structural analysis, capable of providing information about the structure of a material at the atomic level.

Powder X-ray diffraction patterns were obtained using a Bruker AXS D8 Advance instrument with the following operating conditions. 40kV and 30mA with a Cu radiation at λ = 1.5406A°. The relative intensity was recorded in a scattering range (2θ) of 3∞- 90∞. The X-ray diffractometry was done at CUSAT, Kochi.

5.11.1. 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^{0}C.

Fig. 14: Brook Field DV-I viscometer

5.11.5 Ash content

Ash of the crawfish chitosan was calculated according to the standard (AOAC, 1990). method. Placed 2.0g of chitosan (triplicate) into previously ignited, cooled, and tarred crucible. The samples were heated in a muffle furnace preheated to 600^{o}C for 6 hr. The crucibles were allowed to cool in the furnace to less than 200∫C and then placed into desiccators with a vented top. Allowed them to cool and weighed crucible and ash.

Calculation: $\frac{\text{(Weight of residue, g)}}{\text{(Sample weight, g)}} \times 100 = \%\ \text{Ash}$

5.11.6 Moisture content (Tajik H, *et al*., 2008)

Moisture content of the crawfish chitosan was determined by the gravimetric method. The water mass was determined by drying the sample to constant weight and measuring the sample after and before drying. The water mass (or weight) was the difference between the weights of the wet and oven dry samples. Procedures were as follows: weighed and recorded weight of dish, placed 1.0g of chitosan sample in duplicates in the metal aluminium dish, recorded weight of dish with sample, then placed the sample with the lid (filter paper to prevent or minimize contamination) in the oven. Adjusted the oven temperature to 60^{o}C, and dried the samples for 24 hrs or overnight. Took the sample from the oven and placed it in a desiccator until it cools to room temperature. Weighed the sample, and recorded this weight as weight of dry sample.

Calculated moisture content as:

$\frac{\text{(wet weight, g - dry weight, g)}}{\text{(wet weight, g)}} \times 100 = \%\ \text{of moisture content}$

5.12 Antioxidant activity by DPPH method (Yena MT 2007).

Principle

DPPH is a free radical, stable at room temperature, which produces a violet 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

Chitosan sample (0.1-10 mg /ml)in 2g/l acetic acid solution (4ml) was mixed with 1ml of ethanolic solution containing DPPH (sigma) radicals, resulting in a final concentration of 10 mmol/l DPPH. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank.

The scavenging ability was calculated as follows:

Scavenging ability % =

[(ΔA_{517} of control — ΔA_{517} of sample) /ΔA_{517} of control] x 100

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

5.13 SCREENING FOR ANTIMICROBIAL ACTIVITY

(Mackie and McCartney 1996)

Media used

Mueller Hinton agar (For Antibacterial)

Ingredients

Peptic digest of animal tissue	-	5gms
Sodium chloride	-	5gms
Beef extract	-	1.50gmd
Yeast extract	-	1.50gms
Distilled water	-	1000ml

Preparation of Media

The ingredients were dissolved in distilled water with aid of heat; pH was adjusted to 7.2 to 7.6 by using dilute alkali /dilute acid.

Potato Dextrose agar (For antifungal)

Ingredients

Glucose	- 1%
Malt extract	- 0.3%
Yeast extract	-0.3%
Peptone	-0.5%
Agar	-2.0%
Distilled water up to	-100 ml
pH	3-5

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 15 psi (121°c) for not less than15 minutes.

Organisms used

Staphylococcus aureus NCIM 5021, *Pseudomonas aeruginosa* NCIM 5021, *Escherichia coli* NCIM 2911, *Bacillus subtilis* NCIM 2010, *Micrococcus luteus* NCIM 2704 and *Proteus vulgaris* NCIM 2027 were procured from National Chemical Laboratory, Pune and stored in the Pharmaceutical Biotechnology Laboratory, College of Pharmacy, SRIPMS, Coimbatore-44. (Clinical isolate of *E.coli, Klebsiella pneumoniae, S.aureus* and *P.aeruginosa* were obtained from Sri Ramakrishna hospital, Coimbatore-44). The strains were confirmed for their purity and identity by Gram's staining method and their characteristic biochemical reactions. The selected strains were preserved by subculturing them periodically on nutrient agar slants and storing them under frozen conditions. For the study, fresh 24 hrs broth cultures 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 sampling was 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 Mueller Hinton broth for bacteria and potato dextrose broth for fungi from preserved frozen slants. It was incubated at 37°C for 18-24 hrs and used after standardization.

Samples used : Saturated solution of isolated chitosan samples and Commercial chitosan (Sigma)

(Mucor rouxii MTCC 386 and *Mycotypha africana* NCIM 1230*)*

Standard used : Ciprofloxacin (5μg/disc)

Vehicle used : 1 % acetic acid

ANTIMICROBIAL SCREENING BY KIRBY-BAUER METHOD (Mackie and McCartney., 1996)

Mueller Hinton Agar and Potato Dextrose agar plates were prepared aseptically to get a thickness of 5-6 mm. The plates were allowed to solidify and inverted to prevent the condensate falling on the agar surface. The plates were dried at 37°C before inoculation. The organism was inoculated in the plates prepared earlier, by dipping a sterile swab in the previously standardized inoculum, removing the excess of inoculum by pressing and rotating the swab firmly against the sides of the culture tube above the level of the liquid and finally streaking the swab all over the surface of the medium 3 times, rotating the plates through an angle of 60° after each application. Finally the swab was pressed round the edge of the agar surface. It was allowed to dry at room temperature, with the lid closed. The sterile disc containing test drug, standard and blank were placed on the previously inoculated surface of the Mueller Hinton and Potato Dextrose agar plate and it was kept in the refrigerator for one hour to facilitate uniform diffusion of the drug. Plates were prepared in duplicate and they were then incubated for 18-24 hrs. Observations were made for zone of inhibition around the drug and compared with that of standard. All the two isolated chitosan samples were tested for antimicrobial activity in duplicates.

VI. RESULTS AND DISCUSSION

6.1 Strain identification

All the two procured strains were confirmed, its macroscopic and microscopic morphology and further work was carried out.

6.2 Chitosan identification in fungi

The chitosan containing fungi were identified under light microscope by novel staining method using Ponceau S and Stains all.

All the two procured strains were stained by Ponceau S and Stains all. It confirmed the presence of chitosan.

Isolated chitosan

(a)

(b)

(c)

Fig.15 (a) Chitosan from *M. atricana* (b) Chitosan from *M. rouxii*

(c) Commercial chitosan

6.3 Growth profile

Graph 1: Growth curve of *Mucor rouxii* MTCC 386 and *M.africana* NCIM 1230

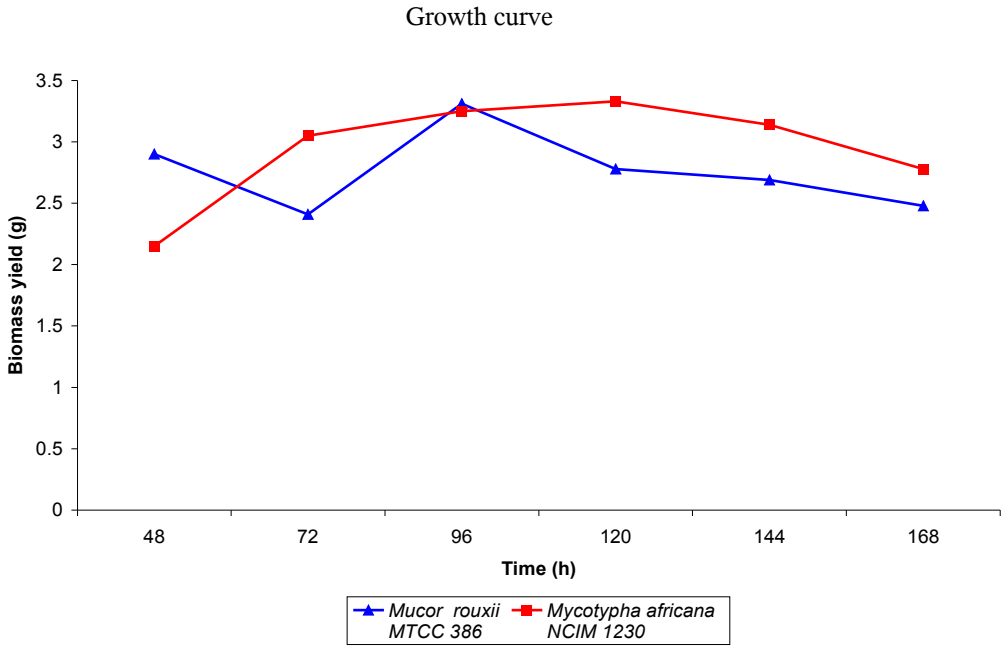


Table No. 2: Weight of biomass, extractable chitosan, chitin and degree of deacetylation of *Mucor rouxii* MTCC 386

S.No	Time (hrs)	Weight of biomass(g)	Weight of chitosan (g)	Weight of chitin (g)	Degree of deacetylation
01	48	2.29±0.05	0.180±0.03	0.31±0.02	85.68±1.43
02	72	2.41±0.01	0.190±0.10	0.32±0.01	92.30±1.75
03	96	3.31±0.002	0.230±0.08	0.52±0.05	92.50±1.36
04	120	2.78±0.07	0.210±0.05	0.36±0.09	82.64±1.82
05	144	2.69±0.01	0.170±0.09	0.60±0.26	90.82±1.57
06	168	2.48±0.03	0.160±0.01	0.50±0.01	86.28±1.06

Values are mean ± S.E.M (n=3)

Graph No.2 Extractable chitosan from *M.rouxii* MTCC 386 with incubation time.

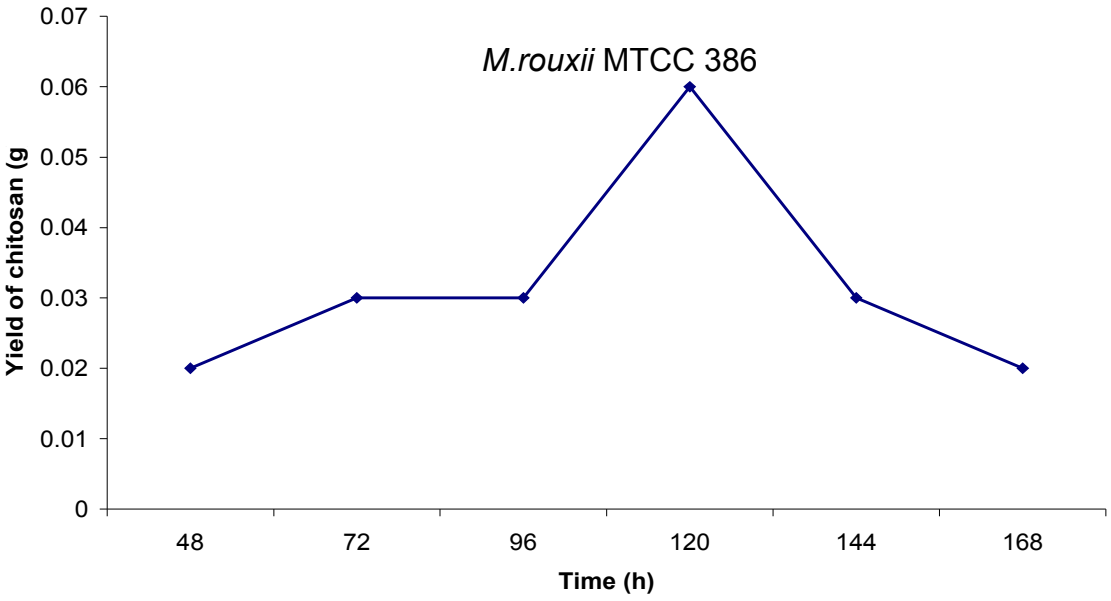
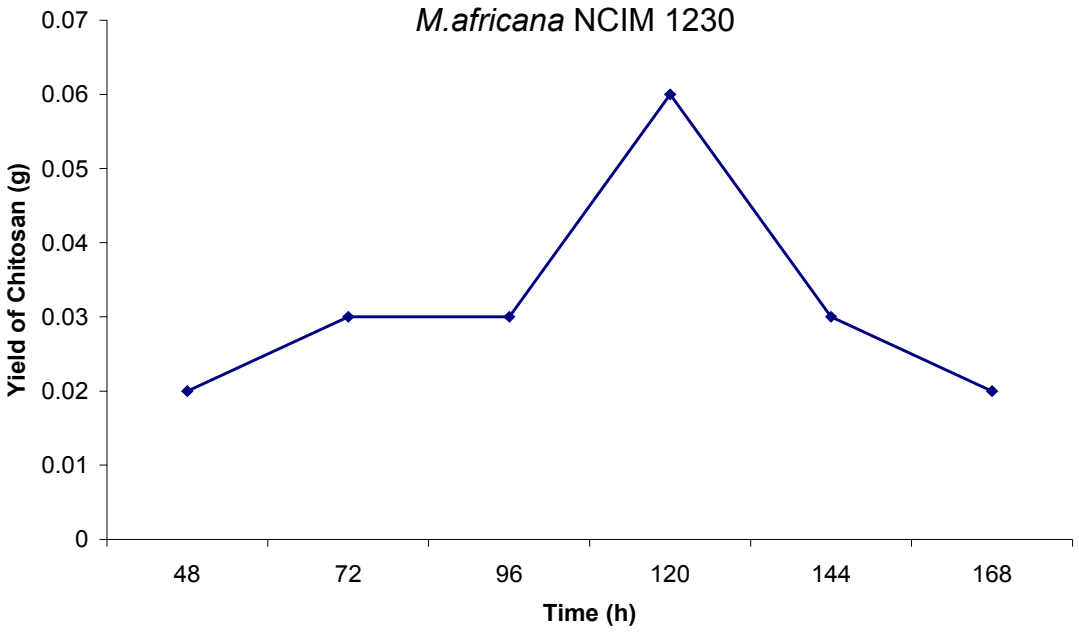


Table No. 3: Weight of biomass, extractable chitosan, chitin and degree of deacetylation of *Mycotypha africana* NCIM 1230

S.No	Time (hrs)	Weight of biomass (g)	Weight of chitosan (g)	Weight of chitin (g)	Degree of deacetylation
01	48	2.15±0.06	0.020±0.01	0.62±0.51	83.38±1.23
02	72	3.05±0.01	0.030±0.005	086±0.01	86.24±1.48
03	96	3.25±0.09	0.030±0.07	1.48±0.03	92.08±1.94
04	120	3.33±003	0.060±0.09	1.26±0.09	90.52±1.25
05	144	3.14±0.04	0.030±0.02	1.34±0.01	88.46±0.88
06	168	2.78±0.01	0.020±0.09	1.28±0.04	85.16±0.68

Values are mean ± S.E.M (n=3)

Graph No. 3 Extractable chitosan from *M.africana* NCIM 1230 with incubation time.



Mucor rouxii NCIM 386 had the highest growth rate with a maximal mycelial dry weight of 3.31g with 0.230 g of chitosan at 96th hours of cultivation, while *Mycotypha africana* NCIM 1230 grew equally with a maximal biomass of 3.33g with 0.06g of chitosan at 120th hrs of cultivation respectively.

The dry weight of mycelia (biomass) and extractable chitosan of the two genus increased with time. The fungal biomass increased rapidly during the first 72 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 curve might be due to physiological changes in the fungal cell wall (McGahren *et al.,* 1984). Chitosan is produced in the fungal cell wall by the enzyme chitin deacetylase. 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 graph 2, 3 indicate that the late exponential growth phase of the fungus would give the best yield for chitosan.

Therefore, both the fungi should be harvested at their late exponential growth phase and the content of extractable chitosan was determined. This is 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 harvesting phase was determined for both the dimorphic fungi used in this study. The harvesting time of *Mucor rouxii* was at 96 hours, and that of *Mycotypha africana* was at 120 hours.

6.4. Medium optimization with relation to chitosan production

The maximal extractable chitosan content was determined for different carbon and nitrogen sources by harvesting at their late exponential phase and the results presented in Table no 4 and 5.

Table no. 4; Comparison of yield of chitosan for different carbon sources

Graph No. 4: Comparison of yield of chitosan from different carbon sources

Medium optimization

Table No. 5: Comparison of yield of chitosan for different nitrogen sources

Strains	Nitrogen source				Carbon source	Mineral salts					Yield (g)	
	SBM	Peptone	C.S.L	Urea	Glucose	K_2HPO_4	$MgSo_4$	$(NH4)_2SO_4$	NaCl	$CaCl_2$	Chitosan	Chitin
M.rouxii	X				X	X	X	X	X	X	0.030	0.068
		X			X	X	X	X	X	X	0.230	0.520
			X		X	X	X	X	X	X	0.225	0.509
				X	X	X	X	X	X	X	0.030	0.068
M.africana	X				X	X	X	X	X	X	0.040	0.62
		X			X	X	X	X	X	X	0.060	1.26
			X		X	X	X	X	X	X	0.070	1.06
				X	X	X	X	X	X	X	0.040	0.27

Graph No. 5: Comparison of yield of chitosan from different nitrogen sources

Medium optimization

The inclusion of glucose as a carbon source and peptone as a nitrogen source led to the highest yield of chitosan and chitin in *Mucor rouxii* MTCC 386.

The inclusion of maltose as a carbon source and corn steep liquor as a nitrogen source led to the highest yield of chitosan and chitin in *Mycotypha africana* NCIM 1230.

The results confirmed that the chitin and chitosan content of the fungi depends on the fungal strains, mycelial age and the composition of the growth medium. (Chatterjee S., et al., 2005)

Table No. 6: Comparison of yield of chitosan for different pH

Strains	pH value			Weight of biomass (g)	Yield (g)	
	3	5	7		Chitin	chitosan
M.rouxii	X			0.38±0.006	0.08±0.03	0.010±0.04
		X		3.31±0.04	0.52±0.01	0.230±0.09
			X	1.71±0.07	0.30±0.09	0.060±0.002
M.africana	X			2.72±0.01	1.13±0.04	0.020±0.01
		X		3.33±0.10	1.26±0.001	0.060±0.03
			X	2.15±0.004	0.62±0.24	0.030±0.009

Graph No. 6: Effect of pH on chitosan yield

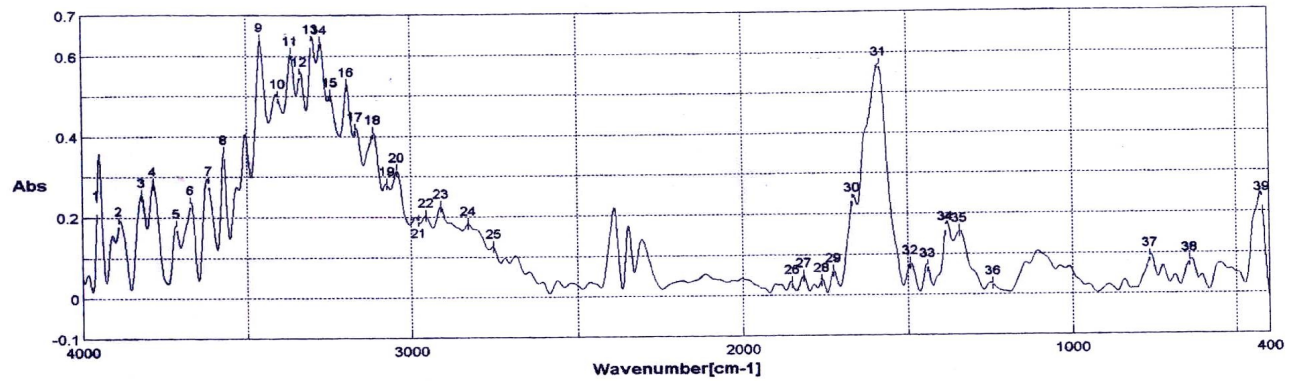
pH Optimization

This study identified that the optimum pH for the chitosan production by *M.rouxii and M.africana* at pH 5, and this may be due to the activity of chitin deacetylase enzyme is more at pH 5 that converts chitin to chitosan in the fungal cell wall.

6.5 Characterization of chitosan by FT-IR and ^{1}H NMR spectroscopy, X-ray diffraction studies, Viscometry, Ash content, Moisture content

6.5.1 FT-IR spectroscopy

To prove that the acid extractable material contains chitosan, its FT-IR spectra were measured in comparison with IR spectrum of commercial chitosan from Sigma. All the two isolated chitosan shown similar FT- IR spectrum to that of the commercial chitosan. The result indicated 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 90-93% but the commercial chitosan showed only 79%.



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Sample Name	commercial chitosan		
Comment			

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1	3955.29	0.224509	2	3887.79	0.183205	3	3816.44	0.255559	4	3780.76	0.283147	5	3713.26	0.17806
6	3668.91	0.23511	7	3614.91	0.28298	8	3570.56	0.360585	9	3457.74	0.639864	10	3404.71	0.498945
11	3364.21	0.605325	12	3337.21	0.553594	13	3301.54	0.634944	14	3274.54	0.633917	15	3243.68	0.50145
16	3194.51	0.527521	17	3167.51	0.416235	18	3113.51	0.40866	19	3073.01	0.282031	20	3042.16	0.315194
21	2979.48	0.191388	22	2956.34	0.203164	23	2911.99	0.22602	24	2830.99	0.182768	25	2754.81	0.124418
26	1850.36	0.0322087	27	1814.69	0.0484793	28	1761.65	0.0354733	29	1725.01	0.0590034	30	1667.16	0.23253
31	1582.31	0.567711	32	1492.63	0.0769829	33	1438.64	0.0699659	34	1384.64	0.160303	35	1340.28	0.156358
36	1241.93	0.0260704	37	762.709	0.0919725	38	642.179	0.0799941	39	422.334	0.227225			

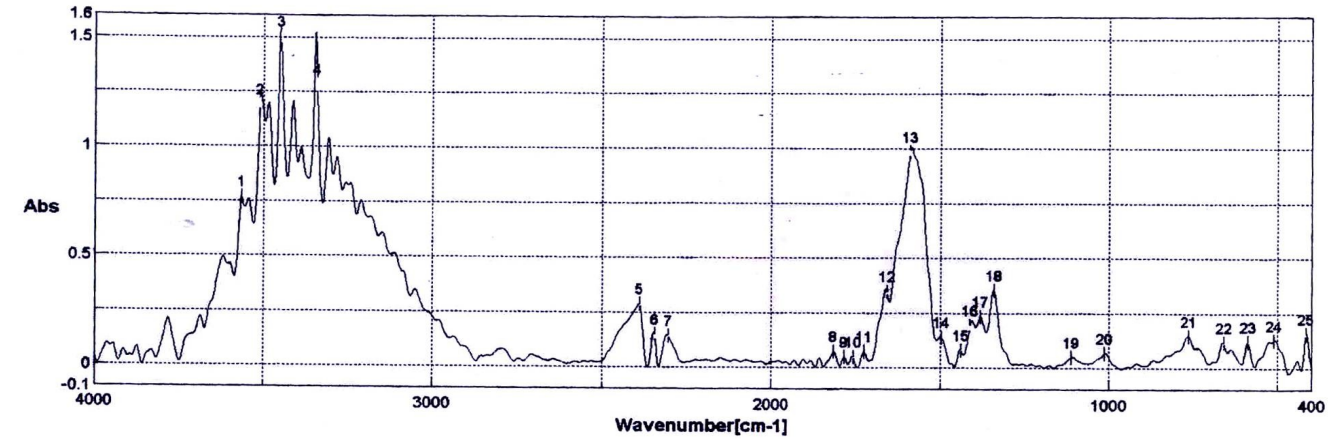
Fig. 16 IR Spectrum of commercial chitosan from sigma chemicals

The degree of deacetylation was calculated using the following formula

DDA = 118.833 – [40.1647(A1655/A3450)]

= 118.833 – [40.1647 (0.23253/0.23511)]

= 79%



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Comment			

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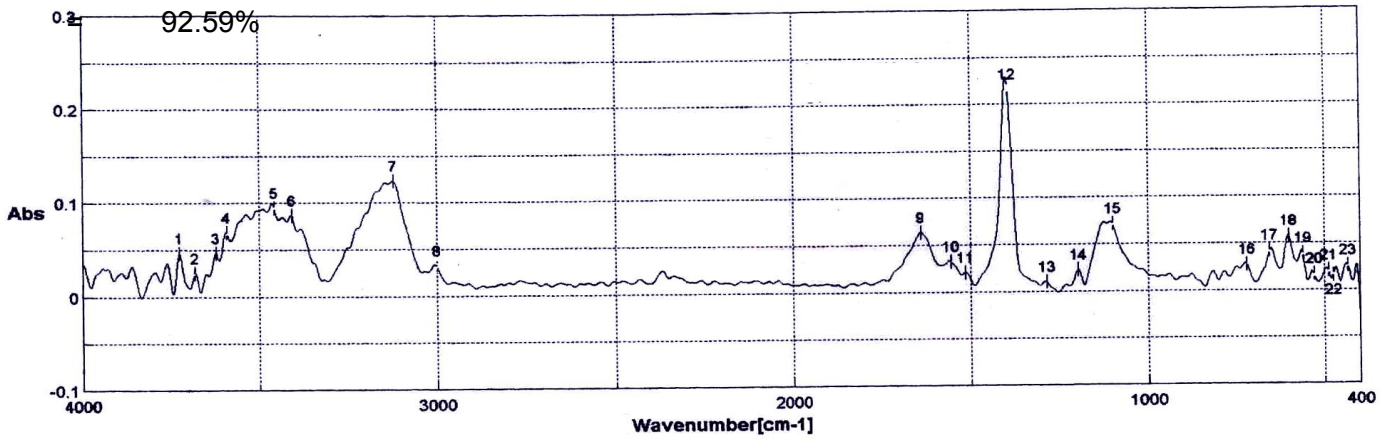
1	3565.74	0.768351	2	3511.74	1.18843	3	3449.06	1.5017	4	3342.03	1.28109	5	2388.41	0.286874
6	2343.09	0.147861	7	2302.59	0.141539	8	1814.69	0.0717487	9	1783.83	0.0472329	10	1756.83	0.0489991
11	1725.01	0.0719659	12	1658.48	0.346781	13	1590.99	0.983053	14	1497.45	0.139711	15	1438.64	0.0788345
16	1411.64	0.192513	17	1380.78	0.234043	18	1340.28	0.353609	19	1111.76	0.0492295	20	1013.41	0.0679006
21	762.709	0.146303	22	659.536	0.116801	23	588.182	0.118393	24	512.008	0.126688	25	417.513	0.159036

Fig. 17 IR Spectrum of isolated chitosan from *Mucor rouxii* MTCC 386

The degree of deacetylation was calculated using the following formula

DDA = 118.833 – [40.1647(A1655/A3450)]

= 118.833 – [40.1647 (0.98305/1.5017)]



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Sample Name	M.africana		
Comment			

No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs	No.	cm-1	Abs
1	3726.76	0.0461795	2	3682.41	0.0249159	3	3623.59	0.0463544	4	3	0.0682635	5	3457.74	0.0946433
6	3408.57	0.086175	7	3122.19	0.123305	8	3001.66	0.0319848	9	6	0.0637243	10	1555.31	0.0324561
11	1514.81	0.0208092	12	1394.28	0.215367	13	1286.29	0.010093	14	1	0.02271	15	1098.26	0.071297
16	718.354	0.0272109	17	655.679	0.0418738	18	601.682	0.0574213	19	4	0.0392015	20	530.328	0.0166662
21	489.831	0.0202212	22	476.331	0.0155133	23	435.834	0.0253963						

Fig.18 IR Spectrum of isolated chitosan from *Mycotypha africana* NCIM 1230

The degree of deacetylation was calculated using the following formula

DDA = 118.833 – [40.1647(A1655/A3450)]

= 118.833 – [40.1647 (0.06372/0.0946)]

= 90.95%

6.5.2. ^{1}H NMR spectroscopoy

The ^{1}H NMR spectra of commercial chitosan was compared with that of the spectra of isolated fraction from two fungal biomass proved that both contains chitosan. H1 proton of the deacetylated polymer resonated at 5.21ppm. Two deacetylated proton of the polymer resonated at 3.52ppm. Signals from proton H2-H6 resonates between 3.9-4.2 and the above results complies with the report of (M.Lavertu et al.2003).

The low intense peak of proton of acetyl group at 2ppm indicates that the isolated fraction are deacetylated more.

CECRI

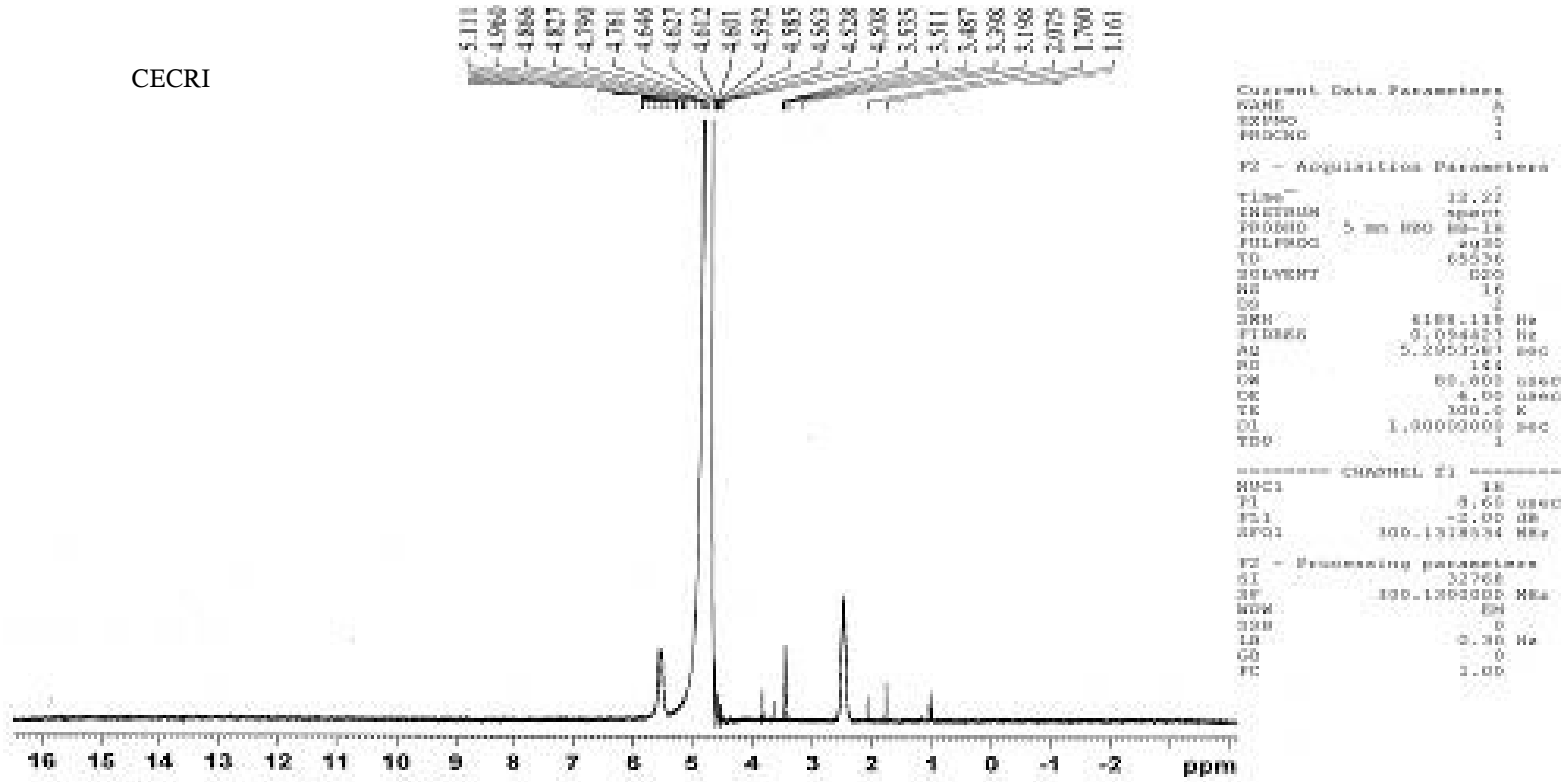


Fig. 19 ^{1}HNMR spectrum of commercial chitosan from Sigma chemicals

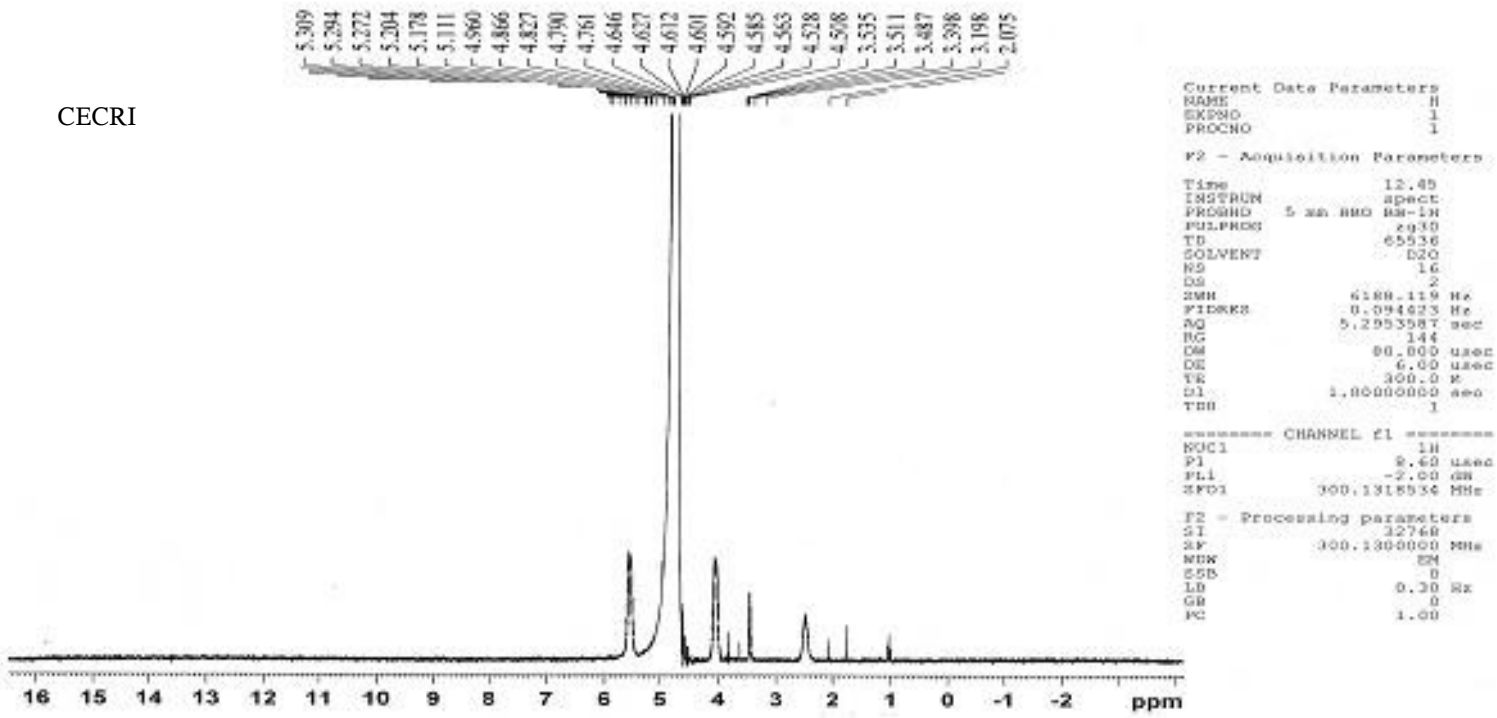


Fig. 20 [1] HNMR spectrum of isolated chitosan from Mucor rouxii MTCC 386

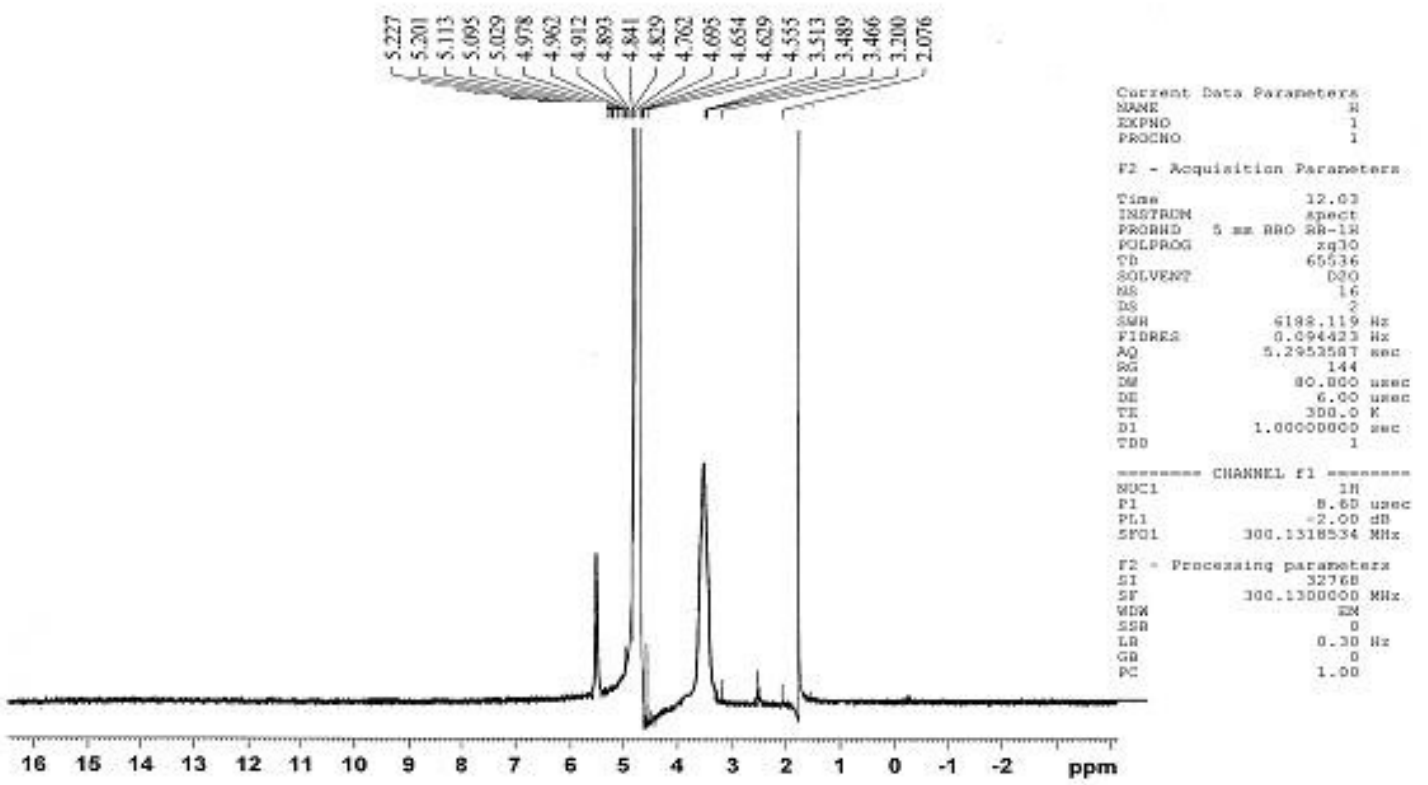


Fig. 21 ^{1}HNMR spectrum of isolated chitosan from Mycotyph africana NCIM 1230

6.5.3 X-ray diffraction

The X-ray diffraction patterns were characterized by peaks around 2θ = 9-10^{o} and 2θ = 19-20^{o}.This confirmed that the samples are found to be chitosan.

The X-ray diffraction patterns of both the fungal chitosans S1 and S2 are similar to characterized by peaks at 2θ = 10^{o} and 2θ = 19^{o} with additional peak at 2θ = 29^{o}-36^{o}.

The X-ray diffraction pattern of the commercial chitosan S3 is characterized by the peak around 2θ = 10^{o} and 2θ = 20^{o}.

The origin of the material strongly affected its crystallinity, depending on the kind of chain arrangement.

The comparison of the X-ray diffraction patterns of the fungal chitosan S1(*Mycotypha africana*) , S2 (*Mucor rouxii*) and S3 (commercial chitosan) showed that less deacetylated the higher its crystallinity.The commercial chitosan is more crystalline than those from fungal biomass. Obviously, the peak at 10° was significantly smaller for the chitosan of fungal source than for the commercial chitosan.This is consistent with the results of Jaworska *et al., (*2003).

The additional intense peak, which was observed for fungal chitosan at 2θ = 30^{o} is consistent with the result of chatterjee *et al.,* (2005).

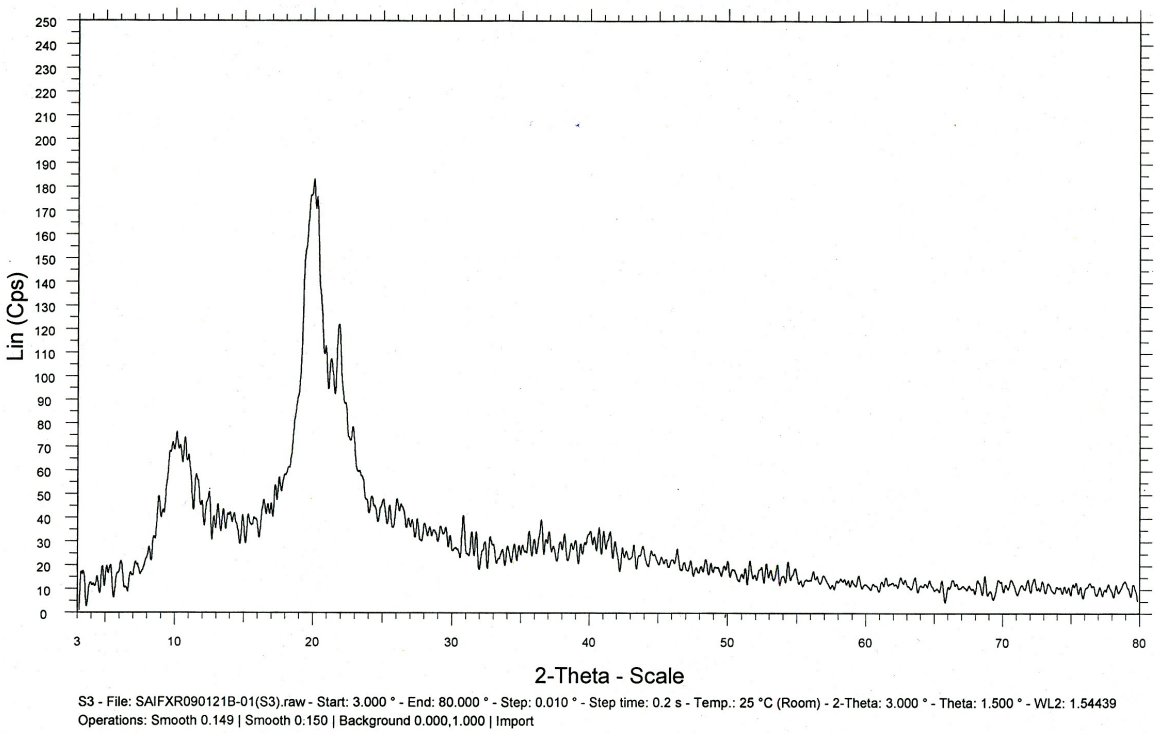


Fig. 22 X-ray diffraction pattern of commercial chitosan of sigma chemicals

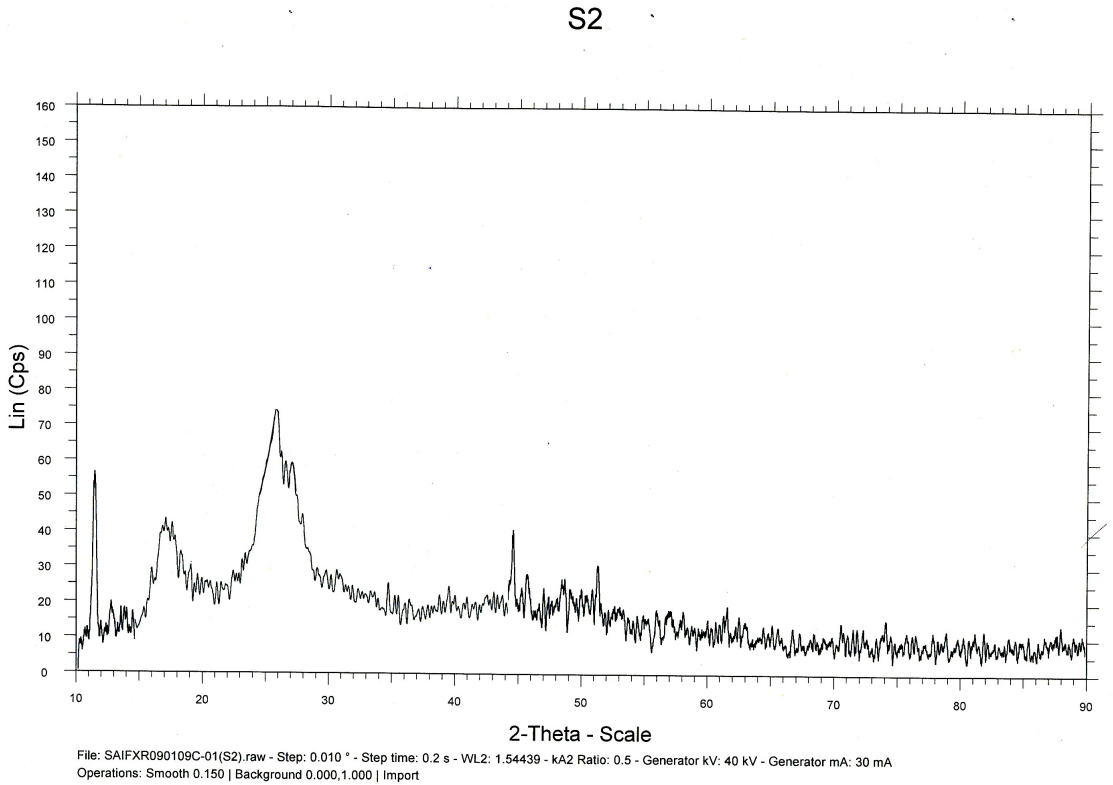


Fig. 23 X-ray diffraction pattern of isolated chitosan from *Mucor rouxii* MTCC 386

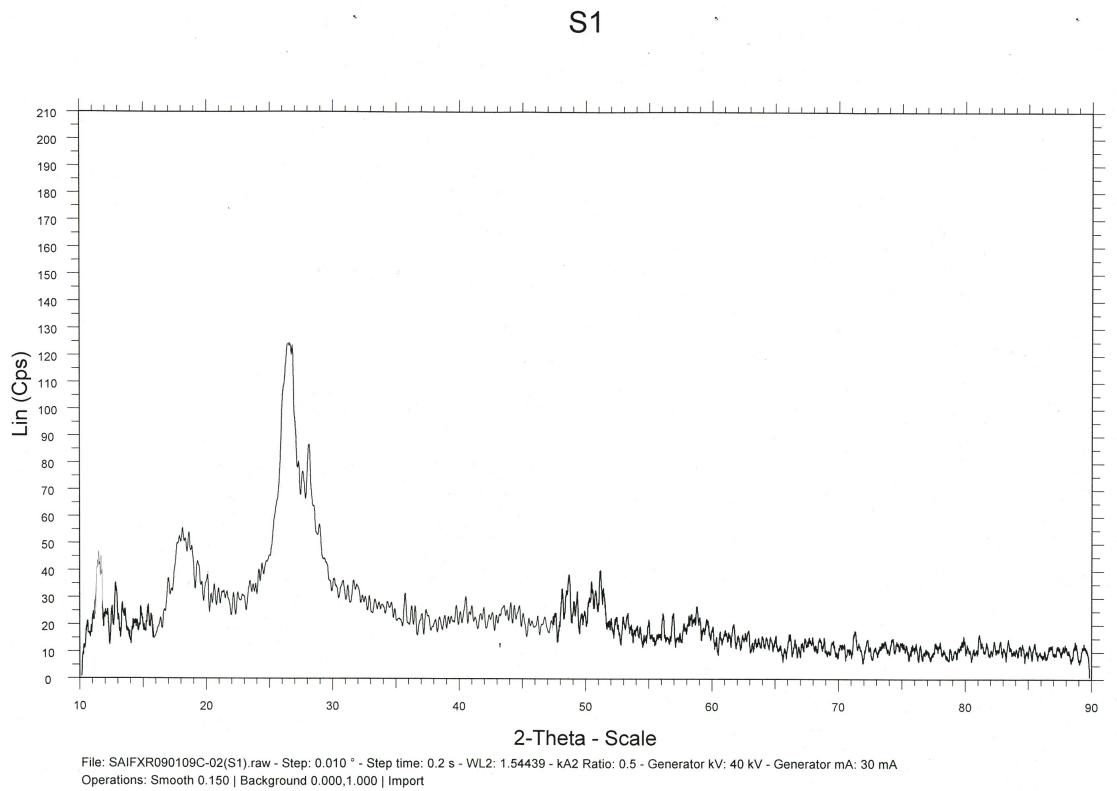


Fig. 24 X-ray diffraction pattern of isolated chitosan from *Mycotypha africana* NCIM 1230

6.5.4 Viscometry

Table no 7: Viscosity of isolated and commercial chitosan

S.No	Samples	Viscosity (cP)
01	Commercial chitosan	96.3
02	*Mucor rouxii*	8.7
03	*Mycotypha africana*	6.9

The viscosity of the isolated fungal chitosan was in the range of 6-9 cP where as commercial chitosan having the viscosity of 96.3 cP. The viscosity of fungal chitosan was considerably lower than commercial chitosan highly viscous solutions are not desirable for industrial handling. A low viscosity chitosan from fungal mycelia as obtained in this work may facilitate easy handling in industries. Results were similar to those reported by Salwa A Khalaf, (2004).

6.5.5 Ash content

Table 8: Ash content of isolated and commercial chitosan

S.No	Samples	Ash content (%)
01	Commercial chitosan	0.92
02	*Mucor rouxii*	0.65
03	*Mycotypha africana*	0.43

The low ash content indicates that fungal chitosan are of with improved quality and clarity so, it can be used for medical and dietary purpose.

6.5.6 Moisture content

Table No.9: Moisture content of isolated and commercial chitosan

S.No	Samples	Moisture content (%)
01	Commercial chitosan	8.82

02	*Mucor rouxii*	4.32
03	*Mycotypha africana*	7.43

The moisture content of the isolated chitosan and commercial chitosan was within the pharmaceutical specification (<10%). This indicates these polysaccharides are of high quality and can be used for pharmaceutical purposes.

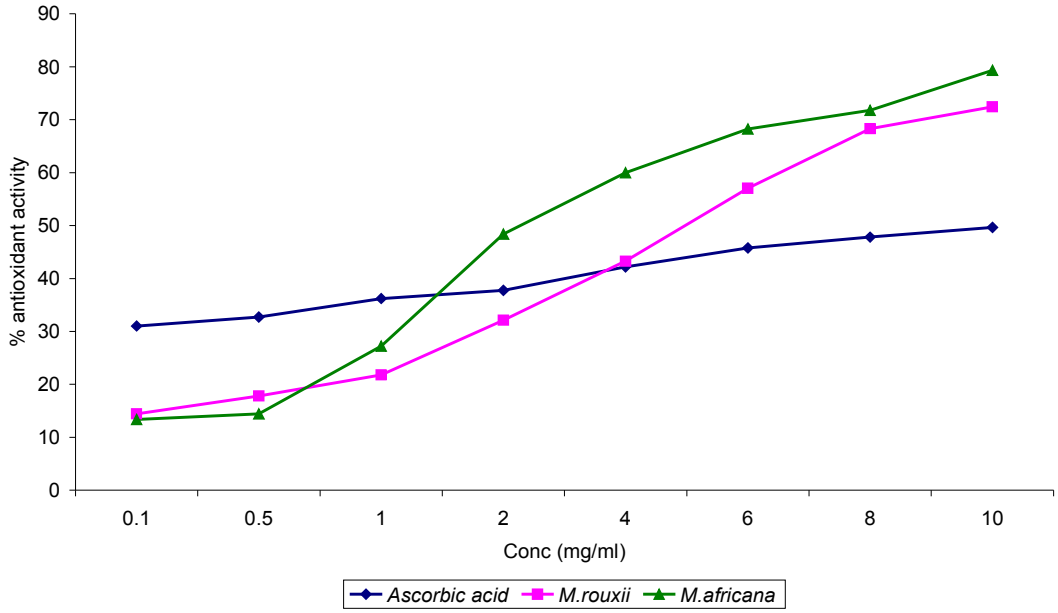
6.6 Antioxidant Activity

Table No. 10 : Antioxidant activity of isolated chitosan

S.No	Conc of sample (mg/ml)	Percentage of Antioxidant activity		
		Ascorbic acid	*M.rouxii*	*M.africana*
01	0.1	31.03±0.40	14.42±0.36	13.38±0.008
02	0.5	32.72±0.14	17.80±0.27	14.38±0.01
03	1.0	36.22±0.57	21.78±0.26	27.21±0.05
04	2	37.76±0.63	32.09±0.32	48.41±0.02
05	4	42.18±0.30	43.27±0.29	59.98±0.15
06	6	45.77±0.31	57.04±0.38	68.25±0.16
07	8	47.80±0.28	68.32±0.25	71.76±0.44
08	10	49.64±0.37	72.46±0.35	79.36±0.26
EC_{50} value	-	7.9mg/ml	5.4mg/ml	4.5mg/ml

Graph No. 7 Antioxidant activity of isolated fungal chitosan and ascorbic acid

Anti oxidant activity

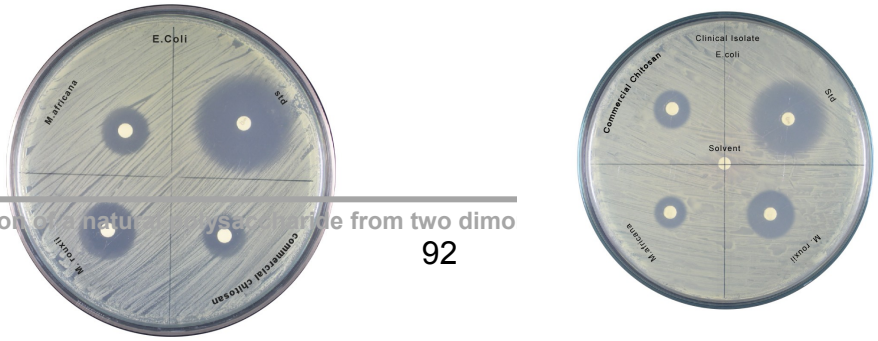


The isolated chitosan reduced DPPH to yellow colour product in concentration dependent manner. The highest DPPH radicals scavenging activity was detected in *Mycotypha africana* NCIM 1230 with EC_{50} of 4.5mg/ml, followed by *Mucor rouxii* MTCC 386 (5.4 mg/ml), when compared with ascorbic acid (7.9 mg/ ml).

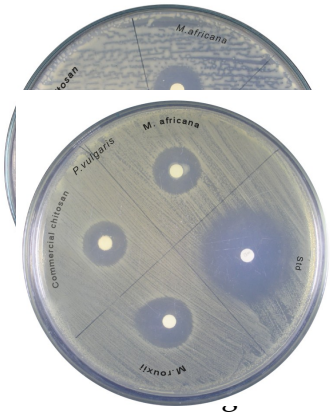
The DPPH scavenging effect of isolated chitosan was more than that of standard compound, ascorbic acid. This showed a good free radical scavenging activity of the fungal chitosan.

6.7 ANTIBACTERIAL STUDIES

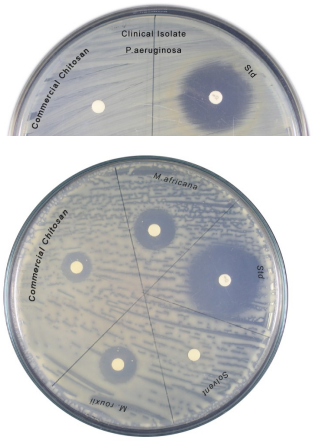
Fig. 25: Anti bacterial activity of isolated and commercial chitosan on Muler Hinton agar by disc diffusion method



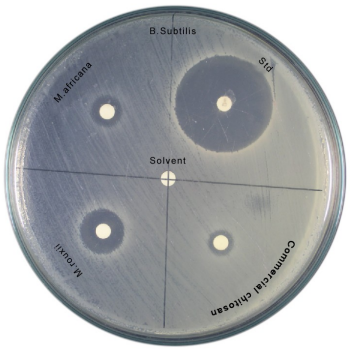
E. coli



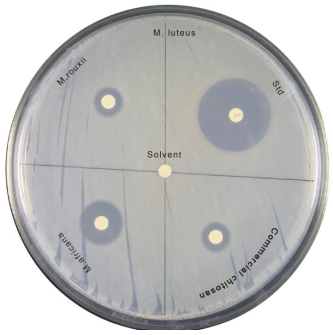
P. vulgaris



K. pneumoniae



B. subtilis



M. luteus

S. aureus

Clinical isolate *S. aureus*

Table No. 11: Antibacterial activity of isolated and commercial chitosan

Samples tested	Diameter of Zone of inhibition (mm)									
	Gram negative bacteria						Gram positive bacteria			
	E.coli	Clinical Isolate *E.coli*	*P.aeruginosa*	Clinical isolate *P.aeruginosa*	*P.vulgaris*	Clinical isolate *K..pneumoniae*	*B.subtilis*	*M.luteus*	*S.aureus*	Clinical isolate *S.aureus*
Commercial chitosan	19	14	10	-	20	17	12	12	10	-
M.rouxii	27	20	14	-	26	19	19	14	-	-
M.africana	21	16	14	-	23	22	14	12	14	-
Ciprofloxacin	39	36	35	30	40	37	39	33	32	34

The isolated fungal chitosan and commercial chitosan were tested for their anti bacterial activity against Gram positive and Gram negative organisms by disc diffusion method using Ciprofloxacin (5μg/disc) as standard.

Gram Positive Organisms

Both *Micrococcus luteus* and *Bacillus subtilis* were found to be sensitive to all the isolated fungal chitosan and commercial chitosan. The chitosan did not show sensitivity against clinical isolate *Staphylococcus aureus* as well as the *Staphylococcus aureus* NCIM 5021 not sensive to chitosan isolated from *M.rouxii*

Gram Negative Organisms

Gram negative organisms such as *E.coli*, clinical isolate *E.coli*, *P.vulgaris* and *K.pneumoniae, Pseudomonas aeruginosa* and *P.vulgaris* was found to be highly sensitive to all the isolated fungal and commercial chitosan. Clinical isolate P.aeruginosa did not show any zone of inhibition against both isolated 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).

Our aim is to produce chitosan a natural polysaccharide, from a dimorphic fungus and we identified, that *Mycotypha africana* NCIM 1230, a dimorphic fungi is able to produce the polysaccharide and is compared with another dimorphic fungi *Mucor rouxii* MTCC 386 which was used as a reference.

The chitosan produced using the above two species was comparable to the commercial chitosan and some of its characteristics were even superior to the commercial source like the present finding typically has at least 80-95% of deacetylation. This level of deacetylation provides high quality chitosan with consistent functional properties.

In medium optimization the yield of chitin and chitosan content of fungi depends on the fungal strains, mycelial age, and composition of the growth medium. In our study the inclusion of glucose as a carbon source and peptone as a nitrogen source led to the highest yield of chitin and chitosan in *Mucor rouxii* MTCC 386 and the inclusion of maltose as a carbon source and corn steep liquor as a nitrogen source led to the highest yield of chitin and chitosan in *Mycotypha africana* NCIM1230.

The present study clearly indicated that pH 5 is optimum for chitosan production by two dimorphic fungi such as *M.rouxii* MTCC386 and *M.africana* NCIM1230, due to the more activity of chitin deacetylase enzyme in that particular pH.

The profile of both isolated and commercial chitosan showed

similar FT-IR and NMR spectra.

The comparison of the X-ray diffraction patterns of the fungal chitosan S1(*Mycotypha africana*) , S2 (*Mucor rouxii*) and S3 (commercial chitosan) showed that less deacetylated the higher its crystallinity. The commercial chitosan is more crystalline than those from fungal biomass. The viscosity of the isolated fungal chitosan was in the range of 6-9 cP where as commercial chitosan having the viscosity of 123 cP (Table no.7) the results was correlated as described (Pochanavanich and Suntornsuk. 2002).

The low ash content indicates that fungal chitosan are of with improved quality and clarity so, it can be used for medical and dietary purpose.

The moisture content of the fungal chitosan from two dimorphic fungi and commercial chitosan are within the pharmaceutical grade specification i.e <10%.

The DPPH free radical scavenging effect of isolated chitosan was more than that of standard compound, ascorbic acid.This shows a good free radical scavenging activity of the fungal chitosan.

The isolated chitosan from both of the dimorphic fungi showed good antibacterial activity against Gram positive bacteria and Gram negative bacteria, including pathogenic organisms.

Based on the results it may be concluded that any dimorphic fungi

can be able to produce chitosan irrespective of the morphological form. Among the two dimorphic fungi *M.rouxii* MTCC 386 yields more chitosan than *M.africana* NCIM 1230 and could be the perfect alternative source for commercial chitosan. The less crystalline nature of the fungal chitosan indicated that it is deacetylated more and can be widely used for different applications. This work suggested that the chitosan production from dimorphic fungi can be further optimized and may be genetically modified in near future to improve the yield in a large scale production.

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ABBREVIATIONS

Chitosan	Poly-(1,4- β-D- glucopyranosamine)
CC	Crustacean chitosan
cP	centipoise
CSL	Corn Steep Liquor
c.s	Corn starch
DPPH	1,1-diphenyl-2- picrylhydrazyl hydrate
DDA	Degree of Deacetylation
EC	Effective concentration
EPS_s	Exopoly saccharides
FC	Fungal chitosan
FT-IR	Fourier Transform Infrared Spectroscopy
GlcNAc	N-acetyl glucosamine
^{1}HNMR	Proton Nuclear Magnetic Resonance
KDa	Kilo Dalton
MIC	Minimum Inhibitory Concentration
MTCC	Microbial Type Culture Collection
NCIM	National Collection of Industrial Microorganism
NAC	N- alkyl chitosan
OM	Outer membrane
PDB	Potato Dextrose Broth
PBS	Phosphate Buffer Saline
Ponceau S	3-hydroxy-4{(2-sulfo-4-((-4sulfophenyl (azo) phenyl) azo) 2,7 naphthaleen disulphonic acid tetra sodium salt
SSF	Solid State Fermentation
Stains all	1- Ethyl-2-[3-(1-ethylnaptho[1,2-d]thiazolin-2-ylidine)-2-methyl propenyl] naphtha [1,2-d]thiazolium bromide,3,3'-diethyl-9-methyl-4,5,4',5'-dibenzothiacarbocyanine bromide
sbm	Soybean meal
SMF	Submerged Fermentation
SOD	Super Oxide Dismutase
TEM	Transmission Electron Microscope
UDP	Uridyl diphosphate