DEVELOPMENT AND EVALUATION OF BETA GLUCAN BASED BIOACTIVE WOUND DRESSING MATERIAL

PROJECT REPORT

Submitted by

VINISH.C.VIJAYAN

Register No: 1020203017

in partial fulfilment for the award of the degree

of

MASTER OF TECHNOLOGY

in

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY

(An autonomous institution affiliated to Anna University of Technology, Coimbatore)

COIMBATORE -641 049

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DEPARTMENT OF BIOTECHNOLOGY

PROJECT WORK

PHASE II

APRIL 2012

This is to certify that the project entitled

DEVELOPMENT AND EVALUATION OF BETA GLUCAN BASED BIOACTIVE WOUND DRESSING MATERIAL

is the bonafide record of project work done by

VINISH.C.VIJAYAN

Register No: 1020203017

of M.Tech (Biotechnology) during the year 2011-2012

……. ……..

Project Guide **Head of the Department**

Submitted for the project Viva-Voce examination held on.....

…….. ……

Internal Examiner External Examiner

DECLARATION

I affirm that the project work titled "**DEVELOPMENT AND EVALUATION OF BETA GLUCAN BASED BIOACTIVE WOUND DRESSING MATERIAL"** being submitted in partial fulfilment for the award of M.Tech (Biotechnology) is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other university.

VINISH.C.VIJAYAN

Reg.No:1020203017

I certify that the declaration made above by the candidate is true.

Signature of the Guide

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ACKNOWLEDGEMENT

I wish to express my sincere thanks to **Dr.S.Ramachandran**, Principal, Kumaraguru College of Technology, Coimbatore and **Dr.A.Manickam,** Dean, Biotechnology, Kumaraguru College of Technology. Coimbatore.

I wish to express my warm and sincere gratitude to my guide **Dr. N. Saraswathy**, Associate Professor, Department of Biotechnology, whose encouragement; guidance and support from the initial to the final level enabled me to develop an understanding of the subject.

I would like to extend my thanks to **Dr.P. Ramalingam**, Associate professor and **Dr. K.Rajasree,** Department of Biotechnology, whose expertise, understanding, and patience, added considerably to my graduate experience and for the assistance he provided at all levels of the research project.

I am grateful to the **teaching and non-teaching staff members** of the Department of Biotechnology for their kind and patient help throughout the project.

I am indebted to my many student colleagues for providing a stimulating and fun environment in which to learn and grow.

All my **family members** have contributed significantly to bring this day in my life. My **parents** remained a constant source of strength throughout my educational career and to achieve my goals.

In the end I am thankful to the **Almighty** for his blessings without which nothing would have been possible.

VINISH C VIJAYAN

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ABSTRACT

ABSTRACT

Polysaccharides, especially β-glucan, are considered responsible for mushroom`s biological activity. The beta-glucan was first rendered soluble from fruiting bodies of mushroom by alkali extraction (2% NaOH) and precipitated by alcohol precipitation to collect fraction–1. Alkali extraction was included into the isolation procedure. The beta-glucan was first rendered soluble from fruiting bodies of mushroom by alkali extraction (2% NaOH) and precipitated by alcohol precipitation technique (Ethanol) [Fraction–1]. The water soluble component contained mainly heteroglycans, glycoproteins. The fraction-1 contained 8.03 % of the protein .To remove the residual proteins, the sample is chromatographed in a DEAE Sephacel anion exchange column which resulted in a reduced to 0.24% protein in the subsequent fraction [Fraction – 2]. Further purification by Con A chromatography gave mannan-free crude beta-glucan. Mannan is known to bind to concanavalin A (ConA), a class of lectin. The fraction – 3 was completely free of proteins and mannan. The recovery of the water soluble beta-glucan was about 19.6 %(wt/wt) of the starting materials expressed as total carbohydrate. The further purified fraction were analysed to and fraction 1(42% carbohydrates and 8% proteins), fraction 2 (15% carbohydrates and 0.24% proteins) and fraction 3 (15% carbohydrates). The fraction 3 after ConA chromatography is analysed for the presence of carbohydrates by anthrone method. The test showed positive result for presence of carbohydrates. The increase in absorbance is an indication of the presence of beta-glucan. The congo red interacts with the beta-glucan and not with any other polysaccharides. So this confirms the presence of beta-glucan in the extracted sample from fruiting bodies of mushroom. The IR bands in the region of 950 – 1250 cm^{-1} is mainly due to C-C and C-O stretching vibrations in pyranoid rings, indicating the presence of polysaccharides as the major component. The cell viability was examined and results depicted in table 4.4. There was no significance difference in cell viability in lower concentration (18.75 – 75µg/ml), suggesting the crude beta-glucan non Cytotoxicity. The lower concentration range (below 20µg/ml) is expected to proliferate the cells, and thereby will confirm the non-toxicity nature of the crude beta-glucan. Free radical scavenging capacities of the crude beta-glucan, measured by DPPH assay revealed that the crude extract has 22.05 % scavenging activity. The optimised film contained 2% Chitosan, 1%acetic acid, 1% lactic acid and 0.1 – 2% beta-glucan. Higher concentration of beta-glucan gave flexibility and can withstand higher stress levels. Flexible films were obtained when 0.1 to 2 % beta-glucan is incorporated, beyond 2% high viscous solutions makes spreading difficult. It can be observed that the maximum swelling ability increased with increase in beta-glucan concentration up to a certain limit. At the first stage of the curve, swelling rate was very high, and the water could be easily absorbed to the film. It can be observed that the film attains swelling saturation after 6hrs. The fluid uptake ability of chitosan film was 84.45%, which increased to 93.87% with the addition of beta-glucan. The poor inhibitory activity of the film-forming solution could be explained by the limitation of the diffusion of chitosan in agar medium. All the films showed zone of inhibition for *E.coli and Staphylococcus.*

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LIST OF ABBREVATIONS

S.NO.	ABBREVATIONS	EXPANSION
1	M	Molar
2	OD	Optical density
3	mM	Millimolar
4	nM	Nanometer
5	µg	Microgram
6	µl	Microlitre
7	mg	Milligram
8	ml	Millilitre
9	h	Hour
10	l	Litre
11	min	Minutes
12	<	Less than
13	>	Greater than
14	g	Gram
15	mol	Moles

INTRODUTION

CHAPTER 1

INTRODUCTION

Wound healing is an intricate biological process and ordered cascade of events, which begin at the moment of injury, namely haemostasis, inflammation, proliferation and continue till maturation or remodeling. The use of wound dressings, offers tremendous potential in therapeutic approach to accelerate the wound healing. An ideal wound dressing material should provide optimised healing environment. The ideal wound dressings should have the following properties: (1) Provide a moist wound healing environment. (2) Provide oxygen permeability for respiration (3) Provide thermal insulation. (4) Be removable without causing trauma to the wound. (5) Remove drainage and debris. (6) Be free of particulate and toxic constituents. (7) Promote tissue reconstruction process. (8) Afford protection from secondary infection (Wittaya-areekul and Prahsarn, 2006). Depending on the state of wound, a wound can be treated by means of dressings that are passive or active. Passive dressings are used for the treatment of low extruding wounds and active dressings for the treatment of chronic wounds because they perfectly adapt to the different stage of cicatrisation and provide optimum therapeutic efficiency (Zaher *et al.*, 2009). Bioactive dressing augment healing process; either by delivery of bioactive compounds or dressing scaffold`s endogenous activity. These materials include proteoglycans, collagen, non-collagenous proteins, alginates or chitosan. Moreover, an effectual wound dressing should promote a rapid healing of the wound and once healed, the detachment of the dressing should not cause secondary trauma to neo-tissue (Mackay and Miller, 2003).

Hydrogels are promising substrates for numerous biomedical applications, including wound dressing, drug delivery systems and tissue engineering scaffolds. In particular, hydrogels are increasingly explored as bioactive scaffold materials for wound dressings because of the similarity of the three dimensional microstructure with that of the extracellular matrix (ECM). The successful use of a hydrogel in these applications greatly depends on the ability to control physical and chemical properties of the hydrogel in a preferred manner. Among these properties hydrogel mechanical property, swelling and degradation rates are major concerns in designing the materials for the application as bioactive wound dressing. A variety of synthetic and naturally derived materials may be used to form hydrogels for wound dressing scaffolds. Wound dressing based on chitosan material is well known, in literature as well as from commercial point of view, in wound management.

Chitosan is the deacetylated derivative of chitin which is a water insoluble polymer, (N-acetyl-d-glucosamine); found in nature, present in insect exoskeletons, outer shells of crabs, shrimps, lobsters etc. and fungal cell walls. Chitin and chitosan are names that do not strictly refer to a fixed stoichiometry. Chemically, chitin is known as poly-*N*-acetylglucosamine, and in accordance to this proposed name, the difference between chitin and chitosan is that the degree of deacetylation in chitin is very little, while deacetylation in chitosan occurred to an extent but still not enough to be called polyglucosamine. Chitosan is the second abundant polysaccharide next to cellulose but it is the most abundant natural amino polysaccharide and is estimated to be produced annually almost as much as cellulose. Chitosan is a FDA certified GRAS material and has been widely utilized in pharmaceuticals, tissue engineering and medical textiles (Manjeti *et al*., 2000). Due to its unique polymeric cationic character and its gel or film forming properties, chitosan is a preferred material for medical and pharmaceutical applications. In addition to good film and gel forming properties, one of chitosan's most promising features is its excellent ability to produce three-dimensional scaffolds. Chitosan is typically not soluble in water, but chitosan solutions can be obtained in acidic aqueous media (pH < 6), which protonates its amino groups, rendering the polymer positively charged and, thereby, overcoming associative forces between chains. Chitosan has one primary amine and two free hydroxyl groups for each monomer with a unit formula of $C_6H_{11}O_4N$. This natural biopolymer is a glucosaminoglycan and is composed of two common sugars, glucosamine and N-acetylglucosamine, both of which are constituents of mammalian tissues. Chitosan has been proved and regarded to be biodegradable, non-toxic and has some important biological properties, such as antimicrobial activity. Chitosan has many advantages for wound dressing material: biocompatibility, biodegradability (Tomihada and Ikada, 1997), anti-microbial activity and accelerated wound healing (Ueno *et al*., 2001 and Suzuki *et al*., 1994). Because of these reasons, chitosan gained importance in wound healing management in recent years. However poor mechanical strength of chitosan itself limits its further use in wound management (Wu *et al*., 2004). Addition of polysaccharides will improve strength and elasticity. Recently, much attention has been given to chitosan as a potential polysaccharide source. Chitosan can be degraded by soil microorganisms and water microorganisms. This makes chitosan environmental friendly. This was acknowledged by the US Environmental Protection Agency when it exempted chitosan from tolerance level testing.

Mushrooms have beneficial effects on health and in treatment of disease through their immunomodulatory, anti-neoplastic and lipid reducing properties. The dry matter of the fruiting bodies of mushroom is about 5-15 %, they have a very low fat content and contain 19-35% proteins. Mushroom fruiting bodies are rich in vitamins, mainly B_1, B_2, C and D_2 (Mattila *et al*., 2000 and Manzi *et al*., 2004). The content of carbohydrates, which are mainly present as polysaccharides or glycoproteins, ranges 50 – 90%; the most abundant polysaccharides are alpha-and beta-glucans and other hemicelluloses. Mushroom polysaccharides are mainly present mostly as glucans with different types of glycosidic linkages, such as branched (1-3), (1-6) beta-glucans and a few heteroglycans (Wasser, 2002). The importance of the polysaccharides has attracted much attention in the field of functional foods, especially, commonly cultivated mushroom of genus *Pleurotus* are interesting because of their beta-glucan demonstrating significant immunomodulative properties. Pleuran, a specific glucan isolated from *Pleurotus* sp., has suppressive effect on tumours (Kuniak and Karacsonyi, 1994).

Beta-glucans are non-starchy polysaccharides composed of mixed linked glucose polymers, linked by a 1-3 linear beta-glycosidic core chain. The branches derived from the glycosidic chain core are highly variable and the 2 main groups of branching are (1–4) or (1–6) glycosidic chains. The molecular structure depends on the source and method of isolation with differences in the distribution and length of side chains. The isolation of beta-glucan typically involves alkali extraction, alcohol precipitation of polysaccharide, purification by chromatographic techniques. The composition of beta-glucans can be evaluated by a variety of methods, normally it is based on an enzymatic and acid hydrolysis and quantification of the free reducing sugar there by released. Molecular mass is determined by liquid chromatography/mass spectrometry (LC/MS) and structural analysis by NMR analysis. This bioactive substance has potential therapeutic properties. Beta-glucan activates the immune response through the immune cells, called macrophages, showing various therapeutic effects. Beta-glucans have also antioxidant attributes and have demonstrated wound healing activity. Although there are several papers related to the extraction and purification of beta-glucan, most of them describe extraction and purification methods from cereals. Moreover, since the beta-glucan content in grains is low (<15%) it is necessary to determine the source having high beta-glucan and to develop, optimise an extraction/purification method that allow reducing operation times and to obtain purified compound. The purified compound will be impregnated on to chitosan based film to test for wound healing activity on animal models.

The objectives of the current study are:

- To optimize extraction procedure for beta-glucan from fruiting bodies of *Pleurotus* sp.
- To characterize purified beta-glucan from *Pleurotus* sp.
- To evaluate *in-vitro* cytotoxicity of beta-glucan using fibroblast cell lines.
- To fabricate, characterize and formulate beta-glucan loaded chitosan film.
- To evaluate bio-active beta-glucan loaded chitosan film on animal models.

LITERATURE REVIEW

CHAPTER 2
LITERATURE REVIEW

2.1 SKIN AND WOUNDS

2.1.1 Skin structure

The skin is covering most of the outer body varying in its thickness and structure. The top layer of skin is the epidermis. Cells in epidermis proliferate and renew the layer regularly. This is important since the skin is the main barrier protecting the body from damaging factors. The underlying layer is the dermis. The dermis is tough for support and nourishes the skin. Fibro elastic tissue is providing the skin its form. Dermis can be regarded as two zones. The upper layer is the thin papillary dermis, and the lower layer the reticular dermis. The deepest layer is the hypodermis. Hypodermis is varying the most, with mainly adipose tissue. Sweat glands, hair follicles, sebaceous glands and nerve fibres intersect all skin layers. Epidermis is considered avascular but the dermis is vascular. This means that epidermis is highly dependent on proper blood flow for its normal function (Sherwood, 2007).

2.1.2 DAMAGED SKIN AND BARRIER PROPERTIES

Damaged skin can severely reduce quality of life and cause unwanted health problems when left untreated. Burns, diabetic ulcers, arterial and venous ulcers can all be challenge to treat. When circulation is reduced, blood flow lowered or dermis damaged, the wound healing takes longer time and the wound might evolve into a chronic one (Bao *et al.,,* 2009).

In regard of development of drug delivery system for damaged skin, the changed barrier function needs to be taken into consideration. pH of the skin is altered. Lipophilic and hydrophilic properties of the skin are most likely different from healthy skin. Permeability of drugs might be unexpectedly high or even low. Atrophy due to degeneration of cells will make drug therapy regimes more difficult to design because of the reduced thickness of the skin (Boateng *et al*., 2008).

2.2 WOUND

A wound is defined as a defect or break in the skin, resulting from physical or thermal damage or as a result of the presence of an underlying medical or physical condition. Based on the nature and repair process of wounds, they can be classified as chronic wounds and acute wounds. Acute wounds are tissue injuries that heal within 8-12 weeks. The primary causes of acute wounds are mechanical injuries (friction contact between skin and hard surfaces), burns and chemical injuries. In the case of burns, the temperature of the source and time of exposure is important to decide the degree of wound. Burn wounds need normally specialist care because of associated trauma (Boateng *et al.,,* 2008). Chronic wounds heal slowly and leave serious scars. There can be different reasons that chronic wound do not heal as fast as acute wounds. Among most common are diabetes, infections and poor primary treatment. The common chronic wounds are: Venous ulcers, Arterial ulcers, Diabetic foot ulcers, Pressure ulcers, Vasculitis, Pyoderma gangrenosum (Fonder *et al*., 2008).

Both acute and chronic wounds can be classified as a complex wound if the wound has these characterizes: Extensive loss of the integument which comprises skin, hair and associated glands, Infection which can result tissue loss, Tissue death or signs of circulations, Presence of pathology,

A wound is colonized when growth and death of bacterial in the wound is balanced by the host. If the host is not able to keep the bacterial growth in balance, the wound will enter the infection phase (bacterial load in excess of 1015). Symptoms for an infected wound are erythema, edema, warmth, pain and exudate. Infections of chronic wounds are often polybacterial with *Staphylococcus aureus* and anaerobs being the most common in chronic wound (Fonder *et al*., 2008).

2.3 WOUND HEALING PROCESS

The wound healing process is a series of independent and overlapping stages. In these stages will both cellular and matrix compounds work to re-establish the integrity of damaged tissue and replacement of lost tissue. These overlapping series can be classified in five stages (Boateng *et al*., 2008): Haemostasis, Inflammation, Migration, Proliferation, Maturation.

Haemostasis: the first response to injury is bleeding. Bleeding is an effective way to wash out bacteria that are on the surface of skin. Afterwards, bleeding activates haemostasis stage that is initiated by clotting factors. The clot dries out and creates a hard surface over the wound that protects tissues underlying.

Inflammation: this stage starts almost at same time as haemostasis. It occurs from between few minutes to up to 24 minutes after injury. In this stage histamine and serotonin are released into wound area and activate phagocytes to enter the wound area and engulf dead cells.

Migration: in this stage the reestablishment of wound begins. The epithelial cells and fibroblasts move into the injured area and grow rapidly under the hard scab to replace the damaged tissue.

Proliferation: this stage has three characteristics. First, the granulation tissue is formed by growth of capillaries. Second, lymphatic vessels enter into wound and the third one, synthesis of collagen starts providing form and strength to the injured tissue.

Maturation: in this stage, the shape of the final scar is determined by formation of cellular connective tissue and strengthening of the new epithelium.

The different stages in wound healing process can be defined in simplified form as The cleansing phase (exsudative phase), The granulation phase (proliferation phase), The epithelialisation phase (differentiation phase) (Goossens and Cleenewerck, 2010). Or as Inflammation, Proliferation, Remodeling (Figure 6; Fonder *at al*., 2008). One factor that can slow the wound healing process is excessive inflammation, especially in burn wounds. This kind of inflammation is not a response to infection and is known as aseptic inflammation. Excessive inflammation is therapeutical challenge in wounds such as burns and chronic wounds (Beukelman *et al*., 2008).

Debridement is the process during which slough, eschar and exudate, bacterial biofilms and callus get removed from the wound bed to enhance the healing process. Drastic methods of debridement can be painful, therefore use of moisture-donating wound dressings will rehydrate desiccated and devitalized tissue and then, separation from healthy tissue will be easier (Fonder *et al*., 2008).

2.4 WOUND DRESSING

From old times people tried to heal wounds. They used crude drug extracts (mostly of plant origin), animal fat and honey to heal wounds. For example in Senegal, the people used the leaves of *Guiera senegalensis* to put on wound. In Ghana the people used extracts of *Commelina diffusa* herb and *Spathodea campanulata* bark to put on wound and heal it. The interesting point is that the researches have shown that some of these extracts and herbs have indeed antibacterial and antioxidant effect (Boateng *et al*., 2008). An ideal material to be applied to wound should be

Nontoxic, Biocompatible, Enhance cellular interaction and tissue development, Be biodegradble and bioresorbable (Huang and Fu, 2010). Moreover, the ideal properties of a wound dressing are:

Providing a moist environment, Creating a protective mechanical barrier and thermal isolation, Protecting against secondary infections, Keeping the wound environment moist,

Absorbing the exudate and bacteria, Promoting debridement, Contributing to simple gas exchange, Decreasing or removing trauma in the defected area, Being acceptable for patient, Not possessing any toxic, irritant or allergic properties, Cost-effectiveness (Goossens and Cleenewerck, 2010). Some of wound dressings can cause allergic reactions when applied to the defected area.

There are three kind of allergic reactions that can appear via wound dressings:
Irritant reactions that originally have mechanical reasons. These reactions can happen because of occlusion or strong adhesion of dressing to the wound Immediate allergic reaction (contact urticaria) Delayed allergic reactions (contact eczema) (Goossens and Cleenewerck, 2010).

2.4.1 Classification of dressings

Dressings can be classified in a number of ways. They can be classified based on their function in the wound (antibacterial, absorbent), type of material employed to produce the dressing (collagen, hydrocolloid), physical form of the dressing (ointment, film and gel), traditional and modern dressings. Some dressings can be placed in several classifications because they fit criteria in several groups. The simplest classification is as traditional and modern dressings and particular focus will be given to hydrogels, one of the most common modern dressings (Boateng *et al*, 2008). Traditional wound dressings can be classified as topical pharmaceutical formulations and traditional dressings.

Topical pharmaceutical formulations: these formulations can be liquids such as solutions and suspensions or semi liquid materials such as ointments and creams. These formulations can be used in the initial stages of wound healing, for example as antibacterials (Boateng *et al.,*, 2008).

Traditional dressings: these are, unlike topical formulations, dry materials as cotton wool and natural or synthetic gauzes. These dressings are more used in chronic wounds and burn wounds because liquid and semi liquid dressings do not remain on the wound over optimal time (Boateng *et al*., 2008). While some clinicians insist that gauzes are as effective as new dressings, some studies show that moisture retentive dressings are associated with faster healing time. Gauzes have been one of the most popular wound dressings. There are several disadvantages with use of gauzes. They can promote desiccation of the wound base, they bind to the wound bed and it causes pain and trauma for patients while dressing change. They do not provide a good barrier against bacterial growth because they are susceptible to full thickness saturation with wound fluid (Fonder *et al*., 2008).

2.4.2 Modern wound dressing

The main aim of modern wound dressing is to create a moist environment for wound to make the healing process facilitated. Modern wound dressings are often classified as hydrocolloid dressings, alginate dressings, hydrogel dressings, dressings in form of gels, foams and films, etc. The modern dressings do not enhance the reepithelialization, but stimulate collagen synthesis and promote the angiogenesis. They can provide a pain relief felling to patient. The modern dressings can inhibit bacterial growth by maintaining a barrier against external contamination and some of them by decreasing pH at the wound surface. They provide a moist environment for the wound bed to enhance the healing process. Dry wound healing process would not only delay the wound healing process, but can cause further tissue death (Fonder *et al*., 2008).

2.5 HYDROGELS

In general, hydrogels can be prepared from either synthetic polymers or natural polymers. The synthetic polymers are hydrophobic in nature and chemically stronger compared to natural polymers. Their mechanical strength results in slow degradation rate, but on the other hand mechanical strength provides the durability as well. These two opposite properties should be balanced through optimal design (Tabata, 2009). Polymeric gels are the liquid-solid systems. It means that they have a solid matrix that swells in water and forms a three dimensional network. These polymers do not dissolve in the liquid. Creating this three dimensional network is a result of crosslinking that is again a result of chemical bindings. There are a numerous monomers that have been used to prepare hydrogels (Kopecek and Yang, 2007). The main features of hydrogels influencing their use in wound treatment are: Shape stability and softness similar to that of the soft surrounding tissues, Chemical and biochemical stability, Absence of extractable, High permeability for water-soluble nutrients and metabolites across the biomaterial Tissue interface (Kopecek, 2009), Hydrogels can be the most suitable dressing in debridement stage of a chronic wound (Vaneau *et al*., 2007).
To improve the mechanical properties of hydrogels several manufacturing methods were proposed, among which three resulted in significant improvements in the mechanical properties. Namely, Double network hydrogels, Hydrogels containing sliding cross-linking agents, Nanocomposite hydrogels (Kopecek, 2009).

Double network hydrogels: in this method, two hydrogels are combined together.
One of them is a highly cross-linked polyelectrolyte and the other one is a loosely cross-linked or maybe uncross-linked natural hydrogel. This combination will result in an effective relaxation of locally applied stress and dissipation of crack energy (Kopecek, 2009).

Hydrogels containing sliding cross-linking agents: in this method two cyclodextrin molecules get cross-linked. These molecules will create double rings that can move slightly along the PEG chains. This will result in an excellent mechanical property for hydrogel. It will provide a hydrogel with a high degree of swelling and a high stretching ratio without fracture (Kopecek, 2009).

Nanocomposite hydrogels: in this method polymer *N*-isopropylacrylamide (NIPAAm) that is clay-contained is combined with hectorite [Mg5.34Li0,66Si8O20(OH)4]Na0,66 as a multifunctional cross-linker. The mechanical property of the hydrogel was enhanced and the tensile module and strength were proportional with clay content (Kopecek, 2009).

Some additional advantages of hydrogels as wound dressings are:
Suitable rheological properties, Good tissue compatibility, Convenience in handling, Ease of application, excellent biocompatibility due to their high water content (Kopecek, 2009). Because of the water medium that is filled in the capillary space of hydrogels, they have good electrical conductivity that can be a benefit using in a combination with iontophoresis penetration method (Liu *et al*., 2008). Polymeric systems such as hydrophilic gels, express non-Newtonian pseudoplastic behaviour, which contributes to their spread ability when applied on a biologic surface. As the degree of pseudoplasticity increases, easiness of spread ability augments (Neves *et al*., 2009).

2.5.1 Classification of hydrogels

Chemically cross-linked hydrogels: Radical polymerization is usually applied to make these polymers. When these types of hydrogels come in contact with H2O molecules, they begin to swell up and spread their network (Jagur-Grodzinski, 2009).
Physically cross-linked hydrogels: Physically cross-linked hydrogels do not need introduction of an external cross-linking agent. Cross-linking agents are usually nondegradable and can be toxic and a removal of their residuals may be needed before they can be used in biomedical or pharmaceutical purpose. The physically crosslinked hydrogels are usually biodegradable. Their amorphous hydrophilic phase is held together by highly ordered aggregated chain segments held together by secondary molecular forces such as hydrogen bonding, Van der Waals forces or hydrophobic interaction (Jagur-Grodzinski, 2009).

There are several other classifications for hydrogels. They can also be classified based on the nature of the network: homopolymer, copolymer, interpenetrating, or double networks; physical structure: homogeneous (optically transparent), microporous, and macroporous hydrogels; or in relation to their fate in the organism: degradable and nondegradable hydrogels (Kopecek, 2009).

2.5.2 Characteristics of hydrogels

Hydrogels can be divided into several groups based on their stimuli-sensitivity.
Stimuli-sensitivity is related to how different groups of hydrogels express varying degrees of response (continuous or discontinuous changes in swelling) to minor changes in environment conditions, such as pH, temperature, ionic strength, quality of solvent, or biorecognition (Kopecek, 2009). Temperature responsive hydrogels: examples of temperature responsible hydrogels can be hydrogels containing polymers such as chitosan PEG-poly, *N*isopropylacrylamide hydrogel (PNIPAA), methyl cellulose and tetronics. These hydrogels are characterized by temperature dependent sol-gel transition Tgel, which corresponds to the lower critical solution temperature (LCST), and by the gel-sol transition temperature Tp (upper critical solution temperature, UCST), which corresponds to dissipation or precipitation of a gel (Jagur-Grodzinski, 2009). When the temperature is below LCST, the H2O molecules make hydrogen bonds with the polar groups of the polymer.

These bonds shape kind of hydrophobic groups as iceberg water. When the temperature increases above the LCST, these hydrogen bonds are released to the bulk with a large gain in entropy resulting in collapse of the polymer network. They can be used in sustained drugs, gene delivery and tissue engineering (Kopecek, 2009). pH responsive hydrogels: in pH responsive hydrogels, the functional group of the polymer gets introduced to a week acidic group such as acrylic acid or week basic groups such as amines. Changes in pKA and pH value of these polymers make sudden swelling. Some polymers have carboxylic acids as their functional groups. These polymers accept hydrogen at low pH but exchange it for other cations above the pKA value. They become ionized at higher pH. The hydrodynamic volume and swelling capacity of these polymers increase sharply when these carboxylic groups become ionized and the highest plateau approaches near pH 7 (Jagur-Grodzinski, 2009). Analyte responsive hydrogels: the analyte responsive hydrogels should function under physiologically relevant temperature, pH and ionic strength. Mono and

disaccharides, enzymes, antigens and various ions are among the stimulus for analytic responsive hydrogels (Jagur-Grodzinski, 2009).

2.5.3 Biomedical Applications Of Hydrogles

Hydrogels that are used for biomedical purposes should be biocompatible and often biodegradable. Drug delivery: right after hydrogels were discovered, their use as anticancer and antibiotic deliver systems was studied. Hydrogels have a porous network. Often we can control the porosity of hydrogels by controlling the density of cross-links or by changing the swell affinity of hydrogels in the environment. This porosity property of hydrogels helps the release of drugs from hydrogels. The release of drug from hydrogels can be controlled by controlling the diffusion coefficient of drugs through hydrogel matrix. We can also make a depot formulation of hydrogel-drug. The depot formulation can be made by trapping drugs into liposomes and incorporating liposomes in the hydrogel (Kopecek, 2009). Hydrogels for tissue engineering and regenerative medicine: hydrogels enable the incorporation of growth factors and control over their release. The release rate is controlled by degree of cross-linking of hydrogel. The protein will diffuse out of hydrogel through the water pathways. Gelatin hydrogel was able to release the incorporated growth factor for up to 3 months (Tabata, 2009).

There are many kinds of tissue engineering scaffolds. Among all of these hydrogels are the most popular candidates due to: Their structure is similar to the natural ECM, Their good biocompatibility, Tunable viscoelasticity, High water content, high permeability of oxygen, Essential nutrients (Jia and Kiick, 2009).

2.6 CHITOSAN

2.6.1 Structure and properties

Chitosans are linear binary heteropolysaccharides composed of (1-4)-linked 2-acetamido-2-deoxy-β-D-glucopyranose (GlcNAc; A-unit) and 2-amino-2-deoxy-β-Dglucopyranose(GlcN; D-unit) in varying composition and sequence. The structure is schematically illustrated in figure no: 2.1. Chitosans are produced from chitin by complete or partial de-N-acetylation. They are also found in nature, to a lesser extent than chitin, in the cell walls of fungi. Chitin is one of the most abundant biopolymers in nature, occurring mainly as a structural polysaccharide in the exoskeleton of animals with an outer backbone (crustaceans and insects). Chitin is also found in microorganisms such as certain species of fungi, yeast and green algae. There is no distinct border between chitin and chitosan. It has been proposed that chitin and chitosan can be distinguished by their insolubility or solubility in dilute aqueous acid solutions. The main resources for commercial utilisation of chitin are from crab and shrimp shell waste, with an annual worldwide chitin production around 10,000 metric tons. It has been shown that A- and D-units in the chitosan chains are randomly distributed and the chemical composition of chitosans can be described by the molar fraction of acetylated units, FA. The amino group in chitosan has a pKa-value ranging from 6.2 to 7, depending on the type of chitosan and conditions of measurement, and will therefore be positively charged in neutral and acidic solutions. FA and the molecular weight average are main factors determining the properties of chitosans, and thereby also suitable applications.

2.6.2 Sources of chitosan

Chitosan is converted from chitin, which is a structural polysaccharide found in the skeleton of marine invertebrates, insects and some algae. Chitin is perhaps the second most important polysaccharide after cellulose and is an abundantly available renewable natural resource. The aquatic species that are rich in chitinous material (10-55 % on a dry weight basis) include squids, crabs, shrimps, cuttlefish and oysters. Mucoraceous fungi, which are known to contain chitin and the deacetylated derivate, chitosan, in cell walls (22 to 44%), have been used for commercial chitin production (Muzzarelli 1977; Muzzarelli *et al.,* 1994). However, in comparison with marine sources, which yield more than 80,000 metric tons of chitin per year (Muzzarelli 1977; Subasingle 1995), chitin production from fungal waste is negligible.

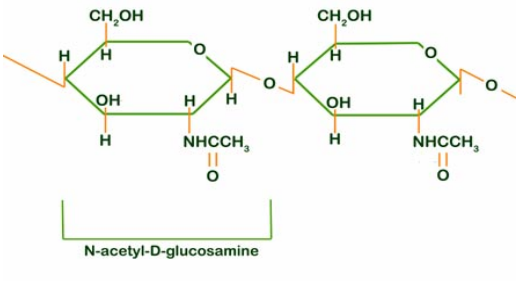


Figure 2.1 Structure of chitosan.

Depending on the sources, the physicochemical properties and functionalities of Chitosan differ (Rhazi *et al*., 2004). For example, chitosan prepared from squid contains β-chitin (amine group aligned with the OH and CH2OH groups) and those prepared from crustaceans contain α-chitin (anti-parallel chain alignment) (Shepherd and others 1997; Felt and others 1999). Despite a wide range of available sources, chitosan is commercially manufactured only from crustaceans (crab, shrimp, krill, and crayfish) primarily because a large amount of crustacean exoskeleton is available as a byproduct of food processing. Disposal of crustacean shell waste has been a challenge for seafood processors. Therefore, production of value-added products, such as chitin, chitosan and their derivatives, and utilization of these value added products in different fields are of utmost interest to food industries. Continual use of new raw materials as a source of chitin would enable production to be significantly increased. Major progress is being made in the development of profitable technology for isolation of chitin and its derivatives (Rashidova *et al.*,2004). However, commercial extraction has been hampered by the corrosive nature of the strong acids and bases used in the manufacture of chitosan, which destroys equipment, requires careful handling by workers, and presents potential environmental hazards (Peniston and Johnson 1980; Leffler 1997).

2.6.3 Film-forming ability of chitosan

Chitosans with higher molecular weight have been reported to have good film-forming properties as a result of intra- and intermolecular hydrogen bonding (Muzzarelli, 1977). A patent was granted to G.W. Rigby in 1936 for the earliest attempt to form films from chitosan. These films were described as flexible, tough, transparent, and colorless with a tensile strength of about 9,000 psi and prepared by a solvent casting methodChitosan films prepared by similar methods were reported later by Muzzarelli *et al*., 1974 , Averbach (1978), Butler *et al*., 1996, Caner *et al*., 1998 and Wiles *et al*., 2000. These films were described to have good gas barrier and mechanical properties. The chitosan film characteristics, however, varied from one report to another. Differences in the sources of chitin used to produce chitosan, chitosan properties, solvents used, methods of film preparation, and types and amounts of plasticizers used affect the quality of the chitosan films (Lim and Wan 1995; Remuñán-López and Bodmeier 1996; Begin and Calsteren 1999; Nunthanid 2001). The film-forming ability of chitosan extracted from crawfish has been reported by Nadarajah and Prinyawiwakul (2002).

2.6.4 Film-forming methods

Edible films are formed by either a wet- or dry-process mechanism. The wet-process mechanism is based on a film-forming dispersion or solution in which polymers are first dispersed or solubilized into a liquid phase, and then dried. Freeze drying is employed to obtain sponge-type scaffolds used in tissue engineering. The wet process is often preferred as it permits the application of films as coatings in a liquid form directly onto food products by dipping, brushing or spraying Peressini *et al*., 2004. Some edible films, such as starch films, can be prepared using a dry-process, such as thermoplastic extrusion. This extrusion process is based on the thermoplastic properties of polymers when plasticized and heated above their glass transition temperature under low water content-conditions. Warburton *et al*., 1993; Psomiadou and others 1997; Arvanitoyannis and Billiaderis 1998).

2.6.5 Film-forming mechanisms

Polymeric solutions form films through a series of phases. When the polymer solution is cast on a surface, cohesion forces form a bond between the polymer molecules (Banker 1996). When the cohesive strength of the polymer molecules is relatively high, continuous surfaces of the polymer material coalesce. Coalescence of an adjacent polymer molecule layer occurs through diffusion. Upon evaporation of water, gelation progresses and allows the polymer chains to align in close proximity to each other and to get deposited over a previous polymer layer (Harris and Ghebre-Sellassie, 1997). When there is adequate cohesive attraction between the molecules, sufficient diffusion, and complete evaporation of water, polymer chains align themselves to form films (Harris and Ghebre-Sellassie 1997).

2.6.6 Film morphology and defects

Polymeric films should be uniform and free from defects for their applications. Uniformity of the films is critical for their functionalities. The processing variables involved in conversion of chitin into chitosan, especially the uniformity of particle size of shells used as a starting material, greatly influence the properties of chitosan No *et al*., 1999, and hence the uniformity of films produced. During the film-forming process, shrinkage of the films due to evaporation of water or rapid drying often causes defects such as cracks or curling in the films (Obara and McGinity 1995). Addition of plasticizers such as glycerol or sorbitol is often used to reduce such defects.

2.6.7 Function of plasticizers in film formation

Films prepared from pure polymers tend to be brittle and often crack upon drying. Addition of food-grade plasticizers to film-forming solution alleviates this problem (McHugh and Krochta, 1994). When a plasticizer is added, the molecular rigidity of a polymer is relieved by reducing the intermolecular forces along the polymer chain. Plasticizer molecules interpose themselves between the individual polymer chains, thus breaking down polymer-polymer interactions, making it easier for the polymer chains to move past each other. The plasticizer improves flexibility and reduces brittleness of the film. Polyethylene glycol, glycerol, propylene glycol, and sorbitol are the most commonly used plasticizers in edible film production (Aydinli and Tutas 2000).

The amount of plasticizer added can cause adverse effects on film properties such as increasing mass transfer through the films. Hence, plasticizers must be used with caution. When the plasticizer concentration exceeds its compatibility limit in the polymer, it causes phase separation and physical exclusion of the plasticizer Aulton *et al*., 1981. This leads to development of a white residue on edible films which has been referred to as "blooming" Aulton *et al*., 1981 or "blushing" Sakellariou *et al*., 1986. The amount of plasticizer used in film formation should also be small enough to avoid probable toxic effects (Nisperos-Carriedo 1994).

2.7 PROPERTIES OF CHITOSAN AND CHITOSAN FILMS

2.7.1 Safety of chitosan films

Chitosan is non-toxic and safe to domestic animals (Hirano and others 1990). According to Rao and Sharma (1997), chitosan films were non toxic and free from pyrogens. Many medical and pharmaceutical applications of chitosan films require sterility of filmsChitosan films can be sterilized by irradiation (Lim and others 1998) and autoclaving (Rao and Sharma 1995), although these processes lead to some degradation of the films.

2.7.2 Biodegradation of chitosan and chitosan films

Many studies have shown that chitin and chitosan are biodegradable polymers. Davies *et al*., 1969 reported that chitosan is most susceptible to hydrolysis by lysozyme at pH 5.2, and the optimum range of pH value is between pH 5.2 and 8.0 (Davies and others 1969; Shigemasa *et al*., 1994. Pangburn *et al*., 1982 studied the effect of deacetylation on susceptibility of chitin and chitosan to lysozyme and found that pure chitin (0% deacetylation) was most susceptible to lysozyme, while pure chitosan (100% deacetylation) was not degraded by lysozyme. Sashiwa *et al*., 1990 studied the relative rates of degradation of six chitosans varying in degree of deacetylation (45%, 66%, 70%, 84%, 91%, and 95%), and reported that 70% deacetylated chitosan degraded most quickly. Shigemasa *et al*., 1994 investigated the effects of preparation methods on Chitosan degradation. They found that for the same molecular weight and degree of deacetylation, homogeneously prepared chitosans were more susceptible to hydrolysis by lysozyme than those heterogeneously prepared.

2.7.3 Mechanical properties of chitosan films

For edible films to be employed as a food packaging material, they should satisfy the requirement of being durable, stress resistant, flexible, pliable, and elastic. Thus, they should possess desirable tensile properties which could bear stresses exerted during various handling processes. Only limited literature is available on mechanical properties of chitosan films. There are variations of physical property values of chitosan films reported in the literature due to different chitosans and testing conditions used. Films produced with low molecular weight chitosan at 3% w/w in 1% acetic acid, with glycerol as a plasticizer at 0.25 and 0.50 mL/g of

chitosan, were reported to have tensile strength (TS) of 15 to 35 MPa and percent elongation at break (%E) of 17 to 76 (Butler and others 1996). Caner and others (1998) reported that films produced by a similar method but with different solvents (acetic, formic, lactic and propionic acid) at 1% and 7.5% concentrations exhibited the TS value range of 12 to 32 Mpa and %E value range of 14 to 70 with an exception of the film made with 7.5% lactic acid having the lowest TS value of 6.85 MPa and the highest %E value of 51. They also reported that increasing the plasticizer content decreased TS and increased %E. Kittur and others (1998) reported a much higher TS value of 70.3 MPa and a lower %E value of 6.2 for a film made of 2% w/w Chitosan in 1 % acetic acid. Variations in mechanical strength of chitosan films are due to the type of chitosan and concentration used, the type of plasticizer and its content, and solvent. The TS values of chitosan films are comparable to those of commercial DDPE and LDPE films but, their %E values are significantly lower than the commercial films (Briston 1988). However, compared to films made of other biopolymers (wheat gluten, corn zein protein and soy protein isolate), chitosan films exhibited significantly higher TS values (Cunningham and others 2000).

2.7.4 Transport properties of chitosan films

In general, edible films and coatings provide the potential to control transport of moisture, oxygen, aroma, oil, and flavor compounds in food systems, depending on the nature of the edible film-forming materials (Donhowe and Fennema 1993; Krochta 1997; Krochta and De Mulder-Johnston 1997). However, when films are formed using biopolymers alone, they are very brittle. To lessen brittleness and to make flexible films, plasticizers are used. However, plasticizers increase the film permeability (Gontard and others 1993), especially for plasticized hydrophilic films. Increased permeability of edible films is undesirable for food applications, so there is a need to minimize the use of plasticizers. Another potential approach to increase film flexibility is reduce polymer molecular weight, thus reducing intermolecular forces along polymer chains and increasing polymer chain end groups and polymer free volume (Sears and Darby, 1982). This approach may permit a decrease in the required amount of added plasticizer in films; consequently, it may minimize permeability of films while producing needed film flexibility (Sothornvit and Krochta 2000)Chitosan films exhibit gas barrier properties. Oxygen permeability of chitosan is as low as many conventional plastic films such as poly vinylidene dichloride (PVdC) and ethyl vinyl alcohol (EVOH) (Webber 2000). Since chitosans obtained from various sources and methods vary in their characteristics, barrier properties of film made of various chitosans also vary. Muzzarelli, *et al.,,* 1974 reported a water vapor transmission rate of 1200 g/m2/d measured at 100 °F and 90% relative humidity for chitosan membranes with 20 µm thickness. Wong and others (1992) reported a water vapor permeability (WVP) value of 0.41 g mm/m2/d/mmHg for chitosan and chitosan-lipid films cast from 1% chitosan solution using formic acid. Butler and others (1996) reported that chitosan films made with plasticizer (glycerol) levels of 0.25 and 0.50 ml/g had a mean WVP of 2.89 × 10–4 g/m/d/mmHg at 25 °C between 0% to 11% RH. Manufacturing biopolymer based films with adequate water barrier properties is a major challenge as many of the bioplymers are hydrophilic by nature (Webber 2000). Butler and others (1996) stated that their chitosan films were extremely good barriers to oxygen, while having higher water vapor barrier properties because of their hydrophilic nature. They also reported that increasing plasticizer concentrations negatively affected barrier properties but improved formation, mechanical, and handling properties. Caner and others (1998) prepared chitosan films using various acid and plasticizer concentrations and reported water vapor permeability coefficients ranging from 1.74 × 10–5 to 7.04 × 10–4 g/m/d/mmHg at 25°C between 50% to 100% RH. They also suggested that storage time had no effect on barrier properties of chitosan films. Attempts to improve vapor barrier properties of chitosan films yielded only limited success. Wong and others (1992) used lipid to form chitosan-lipid composite films to improve moisture barrier properties. Hoagland and Parris (1996) developed a chitosan/pectin-laminated film to alter water vapor permeability and water solubility. Tual and others (2000) produced chitosan films with improved barrier properties by crosslinking chitosan with glutaraldehyde. However, these films were reported to be brittle due to formation of chemical junctions.

2.7.5 ANTIMICROBIAL PROPERTIES OF CHITOSAN AND CHITOSAN FILMS

Chitosan possesses unique properties that make it an ideal ingredient for development of antimicrobial edible film. Chitosan possesses film-forming properties (Averbach 1978), greater and broader spectra of antibacterial activity compared to disinfectants, a higher bacterial/fungal killing rate, and lower toxicity toward mammalian cells (Franklin and Snow, 1981; Takemono and others 1989). Further, Rhoades and Roller (2000) reported that the interaction (binding or chelation) of chitosan with endotoxins of gram-negative bacteria decreased their acute toxicity. Because of the strong chelating ability of chitosan, external chelating agents such as EDTA may not be required, when antimicrobial agents such as nisin are added to chitosan to control gram negative bacteria.

2.7.6 Biomedical applications

There are numerous potential commercial uses of chitosan, and the great commercial interest in chitosan is reflected by the high number of patent applications. The potential use of chitosans and chitosan derivatives includes such diverse fields as agricultural, cosmetic, water-treatment, textile and food applications. Some of the most promising applications of chitosans are in the biomedical and pharmaceutical field, where chitosans have been investigated for a considerable number of applications. Chitosans are biodegradable, biocompatible and have low toxicity. Due to their cationic properties chitosans are bioadhesive and able to interact strongly with epithelial cells and the overlying mucus layer, and hence lead to prolonged contact time between the drug formulation and the adsorptive sites. Combined with the fact that chitosans have the ability to open tight junctions in epithelial cells, thereby further enhancing the absorption of drugs, this makes the use of chitosans in peroral, nasal and ocular drug-delivery applications highly promising. Nasal drug-delivery has recently gained attention for the delivery of challenging drugs that are not

easily administered via other routes than by injection, or where a rapid onset of action is required. With the use of bioadhesive delivery systems containing e.g

Chitosan the delivery of small polar molecules, peptides and proteins and even the large proteins and polysaccharides used in vaccines could be delivered through the nasal route. Chitosan has shown very promising results in this field. Chitosans form complexes with anionic drugs and polyanions such as alginates, xanthan collagen and DNA, and such complexes have been studied for potential use in drugdelivery systems. Microcapsules formed from these polyelectrolyte complexes or by other techniques can exhibit pH- and ion-sensitive swelling behaviour, and controlled drug release systems can be designed. Chitosans can be used for coating of e.g. alginate microcapsules to enhance the bioadhesive properties of the capsules, performing the action in drug-delivery systems. In gene therapy chitosans are of current interest, due to the need for non-viral, non-toxic and biodegradable gene delivery vectors. Chitosans also promote wound healing, and have been shown to exhibit antimicrobial effects. They are also promising as cholesterol lowering agents, and have been suggested as weight reducing agents.

2.8 BETA-GLUCAN

2.8.1 Origin and structure

Beta-Glucan is a biopolymer of glucose that is widely distributed throughout the biosphere (Ruiz-Herrera, 1991). Various types of beta-glucan are commonly found in baker's and brewer's yeast, but also in certain mushrooms, molds, algae and in the bran of oats and barely. beta-glucan derived from various sources have similar structure, but small structure differences influence their biological activity. The innermost layer of the yeast cell wall is built of beta-glucan, proteins, mannan and small amounts of chitin (Vetvicka, 2001).

So, yeast, as a well known microorganism that is used in biotechnology since ancient times, is a good source of beta-glucan. Structure and composition of the yeast cell wall depends on yeast species and strain as well as on culturing conditions (Stone and Clarke, 1992; Klis *et al*., 1997; Lipke and Ovalle, 1998; Nguyen *et al*., 1998; Osumi, 1998; Kath and Kulicke, 1999; Aguilar- Uscanga and Francois, 2003). The beta-glucan component in the *Saccharomyces cerevisiae* cell wall, with the function of maintaining the rigidity and shape of the cell, is oft en named simply glucan or yeast glucan. That polysaccharide consists mainly of a linear central backbone of D-glucose linked in the beta-(1→3) position with glucose side branches (beta-(1→6)-linkage) of various sizes, which occurs at different intervals along the central backbone (Gardiner, 2000). Triple helical multimer provides structure and support to the yeast cell wall.

The native structure of beta-glucanas well as their biological activities could be changed during isolation if harsh procedures are applied. Primary structure, solubility, degree of branching and molecular weight, as well as the charge of their polymers and structure in aqueous media, are responsible for biological activity of beta-glucan.

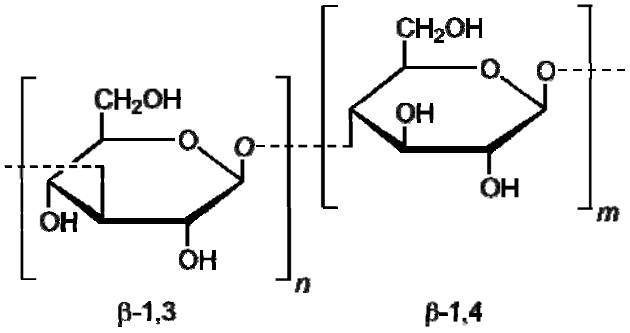


Figure 2.2 Structure of beta-glucan

Solubility of beta-glucan increases as degree of branching decreases, so classification of glucans can be made according to their solubility properties. The available commercial products containing glucan from various sources have several activities due to mentioned differences in structure and conformation (DiLuzio, 1983; Mueller *et al*., 2000). During the complex purification process of beta-(1→3),(1→6)- glucan from cell wall fraction of baker's yeast *Saccharomyces cerevisiae,* other cell components like proteins, lipids, nucleic acids, minerals and mannans are removed to a large extent (Lee *et al*., 2001; Hunter *et al*., 2002). Some of these impurities may induce undesirable side effects (protein induced allergies are sometimes mentioned as a possibility, although it is very rare). It is more important that impurities can interfere with the recognition of the beta -(1→3),(1→6)-glucan active molecule, so the removal of those components is desirable.

Depending on the isolation procedure native structure of beta-glucan could be degraded, resulting in its changed biological activity. Hunter *et al*., 2002 developed the method for the preparation of immunologically active, homogenous, non-aggregated beta-glucan particles of appropriate dimensions for macrophage immune-stimulation. Insoluble yeast cell wall material was subjected to alkaline and acidic treatment followed by sonication and spray-drying of beta-glucan particles. beta-glucan from various sources posses different characteristics and consequently many interesting properties, so immune-potentiation is only one of them (Zeković *et al*., 2005). Branched or linear (1→4)-glucans have limited activity and beta-glucan with a 1,3-configuration with additional branching at the position C-6 of the 1→3 linked D-glucose residues have the highest immune-stimulating activity (Vetvicka, 2001; Freimund *et al*., 2003). Alternative biological sources for the preparation of beta-glucan are barley, oats, mushrooms, algae and bacteria. Those glucans have different structures and consequently diff erent biological activities. Commercial beta-(1→3)-glucans, isolated from bacteria or algae, have no poly-branching (Kedzierska, 2007). The mechanism of beta-glucan biological activity is not yet fully elucidated. Th ere are various opinions which molecular structure could reach the physiological effect (DiLuzio, 1983; Tokunaka *et al*., 2000). Several physicochemical factors (primary structure, conformation, charge of polymers, solubility, particle dimensions) are important for beta-glucan biological activity (Tzianabos, 2000; Vetvicka, 2001, Hunter *et al*., 2002).

Structure essential for biological activity of glucans has not yet been explained in details, but some authors consider that triple helix is the most active conformation (DiLuzio, 1983; Hromadkova *et al*., 2003) while other authors claim that helical structure has no influence on activity at all (Kulicke *et al*., 1997; Ha *et al*., 2002). Vetvicka and Vetvickova (2007) compared the basic immunological activities of a group of commercially available beta-glucan, chosen among those heavily advertised, commonly available and easily obtained in the USA, Europe, Southeast Asia and Japan. Both soluble and insoluble glucans from various sources, including yeast, mushrooms and cereals were included in their study. The tested biological reactions (phagocytosis, surface markers on splenocytes, cytokine synthesis, and stimulation of antibody response) showed that some of the commercial glucans had surprisingly low activity and differed in biological effects. According to their results, it is imperative to find a beta-glucan from a solid vendor that is able to back the claims with solid scientific data.

2.8.2 Beta-glucans: polysaccharide immunomodulators

Immuno-modulators are compounds that interact with the immune system and up regulate or down regulate certain aspects of the host immune response in order to augment or complement a desired immune response. The use of immune-modulators to allow the host to defend itself against invading microorganisms in a more efficacious manner is attractive because it manipulates the host derived immune mechanisms to help the host defend itself and does not involve the use of organism-specific antibiotics, which have recently become a major concern due to emergence of antibiotic resistant strains of several microorganisms (Tzianabos 2000).

Polysaccharides are one class of immune-modulators that comprises of monoclonal antibodies, colony stimulating factors, interferons and several synthetic peptides. Quite a few polysaccharides are recognized as immune-modulators. The list includes but is not limited to Polysaccharide A (PSA), mannans, Hyaluronic acid (HA), and beta-glucan.

Natural products like beta-glucan have been consumed for probably thousands of years especially in China and Japan, and have long been considered to improve general health (Chen and Seviour 2007). However, only recently their importance in health and disease has been recognized (Williams and Di Luzio 1980). Although not medically prescribed as a treatment for any diseases in most countries, they are widely used as food supplements. For example, in Japan, a beta-glucan called lentinan has been approved for clinical treatment of gastric and colorectal cancers (Munemoto *et al*., 2002;). However, the exact functions and molecular mechanisms of beta-glucan have not been elucidated.

The length and distribution of side chain on the backbone may vary among different beta-glucan. They are mainly found as cell wall components in fungi, yeast and bacteria. They are also found in plants and cereals such as barley and oat.

2.8.3 Effects of β -glucans on immune system

Beta-glucan are not found endogenously in animals, so they can act as excellent pathogen-associated molecular patterns and get recognized by immune system as non-self molecules, thereby inducing both innate and adaptive immune responses (Brown and Gordon. 2005). In vitro, large molecular weight or particulate beta-glucan like zymosan (cell wall extract from *Saccharomyces cerevisiae*) can stimulate the phagocytic and antimicrobial

activities of leukocytes. They can also stimulate production of pro-inflammatory cytokines and chemokines, such as IL-6, IL-1β, IL-8, IL-10, IL-12 and TNF-α (Brown.,2006). They also stimulate macrophage phagocytosis of apoptotic cells through upregulation of phosphatidylserine receptor (Fadok *et al*., 2000). Intermediate molecular weight beta-glucan have been shown to show some activity in vivo but their effects are less clear.

Some studies have shown that they can activate leukocytes and enhance the effect of a secondary challenge like LPS (Battle *et al*., 1998), while other studies have reported opposite effect (Nakagawa *et al*., 2003). Very short beta-glucan like laminarin are biologically inactive. In vivo, beta- glucans potentiate host response against variety of conditions, including tumor development and fungal, bacterial or viral pathogens.

Studies reporting immune effects of different beta-glucanare full of contradictions, especially because of the difference in the structural and biological properties of the beta-glucanused in those studies. Also, most of the studies showing biological effects of beta-glucan were done using zymosan or other impure preparations of beta- glucans, which is another reason for discrepancies in the literature. However, recently, a chemically pure beta-glucan, SCG from *Sparassis cripa* was also shown to activate the dendritic cells and stimulate the release of IL-12, TNF-α and IFN- γ, supporting the studies showing beta-glucanas immune stimulators (Saijo *et al*., 2007).

2.8.4 Beta-glucan structure-activity relationship

A number of factors such as molecular weight, solubility, branching and structural conformation are known to influence activity of beta-glucan. Generally, high molecular weight (MW > 100 kDa) beta-glucan are biologically more active, while low molecular weight (MW 5-10 kDa) are biologically inactive (Zekovic *et al*., 2005). With regards to solubility, the extent of solubility of beta-glucan depend on their degree of polymerization (Zekovic *et al*., 2005). While soluble beta-glucan are stronger immunomodulators than insoluble ones (Xiao *et al*., 2004), the latter can become biologically more active, probably after getting degraded into smaller oligomers following oral administration (Hong *et al*., 2004).

In addition to polymerization and solubility, the degree of branching also influences the biological activities of beta-glucan. Unbranched beta-glucan are biologically less active than the branched ones. Generally, a branching frequency of 0.2 (1 in 5 backbone residues) to 0.33 (1 in 3 backbone residues) is considered to be optimal for biological activity (Miyazaki *et al*., 1979). Modifying the glucan side chains also affect its biological activity. For example, sulphated and carboxymethylated derivatives of insoluble scleroglucan showed more activity against sarcoma 180 tumor and gastric carcinoma cells compared to its unmodified, methylated, hydroxymethylated or hydroxypropylated derivatives (Wang *et al.,* 2004).

Another factor contributing to the differences in the biological activities of beta-glucanis their conformation. beta-glucan can exist in triple helix, single helix or random coil conformation. While several studies reported that the triple helix conformation is the most biologically active

conformation (Falch *et al*., 2000), others suggest that the single helix is the most active form (Aketagawa *et al*., 1993). Taken together, all the factors discussed above, either in isolation or in combination, are critical in determining the biological activities of beta-glucan.

2.8.5 Functional importance of beta-glucan

2.8.5.1 Anti-cancer

After 50 years of their first reported anti-tumor property, several animal experiments have shown the useful role beta-glucan on number of tumors (Vetvicka and Yvin 2004). Several clinical trials have also been conducted in this regard but the results are still preliminary and controversial. However, some beta- glucans like lentinan and schizophyllan are currently approved in Japan for clinical use in human cancer treatment (Mizuno *et al*,. 1999). Yeast beta-glucangiven orally with monoclonal antibodies reportedly increased neuroblastoma tumor regression and long-time survival in mice (Yan *et al*., 2005). They act by stimulating granulocytes and macrophages to promote tumor regression and also by triggering cytotoxicity of tumor cells (Yan *et al*., 2005).

Some beta-glucan enhances the effect of chemotherapy and radiation therapy and thereby increase patient tolerance and limit toxic effects of such treatments (Gu *et al*., 2005). In a nutshell, most of the studies have demonstrated that beta-glucancan cause regression in tumor size but not complete eradication of the tumor and can prove beneficial as an adjuvant therapy in cancer. Anti-infective activity of beta–glucans Several beta-glucan like lentinan, zymosan and curdlan were shown to be effective against several viruses (Mayell *et al.,* 2001). Zymosan, a cell wall extract from *S. cerevisiae,* was reported to enhance the effectiveness of vaccine against HIV by stimulating Th cell-mediated immunity through activation of complement system as well as through production of interferon gamma (Ara *et al*., 2001).

Other beta-glucansuch as SCG, PGG and lentinan, are effective against several bacterial infections. For example, lentinan reduced *Mycobacterium tuberculosis* infection in vivo by increasing macrophage number and in vitro by increasing the killing ability of macrophages towards *M. tuberculosis* (Markova *et al*., 2005). Similarly, PGG protected mice against Bacillus anthrax infection (Kournikakis *et al*., 2003). Overall, these and many other studies suggest that beta-glucan treatment, when used as prophylactics, may boost the immune system to fight against subsequent infections. Fungal infections are not so common in healthy individuals, but infections by opportunistic fungi are very common and problematic in immunocompromised individuals. Some beta-glucanactivate host macrophages, neutrophils and dendritic cells to boost immune response against fungal infections (Herre *et al*., 2004b). beta-glucanlike zymosan can protect host cells against *Candida albicans* and *P.carinii* infections (Gantner *et al*., 2005). They can also act synergistically with other anti-fungal agents in several fungal infections. Currently, however, they have not been included in such combination regimens.

2.8.5.2 Activity of beta-glucan on cardiovascular and endocrine systems

Very few studies have reported cholesterol lowering effects of beta-glucan. For example, yeast beta-glucanwere effective in reducing blood cholesterol levels but the mechanism of action is still unclear (Nicolosi *et al*., 1999). Some fungal beta- glucans may also help regulate blood pressure. For example, in genetically deficient rats with spontaneous hypertension (SHR), a diet containing 5% maitake powder reduced the mean systemic blood pressure (Kabir and Kimura 1989). However, the role of beta-glucan in mediating this effect was not elucidated. Thus, based on this and some other studies, the role of β- glucans in reducing hypertension does not seem to be very clear. In addition to their effects on cardiovascular system, several beta-glucanwere shown to reduce blood glucose levels by delaying stomach emptying and thereby decreasing the rate at which glucose is absorbed (Lo *et al*., 2006). Further, feeding whole mushroom maitake powder and its chemically derived fractions to genetically diabetic mice, also reduced their blood glucose levels by increasing insulin sensitivity (Mayell *et al.,,* 2001). Collectively, all the aforementioned studies indicate useful role of beta-glucanon cardiovascular and endocrine systems, although the mechanisms behind their actions are not clear.

MATERIALS AND METHODS

CHAPTER 3
MATERIAL AND METHODS

3.1 MATERIALS

Chitosan (Hi-Media), Acetic acid (Nice chemicals), Latic acid (Loba Cheme), Sodium hydroxide (Sigma), Congo red (Sigma), citric acid (Sigma), DPPH (Hi-Media), Phosphomolybdate (Hi-Media) and mushroom fruiting bodies,. All chemicals were of analytical grade.

3.2 SAMPLE COLLECTION AND PROCESSING

Materials

DEAE Sephacel, ConA agarose column (1.5 x 10 cm), Congo red were products of Sigma– Aldrich.

Protocol

The fruiting bodies of the mushrooms were kindly provided by local breeder, Coimbatore. The mushroom species chosen for analysis was confirmed as *Pleurotus ostreatus*. The mushroom sample was dried in a tray drier at 55°C for 36 hours to reduce the moisture content to 4-5 %. The dried mushroom were finely powered and kept in air tight container in cold room at 4°C until use (Figure 3.1)

3.3 EXTRACTION AND PURIFICATION OF BETA-GLUCAN

Mushroom beta-glucan was isolated according to Lee *et al*., (2001). The isolation was modified for quantitative analysis in the following way (Figure 3.2):

3.3.1 Preparation of soluble beta-glucan:

Five g of dried fruiting bodies was extracted with 100 ml of 2%NaOH at 90°C for Five hours. After cooling the suspension was centrifuged at 3000 x g for 10 min. The resulting supernatant was neutralized with 2M Acetic acid and further treated with 3 volume of ethanol to precipitate beta-glucan. [Fraction-1]

Figure: 3.1: Sample collection and processing.

Raw mushroom

Mushroom cut into small pieces and kept for drying

Powdered mushroom

Stored in air tight container

FIGURE: 3.2 Purification of beta-glucan from fruiting bodies

Mushroom powder

NaOH Extraction

Alcohol Precipitation

FRACTION 1

DEAE-Sephacel Chromatography

FRACTION 2

Con-A Chromatography

FRACTION 3

Rotary evaporator

Crude beta-glucan powder

3.3.2 DEAE chromatography to remove the residual proteins

The precipitated beta-glucan (fraction-1) was dissolved in 3% Acetic acid and centrifuged to remove insoluble beta-glucan. The recovered supernatant was neutralized with 2M NaOH. After this neutralization, the preparation was loaded into DEAE sephacel anion-exchange column to remove residual proteins. The unbound fraction containing the beta-glucan was collected by eluting the c olumn with 3 bed volume of 10mM sodium phosphate buffer at pH 8.0. [Fraction – 2]

3.3.3 Affinity chromatography in an agarose bound concanavalin A column

The fraction free of proteins (Fraction – 2) was concentrated by rotary evaporator and then dissolved in 10 ml of 50mM phosphate buffer (pH – 7.4) containing 0.15M NaCl. It was then applied to ConA agarose column to remove mannan from fraction – 2. The unbound fractions in the column containing water soluble glucan were collected by eluting the column with 50ml of the buffer. The bound material in the column, mannan, was washed with a 50mM phosphate buffer (pH – 7.4) containing 0.25M NaCl to regenerate the column. The protein content and total carbohydrate were quantified at each step. [Fraction – 3]

3.4 ANALYSIS OF CRUDE BETA-GLUCAN

Materials

Congo red, citric acid and sodium hydroxide were products of Sigma-Aldrich.

3.4.1 Colorimetric determination of beta-1, 3-1, 6-glucans with Congo red

The beta-glucan content of the samples was quantified using a modified protocol of Ogawa *et al*., (1972). A spectrophotometer was used at a wavelength of 550 nm for the photometric determinations. A defined volume of sample solution was pipetted into a cuvette and diluted with distilled water to a total volume of 0.7ml. Then 0.6ml of 0.2 mol/l citric acid/sodium hydroxide buffer (pH 7) and 0.1ml of dye solution (0.08g Congo red were diluted in 100 ml buffer) were added. Then the samples were mixed and analysed at 550 nm against 0.7ml distilled water, 0.6 ml buffer and 0.1ml dye solution. Because of the light brownish colour of some fractions, a measurement of the background absorption at 550 nm is necessary. Therefore, the same volume of sample was diluted with distilled water to a final volume of 0.7ml. The solution was immediately neutralised and filled up with distilled

water to a quantitative volume. This stock solution was used for the standard calibration curve in the range of 50–150 mg/ml. All analyses were performed in triplicate.

3.5 TOTAL CARBOHYDRATE AND PROTEIN ANALYSIS

The crude protein, crude total carbohydrate composition of *Pleurotus* sp. was determined according to AOAC methods.

3.5.1 Carbohydrate determination

Total carbohydrate content was determined via the phenol-sulfuric method according to Dubois, Gilles, Hamilton, Rebers, and Smith (1956), using glucose for the standard curve. The qualitative analysis of carbohydrates was achieved by thin layer chromatography (TLC), according to the methods of Moreira, Souza, and Vendruscolo (1998), in reference to glucose and mannose standards from Sigma.

3.5.2 Protein determination

Total soluble protein content was determined via the method described by Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin (Sigma) for the standard curve

3.6 PURITY AND STRUCTURAL ANALYSIS

3.6.1 Infrared spectroscopy [FTIR]

The extracted and purified sample (crude beta-glucan) was sent to SAIF- IIT, Mumbai for FTIR analysis. Fourier-transform infrared (FT-IR) spectroscopy was used for functional group determination of purified beta-glucans extracted from mushroom. The spectra were recorded with a 3000 Hyperion Microscope with Vertex 80 FTIR System (Bruker, Germany) equipped with a Micro ATR (attenuated total reflection). Scan run for each spectrum from 7500 – 450 cm^{-1} at a resolution of 0.5 µm

3.6.2 Nuclear magnetic resonance spectroscopy

The extracted and purified sample (crude beta-glucan) was sent to SAIF-CDRI, Lucknow for NMR analysis. Liquid state nuclear magnetic resonance (NMR) spectra of purified beta-glucan from mushroom were obtained using a Bruker DRX-300 (300 MHz FT NMR with low and high temperature facility -90°C to +80 °C) operating at 300 MHz for ^{1}H and 75 MHz for ^{1}H Beta-glucan samples were dissolved in DMSO.

3.7 ANIMAL CELL LINES STUDIES

The extracted and purified beta-glucan was sent to KMCH pharmacy, Coimbatore for cell toxicity and proliferation analysis.

3.7.1 Cell line

The murine embryonic fibroblasts cell line (NIH 3T3) was obtained from National Centre for Cell Science (NCCS), Pune and grown in Dulbecco's Modified Eagles Medium containing 10% fetal bovine serum (FBS). The cells were maintained at 37^0C, 5% CO2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

3.7.2 Cell treatment procedure

The monolayer cells were detached with trypsin-ethylene diamine tetra-acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium containing 5% FBS to give final density of $1x10^5$ cells/ml. One hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37^0C, 5% CO_2, 95% air and 100% relative humidity. After 24 h the cells were treated with serial concentrations of the test samples. They were initially dissolved in serum free medium and sterilized by filtration using 0.45 µm syringe filter. At the time of sample addition, an aliquot of the sample solution was diluted to twice the desired final maximum test concentration with serum free medium. Additional four serial dilutions were made to provide a total of six sample concentrations. Aliquots of 100 µl of these different sample dilutions were added to the appropriate wells already containing 100 µl of medium, resulting in the required final sample concentrations. Following sample addition, the plates were incubated for an additional 48 h at 37^0C, 5% CO_2, 95% air and 100% relative humidity. The medium containing without samples were served as control and triplicate was maintained for all concentrations.

3.7.3 MTT assay

MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells.

After 48 h of incubation, 15µl of MTT (5mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37^0C for 4h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The percentage cell viability was then calculated with respect to control as follows

$$\% \text{ Cell viability} = \frac{[A]\,\text{Test}}{[A]\text{control}} \times 100$$

3.8 ANTIOXIDANT ACTIVITY

3.8.1 Total antioxidant assay [Phosphomolybdenum assay]

3.8.1.1 Principle

Phosphomolybdenum assay is used to determine the total antioxidant capacity. It is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of green phosphate / Mo (V) complex at acidic pH.

3.8.1.2 Materials

Ascorbic acid, Sulphuric acid, Ammonium molybdate, Disodium hydrogen phosphate.

3.8.1.3 Protocol

The method described by Prieto *et al.,* was used to determine the total antioxidant capacity of the extract. The tubes containing 0.2 ml of the mushroom extract, 2ml of phosphomolybdenum reagent solution (0.6M sulphuric acid, 28mM sodium phosphate and 4mM ammonium molybdate) were incubated at 95°C for 90 min. After the mixture has cooled to room temperature, the absorbance was measured at 695nm using UV/visible spectrophotometer. The antioxidant capacity was expressed was ascorbic acid equivalent (kumar *et al*., 2008).

The total antioxidant activity is calculated by the following formula:

$$\text{Ascorbic acid equivalent } (\mu M/g) = \frac{T}{S} \times C \times \frac{V}{P} \times \frac{RS}{E} \times \frac{1}{MW}$$

T – OD of the test.

S – OD of the standard

C – Concentration of test (µg)

V – Volume of solvent used for extraction

P – Amount of powder (g)

RS – Volume of reagent solution (ml)

E – Volume of extract (ml)

MW – Molecular weight of Ascorbic acid (176.13 g/gmol)

3.8.2 Free radical scavenging assay [DPPH assay]

3.8.2.1 Principle

DPPH scavenging activity is measured by the slight modification of spectrophotometric method. 1,1-diphenyl-2-picryl-hydrazyl radical is scavenged by antioxidants through the donation of protons forming reduced DPPH. The electrons become paired off and the solution losses colour depending on the number of electrons taken up. After reduction, the colour changes from purple to yellow, which is quantified by the decrease of absorbance at 517nm (Shimada *et al*., 1992).

3.8.2.2 Materials

Methanol, DPPH in methanol, Ascorbic acid.

3.8.2.3 Protocol

The mushroom extracts were dissolved in methanol. A solution of DPPH in methanol (0.6mM) was prepared freshly. 3ml of the solution was mixed with 1ml of the samples of varying concentrations. The solution in the tubes were shaken well and incubated in the dark for 30 min at room temperature. The decrease in absorbance was measured at 517nm. The control had equal volume of methanol instead of the extract. 3.1 ml methanol was taken as blank. The percentage inhibition of the radicals due to the antioxidant activity of the extract was calculated using the formula:

$$\textbf{DPPH scavenging activity (\%)} = \frac{\text{Abs}_{515}\ \text{DPPH sol} - \text{Abs}_{515}\ \text{Sample}}{\text{Abs}_{515}\ \text{DPPH sol}} \times 100$$

3.9 PREPARATION OF BETA-GLUCAN LOADED CHITOSAN FILM

3.9.1 Materials

Chitosan, lactic acid, acetic acid, betaglucan.

3.9.2 Protocol

Chitosan (2 g) was suspended in 100 ml of aqueous acetic acid solution (1% v/v) for 6 hour to get a clear solution, which was later filtered to remove undissolved polymer (chitin). To the Chitosan solution, Lactic acid (1%v/v) was added and vortexed for 15 min. The viscous dispersion was kept aside for 30 min for complete expulsion of air bubbles. Films were cast by pouring the polymer solution into the centre of glass moulds and allowed to dry at room temperature. The dry films were cut into strips, wrapped in aluminium foil and stored in cold room (4°C). Preparation of Chitosan films containing beta-glucan followed the same protocol.

3.10 DETERMINATION OF MECHANICAL PROPERTIES OF FILM

3.10.1 Materials

Film, Texture analyser-Instron and thickness meter.

3.10.2 Protocol

The thickness of the film at 10 different positions was measured using a thickness meter. The values are the average of triplicate independent measurement. The mechanical properties of the film were measured using a texture analyser – Instron equipped with a 5Kg load cell. A film strip (8 x 2.5) was held between 2 clamps and pulled by the top clamp at the rate of 30mm/min (Kalapathy *et al*., 2000). The load at the break (N), extension at break (mm) and time at break (sec) were measured when the film broke off. The values are the average of the triplicate independent measurement. The tensile strength and elongation at break were calculated by the equation below.

$$\text{Tensile strength (N/mm}^2\text{)} = \frac{\text{Breaking Force (N)}}{\text{Cross scetional areaof sample (mm}^2\text{)}}$$

$$\text{Elongation at break (\%)} = \frac{\text{Increase in the length at breaking point (mm)}}{\text{Initial length (mm)}} \times 100$$

Figure 3.3 Thickness analyser

Figure 3.4 Texture analyzer- Instron

3.11 FLUID UPTAKE ABILITY (FUA)

3.11.1 Materials

Film, vials, forceps, filter paper, PBS (pH: 7.4).

3.11.2 Protocol

The FUA of the films were determined as described below. Completely dried membrane samples were cut into square-shaped specimens (1 cm x1 cm) and weighed. Then, the specimens were immersed in phosphate-buffered saline (PBS; pH 7.4) buffer, and taken out quickly at specific time intervals, i.e., 1, 2, 3, 4 and 5 hours, respectively, and weighed after blotting with filter paper. The FUA of the film was calculated as follows:

$$\text{FUA (\%)} = \frac{(Ws - Wd)}{Ws} \times 100$$

3.12 ANTI-MICROBIAL TESTING OF FILMS

3.12.1 Disc diffusion method

3.12.1.1 Principle

The agar diffusion assay is one of the methods for quantifying the ability of antibiotics to inhibit bacterial growth. The principle used here is that antibiotic will diffuse from paper disc or cylinder into the agar medium that contains test organisms. Interpretation of the results from this assay relies on the clear zone of inhibition.

3.12.1.2 Materials

Luria Agar, Nutrient Agar were obtained from Hi Media, Ampicillin.

3.12.1.3 Protocol

Antimicrobial activity test was carried out using agar diffusion method. Indicator cultures were *Eschericha coli* and *Staphylococcus aureus,* representing gram negative and gram positive bacteria. One hundred microlitres of the inoculum solution was added to agar plates.

After swabbing the film (1cm x 1cm) was placed in the form of discs separately. The plates were incubated at 37°C for 24hrs.It was then visually examined for zones of inhibition around the film (Kirby Bauer method).

3.12.2 Liquid culture medium assays

3.12.2.1 Materials

Film, Luria broth, Nutrient broth, *Staphylococcus.sp, E.coli*, Spectrophotometer.

3.12.2.2 Protocol

A liquid culture assay was conducted in 100 ml of Luria and Nutrient broth with an inoculation of 5% (v/v) of an overnight culture of *Staphylococcus.sp* and *E.coli.* Cell cultures were incubated at 37°C under stirring. Cell growth was followed by determining OD 600nm (Hitachi Spectrophotometer, U2000). When exponential phases were reached, different content of the film-forming solution were added and antimicrobial activity was followed by determining OD600nm. Experiments with chitosan replaced by the diluant of the film-forming solution (acetic acid, pH 5) were conducted in parallel. Microbial controls were performed from experiments with only microbial strains. All the results are the mean of 3 replicate assays. For the numeration of bacterial cells in flock and supernatant, the same procedure as before was conducted. After Chitosan adding (10% v/v), cultures, initially inoculated by 1% inoculums of *Staphylococcus.sp* and *E.coli*, were left at 37°C for 25 min. Flocculants were recovered by centrifugation (5 min at 2000 rpm, Jouan BR4i) and numeration was carried out on the supernatant. Viable micro-organisms in flocks were recovered by the same volume of tryptose broth or medium with the following detergents or additives (Merck): lecithine 1g/l, Tween 80 5g/l and Triton X100 1g/l. In order to remove viable cells from the flocks, the media were stirred with marbles before spreading. The percentage reduction was calculated as the following relation:

$$\text{\% Reduction} = \frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} X\ 100$$

RESULTS AND DISCUSSIONS

CHAPTER 4

RESULT AND DISCUSSION

4.1 Preparation of beta-glucan from fruiting bodies of mushroom

Polysaccharides, especially β-glucan, are considered responsible for mushroom`s biological activity. The isolation of polysaccharides derived from medicinal mushrooms and their biological activity had been reported (Vinogradov and Wasser, 2005). Beta-glucan research is mostly limited to mycelium and oats. This beta-glucan is already well analysed and identified, while *Pleurotus* sp. is little known about the beta-glucan content. Therefore *Pleurotus ostreatus* is included in present investigation.

4.2 Determination of moisture content

The beta-glucan content of the fruiting bodies of mushroom was found to be much higher when compared to the mycelia. The dry matter of the mushroom fruiting bodies is about 5 to 15 %. The fruiting bodies were dried and moisture content was reduced to 4.14 % (Table 4.1) finely powdered and used for further analysis.

Table 4.1 Moisture content analysis of the mushroom sample

SL.NO	TIME (Hrs)	WET WEIGHT OF MUSHROOM (g)	MOISTURE CONTENT (g)	MOISTURE LOST (g)	% MOISTURE CONTENT
1	0	10.00	8.50	0.00	100
2	4	7.61	6.11	2.39	71.88
3	8	4.94	3.44	5.06	40.47
4	12	3.51	2.01	6.49	23.64
5	16	2.41	0.91	7.59	10.70
6	20	2.18	0.68	7.82	8.00
7	24	1.93	0.43	8.07	5.05
8	28	1.92	0.42	8.08	4.94
9	32	1.92	0.42	8.08	4.94
10	36	1.92	0.42	8.08	4.94

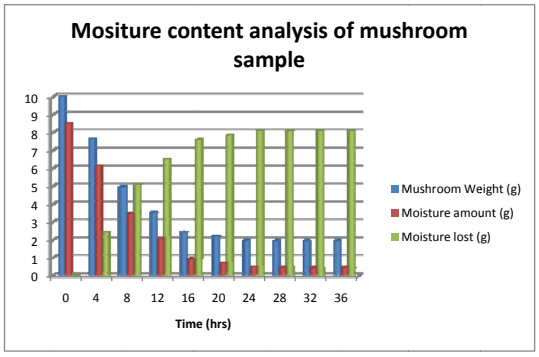


Figure 4.1 Moisture content analysis of fruiting bodies of mushroom

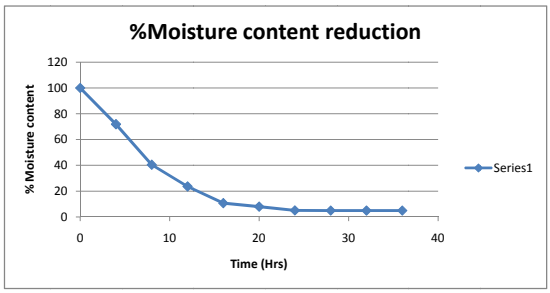


Figure 4.2 % Moisture content reduction in fruiting bodies of mushroom

4.3. Extraction and sequential purification of beta-glucan from fruiting bodies of *P.oestreatus*

4.3.1 Alkali extraction

A suitable procedure for the extraction of glucan from fruiting bodies was worked out. The analysis of solid residues, however, confirms that water insoluable fractions contains large amount of glucans, which can be extracted. Alkali extraction was included into the isolation procedure. The beta-glucan was first rendered soluble from fruiting bodies of mushroom by alkali extraction (2% NaOH) and precipitated by alcohol precipitation technique (Ethanol) [Fraction–1]. The water soluble component contained mainly heteroglycans, glycoproteins. The fraction-1 contained 8.03 % of the protein.

4.3.2 DEAE anion exchange chromatography

To remove the residual proteins, the sample is chromatographed in a DEAE Sephacel anion exchange column which resulted in a reduced to 0.24% protein in the subsequent fraction [Fraction – 2]. Proteins are known to bind on to DEAE column, there by eluting out the beta-glucan. The fractions collected from the DEAE Sephacel column were analysed for protein content using UV spectrometer and the absorbance measured at 280nm for each fraction were plotted (Figure 4.2). The figure shows that protein content is in fractions from 10 to 30, compared to other fractions. Sixty two fractions collected to remove the proteins and then the column was washed with 50mM phosphate buffer and the protein content in the wash out fraction was estimated using UV-Spectrometer and it was found to 0.02 at 280nm. This ensured complete removal of protein from the bound sample in the DEAE column.

4.3.3 ConA affinity chromatography

The fraction collected after DEAE chromatography was concentrated using rotary evaporator and Further purification by Con A chromatography gave mannan-free crude beta-glucan. Mannan is known to bind to concanavalin A (ConA), a class of lectin. The fraction – 3 was completely free of proteins and mannan. The recovery of the water soluble beta-glucan was about 19.6 %(wt/wt) of the starting materials expressed as total carbohydrate.

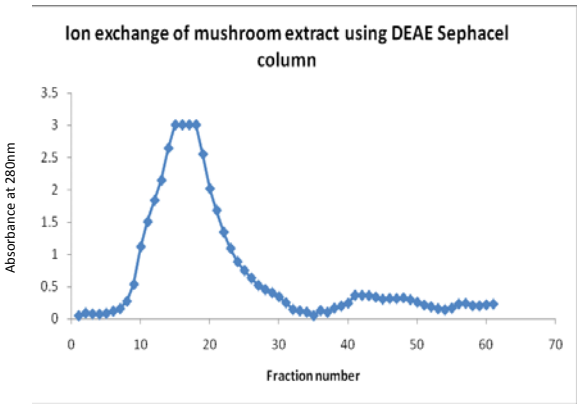


Figure 4.3 Protein measurements by using Ion exchange DEAE Sephacel column

4.4 Total carbohydrate and protein analysis

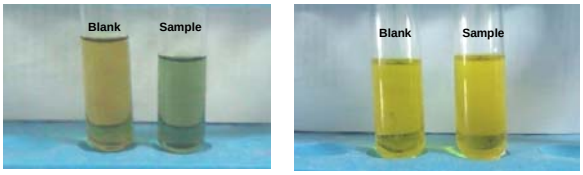
The total carbohydrate and protein analysis was performed using Anthron and Lowry methods. The amount of total carbohydrate and protein initially present in the sample is found to be 67% and 21% respectively. These values were close to the earlier reported for other species *P. sajur caju*.56% and 24 respectively (Chang *et al.,* 1981. The further purified fraction were analysed to and fraction 1(42% carbohydrates and 8% proteins), fraction 2 (15% carbohydrates and 0.24% proteins) and fraction 3 (15% carbohydrates). Sequential steps of purification increased the purity of beta-glucan (Table 4.2)

Table 4.2 Total carbohydrate and protein content for different fractions from fruiting bodies of mushroom

Extract	Total Carbohydrate (% wt/wt)	Protein (% wt/wt)
Sample	**67.068g/100g (67%)**	**21.290g/100g (21.29%)**
NaOH Extraction Fraction 1	**42.560g/100g**	**8.03g/100g**
DEAE Chromatography Fraction 2	**15.876g/100g**	**0.240g/100g**
ConA Chromatography Fraction 3	**15.280g/100g**	**—**

4.5 Confirmation test for carbohydrate and reducing sugars in fraction 3

The fraction 3 after ConA chromatography is analysed for the presence of carbohydrates by anthrone method. The test showed positive result for presence of carbohydrates. The results were shown in figure 4.3. This analysis is a positive conformation for the presence of beta-glucan. The fraction 3 after ConA chromatography is analysed for the presence of reducing sugars by DNS method. The results were shown in the figure 4.4. The test showed result for presence of feeble amount of reducing sugars.



A B

Figure 4.4 A. Anthrone test for total carbohydrates; B. DNS test for reducing sugars

4.6 Colorimetric determination of beta-1, 3-1, 6 glucan with Congo red

The proteoglycans are known for their health benefits (Bush *et al.,* 2007). With the desire to only quantify pure polysaccharides, it can be easily purified by ion exchange chromatography. Thos method is applicable for mushroom extracts. Glucans can be detected with good precision and sensitivity (Nitschke *et al.,* 2011).The total amount of Congo red positive beta-1, 3-1, 6 glucan of the mushroom sample was estimated in the fruiting bodies. Congo red is used for characterization of glucan tertiary structures because of the interaction with the triple helix of beta-1, 3-1, -6 glucan and not with other polysaccharides. An incorporation of the Congo red to the triple helix leads to a barometric shift. The maximum absorption shifted from 493 to 523nm (Muzzarelli *et al.,* 1998). The beta-glucan extracted from the fruiting bodies is detected and observation is provided at table no: 4.3. The increase in absorbance is an indication of the presence of beta-glucan. The congo red interacts with the beta-glucan and not with any other polysaccharides. So this confirms the presence of beta-glucan in the extracted sample from fruiting bodies of mushroom.

Table no: 4.3 Detection of beta-glucan extracted from fruiting bodies of mushroom

Sample	Absorbance Abs 550nm			Mean
	Trial1	Trial2	Trial3	
Water + Congo red	0.426	0.432	0.429	0.429
Beta-glucan + Congo red	0.576	0.582	0.578	0.579

4.7 FTIR Analysis

Infrared spectroscopy is often applied for the structural characterization of beta-glucan (Sandula *et al.,* 1999). FTIR spectra of the fraction obtained from fruiting bodies of *Pleurotus ostreatus* are shown in the figure 4.5. The IR bands in the region of 950 – 1250 cm^{-1} is mainly due to C-C and C-O stretching vibrations in pyranoid rings, indicating the presence of polysaccharides as the major component. The band at 1150 – 1160cm^{-1} was assigned to C-O-C stretching of glycosidic bonds. Other bands and shoulders assigned to beta-glucan were found near 1162 and 1080 cm^{-1}. The bands at 1635 were assigned to amide. The broad band around 3450 – 3370cm^{-1} is the charaterstic adsorption of the hydroxyl group. And a week band at 2000 – 2900cm^{-1} is C-H stretching vibration. These values are similar to those found by Gonzaga *et al*., 2005 with glucans from the fungus *Agaricus blazei*. The absorption peak nearby 844cm^{-1} corresponds to C-H bond in alpha configuration; while C-H bond in beta configuration has an absorption peak near 841cm^{-1} (Schmid *et al.,* 2001 and Hromadkova *et al.,* 2003) but these spectra in this study cannot be clearly identified.

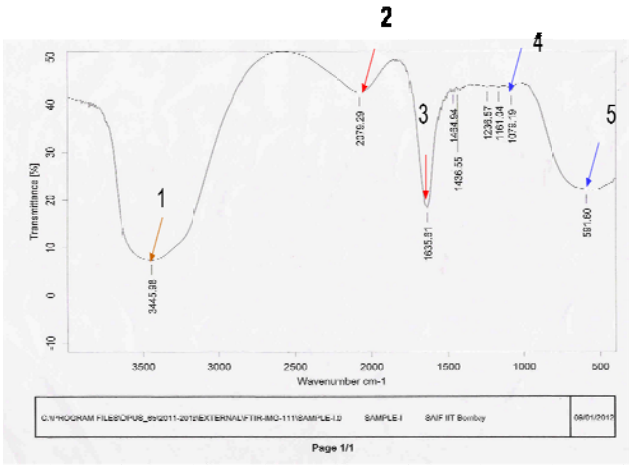


Figure 4.5 FTIR spectrum of crude beta-glucan from fruiting bodies of mushroom

4.8 NMR analysis

The extracted crude beta-glucan from the fruiting bodies was a white power and relatively pure compound (Kameda *et al.*, 2005). The figureno:4.6 shows ^{1}H NMR spectra of beta-glucan extracted from mushroom. The assignment of signal to various carbon types follows that in the literature. The ^{1}H NMR spectrum of extract protons appeared at 1.863, 3.696 and 4.800. Other peaks cannot be clearly identified. These signals are in agreement with results from previous analyses. The ^{1}H NMR shows signal in the anomeric region at 4-6ppm. The signal between 4.1 and 4.6 pp, corresponds to the presence of beta-glucan (Kawagishi *et al.,* 1990 and Gonzaga *et al.,* 2005). There are signal between 1.4 and 2.5 that is related to the glucan-protein structure (Silverstein *et al.,* 1994)

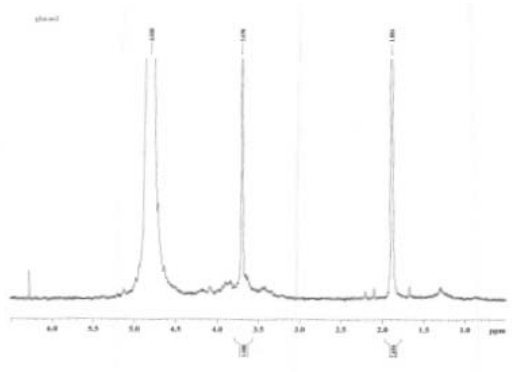


Figure 4.6 ^{1}H NMR spectrum of beta-glucan from fruiting bodies

4.9 Toxicity and Cell viability analysis

Cytotoxicity test are based on the capacity of cells to convert trtrazolium salt (MTT) in a blue compound called formazan. Only live cells have this capacity. The colorimetric MTT test is based on the reduction of yellow tetrazolium salt by mitochondrial reductases in the metabolically active cells. Intracellularly, blue crystals are formed, which are solubilised and then analyzed through UV visible spectrophotometry. Thus, the lower the MTT reductions is, the lower the spectrophotometric signal, and hence, the lower the mitochondrial activity will be (Mosmann et al., 1983).

The Cytotoxicity and cell viability of the prepared crude beta-glucan were evaluated by assessing MTT transformation. The cells were seeded at the same initial density and after 24 h the cells were treated with varying concentration of beta-glucan (18.75 – 300µg/ml). The cell viability was examined and results depicted in table no: 4.4. There was no significance difference in cell viability in lower concentration (18.75 – 75µg/ml), suggesting the crude beta-glucan non Cytotoxicity.

It was noticed that at higher concentration (150 – 300µg/ml) showed a slightly lower absorbance that those of the control, which could be attributed to the large amount of crude beta-glucan. The lower concentration range (below 20µg/ml) is expected to proliferate the cells, and thereby will confirm the non-toxicity nature of the crude beta-glucan. In a study with b-glucan from barley, Angeli, Ribeiro, Angeli, and Mantovani (2009) also reported cytotoxic effects for the same concentration.

Table no: 4.4 MTT assay

	CONCENTRATION OF CRUDE BETA-GLUCAN (µg/ml)					
	Control	**18.75**	**37.5**	**75**	**150**	**300**
ABS Trial 1	0.239	0.236	0.245	0.236	0.202	0.18
ABS Trial 2	0.243	0.241	0.222	0.231	0.197	0.185
ABS Trial 3	0.229	0.244	0.239	0.228	0.211	0.194
ABS Average	0.237	0.240	0.235	0.231	0.203	0.186

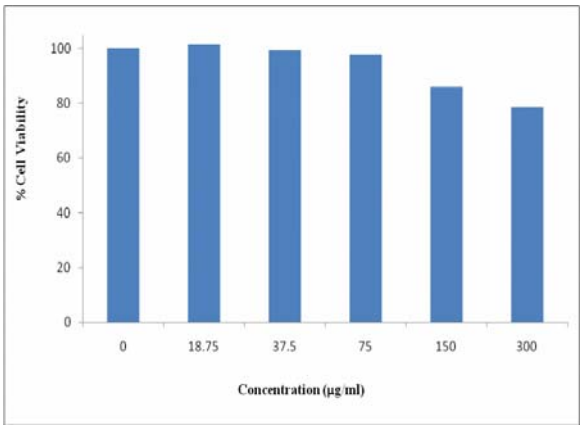


Figure 4.7 Effect of beta-glucan extracted from fruiting bodies of mushroom on cell viability

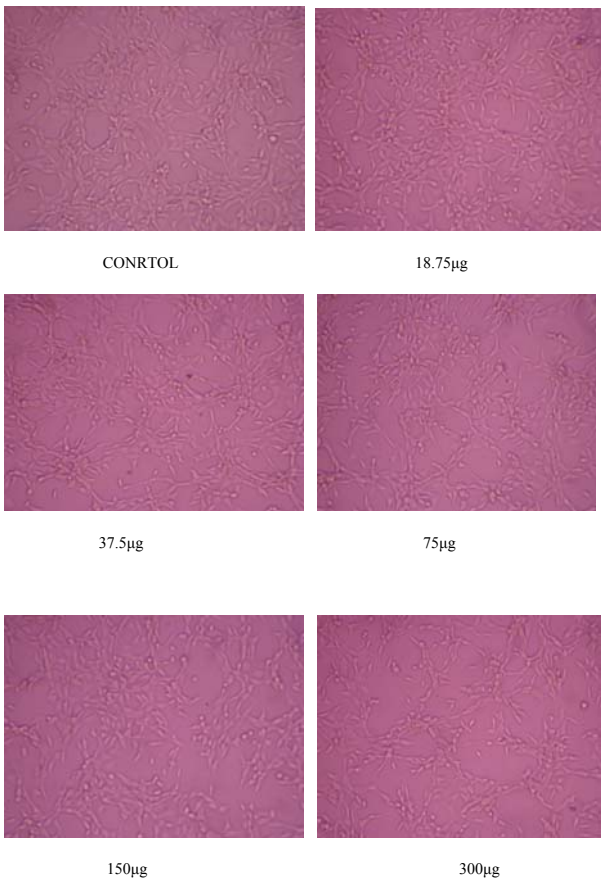


Figure 4.8 Effect of beta-glucan on cells morphology

4.10 Antioxidant activity

Polyphenols are considered to be the major contributors to the antioxidant property of fruits, vegetables and mushroom (Hatano *et al*., 1989). But in this study the beta-glucans contribution or effect of beta-glucan on the antioxidant activity was analysed. The isolated and purified beta-glucan [fraction1, fraction2, fraction3] was analysed for total antioxidant and scavenging activity.

4.5.1 Estimation of total antioxidant activity by phosphomolybdate method

Table 4.5 Total antioxidant activity Standard

Concentration of Ascorbic acid (µg/ml)	Replicate 1 Absorbance @ 695	Replicate 2 Absorbance @ 695	Replicate 3 Absorbance @ 695	Mean ± SD
20	0.118	0.120	0.124	0.121 ± 0.003
40	0.172	0.175	0.169	0.172 ± 0.003
60	0.236	0.239	0.241	0.239 ± 0.003
80	0.283	0.284	0.286	0.284 ± 0.002
100	0.424	0.424	0.425	0.424 ± 0.001
120	0.497	0.495	0.498	0.497 ± 0.002
140	0.569	0.565	0.567	0.567 ± 0.002
160	0.660	0.680	0.685	0.675 ± 0.013
180	0.730	0.731	0.729	0.730 ± 0.001
200	0.810	0.814	0.824	0.816 ± 0.007

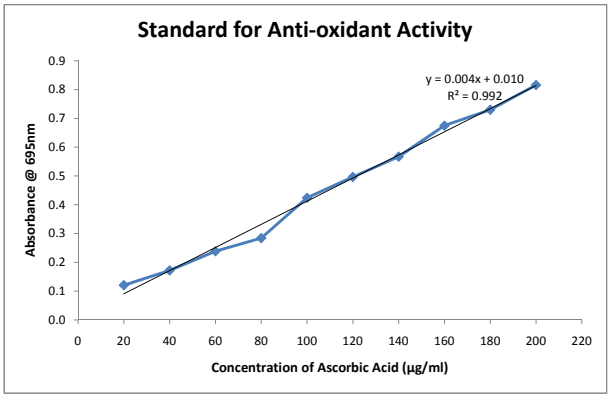


Figure no: 4.9 Standard for total antioxidant activity [phosphomolybdate assay]

Table no: 4.6 Ascorbic acid equivalence for fractions

Extract	**Ascorbic Acid Equivalent (µM/g)**			
	Replicate 1	Replicate 2	Replicate 3	Mean ± SD
Fraction 1	2.598	2.405	2.501	2.501 ± 0.097
Fraction 2	2.117	1.924	2.020	2.020 ± 0.097
Fraction 3	1.250	1.058	1.154	1.154 ± 0.096

4.11 DPPH assay

DPPH, a stable free radical with a characteristic absorption at 517 nm, was used to study the free radical scavenging effects of crude beta-glucan. As antioxidants donate protons to these radicals, the absorption decreases. The decrease in absorption is taken as a measure of the extent of radical scavenging. The DPPH procedure provides a reproducible and practical way for measuring antioxidant activity of different extracts. It has been used to evaluvate the potential of various natural plant and vegetable extracts as antioxidant (Yamaguchi *et al.,* 1998; Yen and Chen, 1995). Free radical scavenging capacities of the crude beta-glucan, measured by DPPH assay, shown in Figure no: 4.8, revealed that the crude extract has 22.05 % scavenging activity.

Table 4.7 Standard for DPPH assay

Concentration of Ascorbic acid (µg/ml)	**Absorbance @ 517nm**			Mean ± SD	% inhibition
	Replicate 1	Replicate 2	Replicate 3		
10	3.135	3.195	3.165	3.165 ± 0.036	7.91
20	2.913	3.013	2.956	2.961 ± 0.050	13.85
30	2.767	2.812	2.794	2.791 ± 0.022	18.79
40	2.436	2.532	2.484	2.484 ± 0.048	27.72
50	2.197	2.213	2.204	2.205 ± 0.008	35.85
60	1.969	1.905	1.954	1.943 ± 0.033	43.47
70	1.555	1.592	1.563	1.570 ± 0.019	54.32
80	1.276	1.265	1.273	1.271 ± 0.005	63.01
90	0.969	1.005	0.979	0.984 ± 0.018	71.36
100	0.713	0.751	0.741	0.735 ± 0.019	78.61
CONTROL	3.437	3.447	3.427	3.437 ± 0.010	

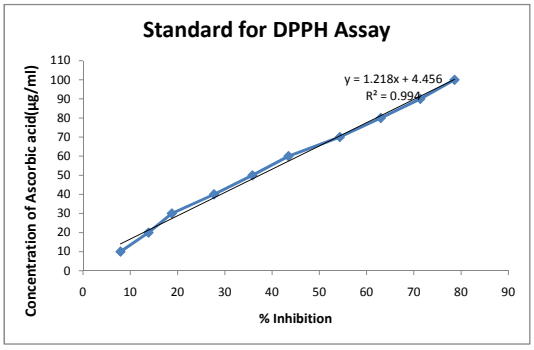


Figure 4.10 Standard graph for DPPH assay

Table 4.8 Scavenging activity of fractions from fruiting bodies *P.osetreatrus*

Sl no	**Sample**	**Absorbance @ 517nm**				**Scavenging Activity %**
		Replicate 1	**Replicate 2**	**Replicate 3**	**Mean ± SD**	
1	Fraction 1	2.280	2.274	2.286	2.280 ± 0.006	33.66
2	Fraction 2	2.401	2.386	2.482	2.423 ± 0.052	29.50
3	Fraction 3	2.620	2.742	2.675	2.679 ± 0.061	22.05

4.12 Beta-glucan loaded Chitosan film

Chitosan was made soluble by the immersion in acetic acid solution, which trigger the salt formation between some amino groups of CS and acetic acid. These salts dissociated will interact with beta-glucan, leading to the formation of a film with beta-glucan–Chitosan complex. Unlike Beta-glucan was soluble in acidic, basic and aqueous solutions and Chitosan which was soluble only in acidic solutions, the sheet does not dissolve in either acidic or basic solutions. The differences in the properties indicate the successful beta-glucan – Chitosan complex formation (Kyoko *et al.,* 2010). The final film is a transparent, colourless sheet obtained by drying at room temperature. Flexible films were obtained when 0.1 to 2 % beta-glucan is incorporated, beyond 2% high viscous solutions makes spreading difficult. At lower concentration (below 0.1 %) the films were brittle and tend to break faster at lower stress levels. Sheets with only beta-glucan dissolve easily and with Chitosan were found to be very brittle. The optimised film contained 2% Chitosan, 1%acetic acid, 1% lactic acid and 0.1 – 2% beta-glucan. Higher concentration of beta-glucan gave flexibility and can withstand higher stress levels.

Table 4.9 Standardization of film

Sl. No	Chitosan (%)	Acetic Acid (%)	Lactic Acid (%)	Temperature (°C)	Casting Time (Hrs)	Nature of Film
1	2	1	0	37	48	Thin, Fragile
2	2	1	0	37	48	
3	2	1	0	37	48	
4	2	1	1	37	48	**Good , Compact**
5	2	1	1	37	48	
6	2	1	1	37	48	
7	2	2	0	37	48	Too Viscous, Unable to spread
8	2	2	0	37	48	
9	2	2	0	37	48	
10	2	2	1	37	48	Viscous , Fragile
11	2	2	1	37	48	
12	2	2	1	37	48	
13	2	3	0	37	48	Very fragile, No elastic property
14	2	3	0	37	48	
15	2	3	0	37	48	
16	2	3	1	37	48	Good elastic Property , Not tearing
17	2	3	1	37	48	
18	2	3	1	37	48	

4.13 Mechanical properties of the film

To investigate the influence of beta-glucan on the mechanical properties of chitosan film, tensile strength and elongation at break were evaluated Maximum load bearing capacities of the film were in the range of 329 gf to 7564.51 gf and the extension ranged from 4.1 mm to 53.46 mm. this observation is in agreement with (Sezer *et al.,* 2004). The flexibility of the film was found to increased with addition of beta-glucan. The tensile strength of the film changed in inverse proportion to its elasticity. Among the chitosan films, 1% beta-glucan loaded film has maximum elongation capacity. The chitosan film was very hard and close to solid, beta-glucan loaded films displayed more flexibility and elasticity. The primary characteristic of any wound healing film is elasticity, which protects the wound surface form wear and tear (Burn, 2001). The addition of beta-glucan gave chitosan film flexibility, which is in agreement with the reports available.

Table 4.10 Thickness of the chitosan and beta-glucan incorporated chitosan film

Trial no	Thickness (mm)			
	Chitosan	Chitosan and Beta-Glucan (1%)	Chitosan and Beta-Glucan (1.5%)	Chitosan and Beta-Glucan (2%)
1	0.10	0.11	0.12	0.14
2	0.10	0.09	0.14	0.11
3	0.10	0.09	0.13	0.15
4	0.09	0.11	0.12	0.13
5	0.08	0.12	0.15	0.12
6	0.09	0.10	0.12	0.12
7	0.08	0.10	0.11	0.13
8	0.09	0.11	0.11	0.11
9	0.10	0.08	0.12	0.14
10	0.09	0.09	0.12	0.14
MEAN	0.092±0.008	0.100±0.012	0.124±0.013	0.129±0.014

Film type: Chitosan
Film size: 12 cm x 2.5cm
Parameters analysed: Extension, break point, time.

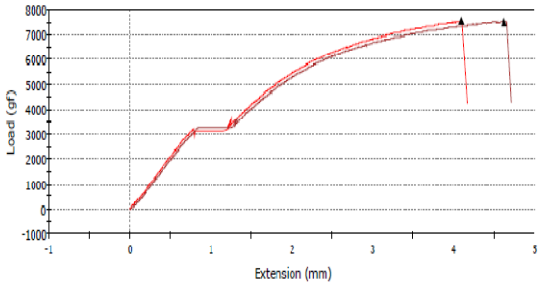


Figure 4.11 Load vs extension for chitosan film

Table 4.11 Determination of tensile strength

Sl.no	Maximum Load (gf)	Extension at Maximum Load (mm)	Time at Maximum Load (sec)
1	7564.51	4.10	443.10
2	7539.65	4.63	444.40
Mean ±SD	7552.080±17.579	4.365±0.375	443.750±0.919

Film type: Chitosan + 1.0% Beta-glucan
Film size: 12 cm x 2.5cm
Parameters analysed: Extension, break point, time.

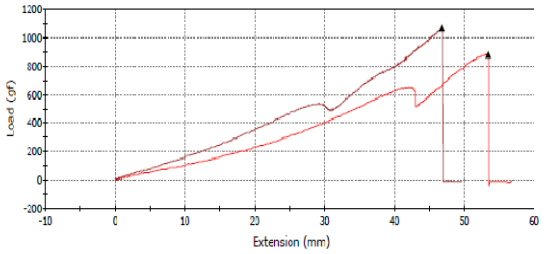


Figure 4.12 Load vs extension for chitosan with 1% beta-glucan

Table 4.12 Determination of tensile strength

Sl.no	Maximum Load (gf)	Extension at Maximum Load (mm)	Time at Maximum Load (sec)
1	881.77	53.46	48.80
2	1064.00	46.81	62.80
Mean ±SD	972.885±128.856	50.135±4.702	55.800±9.899

Film type: Chitosan + 1.5% Beta-glucan
Film size: 12 cm x 2.5cm
Parameters analysed: Extension, break point, time.

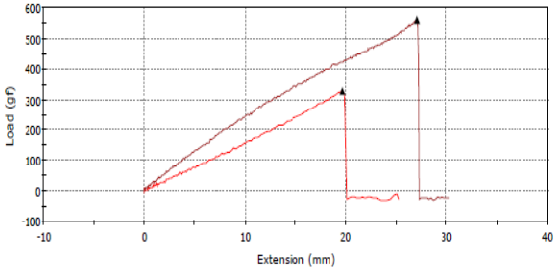


Figure 4.13 Load vs extension for chitosan with 1.5% beta-glucan

Table 4.13 Determination of tensile strength

Sl.no	Maximum Load (gf)	Extension at Maximum Load (mm)	Time at Maximum Load (sec)
1	329.35	19.65	17.70
2	559.77	27.08	33.10
Mean ±SD	444.560±162.932	23.365±5.254	25.400±10.889

Film type: Chitosan + 2.0% Beta-glucan
Film size: 12 cm x 2.5cm
Parameters analysed: Extension, break point, time.

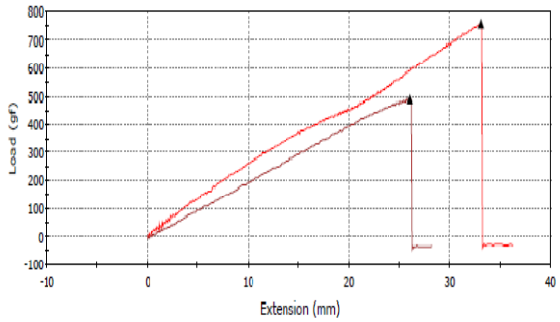


Figure 4.14 Load vs extension for chitosan with 2% beta-glucan

Table 4.14 Determination of tensile strength

Sl.no	Maximum Load (gf)	Extension at Maximum Load (mm)	Time at Maximum Load (sec)
1	757.10	33.07	43.60
2	490.64	26.08	27.90
Mean ±SD	623.870±188.416	29.575±4.943	35.750±11.102

4.14 Fluid uptake ability of chosen and beta –glucan loaded chitosan films (FUA)

In the swelling study when chitosan films were socked in liquid for one hour, small amounts of chitosan were dissolved. Thus, the dissolved amount of chitosan affects swelling study (Balakrishna *et al.*,2005)The beta-glucan incorporated film was stable even after 6 hrs. It can be observed that the maximum swelling ability increased with increase in beta-glucan concentration up to a certain limit. At the first stage of the curve, swelling rate was very high, and the water could be easily absorbed to the film (Choi *et al.,* 1999). It can be observed that the film attains swelling saturation after 6hrs. The fluid uptake ability of chitosan film was 84.45%, which increased to 93.87% with the addition of beta-glucan.

Table 4.15 Fluid uptake ability of Chitosan film

Sl.no	Time (Hr)	Weight of the gel			Mean ± SD	FUA
1	0	0.03	0.03	0.03	0.030±0.000	
2	1	0.14	0.14	0.11	0.130±0.017	
3	2	0.19	0.20	0.19	0.193±0.006	84.45%
4	3	-	-	-	-	
5	4	-	-	-	-	
6	5	-	-	-	-	

Table 4.16 Fluid uptake ability of Chitosan film with 1.0% beta-glucan

Sl.no	Time (Hr)	Weight of the gel			Mean ± SD	FUA
1	0	0.03	0.03	0.04	0.033±0.006	
2	1	0.25	0.26	0.24	0.250±0.010	
3	2	0.36	0.37	0.35	0.360±0.010	92.66%
4	3	0.40	0.41	0.41	0.407±0.006	
5	4	0.43	0.43	0.44	0.433±0.006	
6	5	0.45	0.44	0.46	0.450±0.010	

Table 4.17 Fluid uptake ability of Chitosan film with 1.5% beta-glucan

Sl.no	Time (Hr)	Weight of the gel			Mean ± SD	FUA
1	0	0.04	0.03	0.03	0.033±0.006	
2	1	0.27	0.26	0.26	0.263±0.006	
3	2	0.35	0.38	0.38	0.370±0.017	93.87%
4	3	0.39	0.43	0.42	0.413±0.021	
5	4	0.46	0.47	0.45	0.460±0.010	
6	5	0.49	0.50	0.48	0.490±0.010	

Table 4.18 Fluid uptake ability of Chitosan film with 2.0% beta-glucan

Sl.no	Time (Hr)	Weight of the gel			Mean ± SD	FUA
1	0	0.03	0.03	0.04	0.033±0.006	
2	1	0.26	0.25	0.24	0.250±0.010	
3	2	0.33	0.32	0.31	0.320±0.010	93.56%
4	3	0.42	0.41	0.42	0.417±0.006	
5	4	0.47	0.46	0.48	0.470±0.010	
6	5	0.51	0.51	0.52	0.513±0.006	

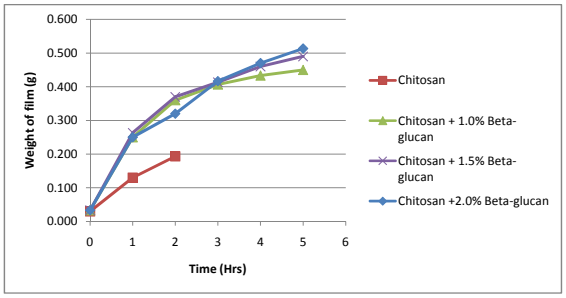


Figure 4.15 Water Absorption capacity

4.15 Antimicrobial activity

Microbial infection is one among the factors that hamper the wound healing process. Wound is commonly infected by 34 and 66% of Gram positive and Gram negative bacteria (Masaadeh *et al.,* 2009). Wounds are very suceptable to *P.aeruginosa* (Abraham *et at.*, 2009).

4.16 Disc diffusion technique

The present study revealed the activity of chitosan films against Gram positive and Gram negative bacteria. None of the preliminary results concerning inhibition zone assays on solid medium, against Staphylococcus.sp and *E.coli*, showed clear zone, whatever the chitosan content was. The poor inhibitory activity of the film-forming solution could be explained by the limitation of the diffusion of chitosan in agar medium. All the films showed zone of inhibition for *E.coli and Staphylococcus.* The results were shown in figure: 4.16 -4.18.

It is well-known that chitosan itself has antimicrobial activity due to its cationic property. This seemingly contradictory result for chitosan films is mainly due to the limits of detection of antimicrobial activity when using the disk method. The appearance and size of the clear zone in the disk method is mainly dependent on the ratio of disk area and size of inoculum, type of solid medium, and contact area. (Jong *et al.*, 2006).

4.17 Liquid culture medium assays

The addition of 10% (v/v) of chitosan solution in liquid medium containing *E.coli and Staphylococcus* in exponential growth phase, dramatically decreased the absorbance at 600 nm (Figure 1a). Treatment with 10% (v/v) acetic acid which was the diluant of chitosan film-forming solution had no effect on the turbidity, showing that the compound which strongly reduced optical density was partially deacetylated chitosan. This may be explained by antimicrobial activity of the polysaccharide or by a flocculation phenomenon obtained by polycationic compound.

According to Hughes (1990), chitosan is an effective flocculant compound, which could be related to polymer/particule interactions and under pH dependence. Chitosan can interact by hydrogen bonding with released polysaccharides extracellular polymers attached to the cell surface which are hence incorporated into the polymer network giving an excellent removal of turbidity.

Figure no: 4.16 Zone of inhibition for Positive control (Amp)

Staphylococcus aureus ***E.coli***

Figure no: 4.17 Zone of inhibition for Chitosan with 2% beta glucan

Staphylococcus aureus ***E.coli***

Figure no: 4.18Zone of inhibition for Chitosan film

Staphylococcus aureus ***E.coli***

Table 4.19 Antimicrobial activity of chitosan and beta-glucan incorporated chitosan against *E.coli*

	Absorbance at 660nm				
	Initial absorbance	Absorbance after 1h	Absorbance after 2h	Absorbance after 3h	Absorbance after 4h
Negative control	0.03	0.039	0.0211	0.457	0.621
Amp (positive control	0.016	0.024	0.043	0.063	0.08
Chitosan film	0.101	0.163	0.141	0.114	0.09
Chitosan + Beta glucan	0.026	0.085	0.062	0.05	0.04

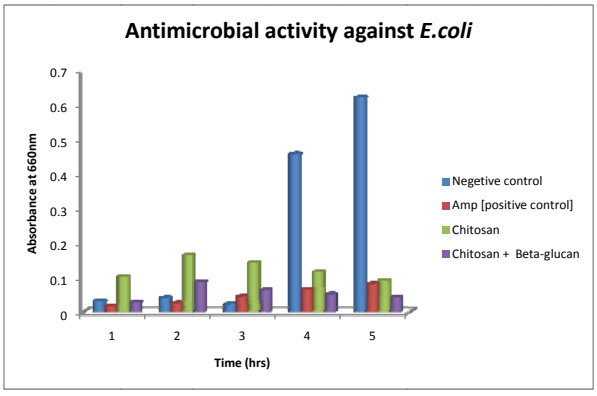


Figure 4.19 Antimicrobial activity of chitosan and beta-glucan incorporated chitosan against *E.coli*

Table 4.20 Antimicrobial activity of chitosan and beta-glucan incorporated chitosan against *Staphylococcus.aureus*

	Absorbance at 660nm				
	Initial absorbance	**Absorbance after 1h**	**Absorbance after 2h**	**Absorbance after 3h**	**Absorbance after 4h**
Negative control	0.016	0.065	0.207	0.36	0.465
Amp (positive control	0.032	0.046	0.031	0.013	0.03
Chitosan film	0.168	0.12	0.013	0.163	0.157
Chitosan + Beta glucan	0.016	0.096	0.03	0.09	0.259

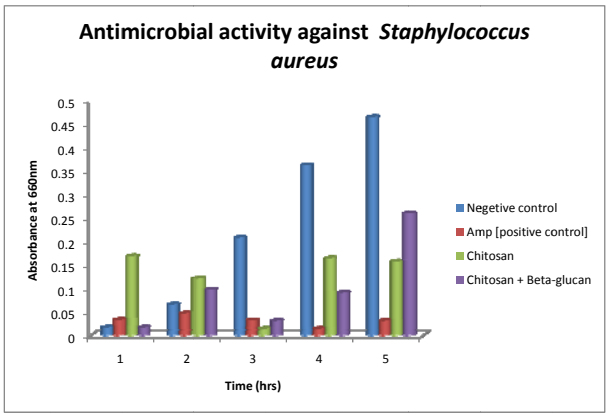


Table 4.20 Antimicrobial activity of chitosan and beta-glucan incorporated chitosan against *Staphylococcus aureus*

CONCLUSIONS

CHAPTER 4

CONCLUSION

- The present project work describes the purification of water soluble beta-glucan from fruiting bodies of *Pleurotus ostreatus* and its incorporation in chitosan film to be used as bioactive wound dressing material.
- *Pleurotus ostreatus* fruiting body was processed to get uniform size powder and used for extraction of beta-glucan by sequential purification steps, namely alkali extraction, DEAE chromatography and ConA affinity chromatography.
- The final fraction after sequential purification showed the presence of total carbohydrate which of devoid of reducing sugar and protein.
- The recovery of the water soluble beta-glucan was about 19.6 %(wt/wt) of the starting materials expressed as total carbohydrate. The further purified fraction were analysed to and fraction 1(42% carbohydrates and 8% proteins), fraction 2 (15% carbohydrates and 0.24% proteins) and fraction 3 (15% carbohydrates).
- The IR bands in the region of 950 – 1250 cm^{-1} is mainly due to C-C and C-O stretching vibrations in pyranoid rings, indicating the presence of polysaccharides as the major component.
- The cell viability was examined and there was no significance difference in cell viability in lower concentration (18.75 – 75µg/ml), suggesting the crude beta-glucan non Cytotoxicity. The lower concentration range (below 20µg/ml) is expected to proliferate the cells, and thereby will confirm the non-toxicity nature of the crude beta-glucan.
- Free radical scavenging capacities of the crude beta-glucan, measured by DPPH assay, revealed that the crude extract has 22.05 % scavenging activity.
- The optimised film contained 2% Chitosan, 1%acetic acid, 1% lactic acid and 0.1 – 2% beta-glucan. Higher concentration of beta-glucan gave flexibility and can withstand higher stress levels.
- The fluid uptake ability of chitosan film was 84.45%, which increased to 93.87% with the addition of beta-glucan.
- The poor inhibitory activity of the film-forming solution could be explained by the limitation of the diffusion of chitosan in agar medium. All the films showed zone of inhibition for *E.coli and Staphylococcus.*

APPENDIX

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SIGNATURE

REFERENCES

CHAPTER 7

REFERENCES

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