Chapter 1
Introduction
and
Review of literature
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1.1.3 Scope of the work
1.1 Introduction

Year ago, before there were plastics and synthetic polymers, in fact, all the way back to the beginning of the earth, nature was using natural polymers to make life possible. Technically, polymers are long chain giant organic molecules assembled from several smaller molecules termed as monomers. Polymer consists of many repeating monomer units in long chains. There are two types of polymers: synthetic and natural. Synthetic polymers are derived mainly from petroleum oil, and can be synthesized in the laboratory. Examples of synthetic polymers include nylon, polyethylene, polyester, teflon, and epoxy. Natural polymers occur in nature mainly as structural components of plants and animals and can be easily extracted from natural substrates since they are often water-based. This group consists of naturally occurring polymers and chemical modifications of these polymers. Cellulose, starch, lignin, chitin, and various polysaccharides are included in this group. These materials and their derivatives offer a wide range of properties and applications. Natural polymers tend to be readily biodegradable, although the rate of degradation is generally inversely proportional to the extent of chemical modification. (fig.1.1)

![Classification and types of polymers](http://www.wellesley.edu/Chemistry/Chem105manual/Lab06/lab06.html)
1.1.1 Chitin
Chitin, a tough and pliable homopolymer of β 1-4 linked 2-acetamido-2-deoxy-D-glucose (N-acetyl-D-glucosamine or GlcNAc), is the most abundant polysaccharide after cellulose, existing in nature (Khousehab & Yamabhai, 2010). It constitutes a major structural component of many biological systems particularly, insects, crustaceans, molluscs, fungi, algae and marine invertebrates (Shaikh & Deshpande, 1993).

1.1.1.1 Historical account
History dates back to 1811 when Henry Braconnot (1780–1855) for the first time isolated from mushrooms, which turned out to be the first polysaccharide identified by man, preceding cellulose by about 30 years (Braconnot, 1811; Labrude & Becq, 2003). Henri Braconnot’s name for chitin was ‘fungine’. In 1823, Odier found the same material in insects and plants, and named it ‘chitine’ (Muzzarelli, 2010). In 1843, Lassaigne demonstrated the presence of nitrogen in chitin (Jeuniaux, 1996). In 1859, Prof. C. Rouget subjected chitin to alkali treatment, which resulted into a substance that could, unlike chitin itself, be dissolved in acids. The term ‘chitosan’ was given to deacetylated chitin by Hoppe-Seiler in 1894 (McKay, 1995).

1.1.1.2 Occurrence
Chitin occurs widely in nature as a structural polymer in the integument of insects and crustaceans and the cell wall of true fungi (Jeuniaux, 1971). The prominence in the mantles of the insects led to its name which comes from the Greek ‘chiton’ means a ‘coat of mail’. The cell wall of fungi is reported to contain 22-44% but in green algae it is only 3-5% (Teng & Whistler, 1973). In arthropods, coelenterates, and nematodes, it accounts for 25-50% of the cuticles (Jeuniaux, 1971).

Chitin usually forms a part of very complex systems. Insect exoskeletons are largely composed of chitin-protein complexes such as the cuticle and the peritropic membrane, whereas crustacean shells reportedly contain large proportions of CaCO₃ in addition to protein (Kramer & Muthukrishnan, 1997) (Table.1.1). Analyses of many isolated chitin samples show that they contain some bound histidine and aspartic acid (Teng & Whistler, 1973; Jeuniaux, 1971)
Similarly in the fungi, chitin is found in close association with polysaccharides (Teng & Whistler, 1973; Shirai et al., 1996). It has been suggested that the presence of chitin or cellulose can be used to establish phylogenetic relationships between groups of fungi, especially the Phycomycetes (Foster & Weber, 1960) thus facilitating the evolutionary studies.

Table 1.1. Distribution and chemical features of the diverse chitinous structures in living organisms (Adapted from Deshpande, 1986)

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Structures</th>
<th>Inorganic constituents</th>
<th>Chitin/ % organic fraction (dry wt)</th>
<th>Main other organic constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fungi</td>
<td>Cell walls</td>
<td>-</td>
<td>Traces to 45</td>
<td>Glucans and mannans</td>
</tr>
<tr>
<td>Algae: chlorophyceae</td>
<td>Cell wall</td>
<td>-</td>
<td>+</td>
<td>Cellulose</td>
</tr>
<tr>
<td>Protozoa</td>
<td>Cyst wall and shell</td>
<td>Silica and iron</td>
<td>+</td>
<td>Proteins and lipids</td>
</tr>
<tr>
<td>Chidaria</td>
<td>Podocyst skeleton</td>
<td>CaCO₃</td>
<td>Traces to 30.3</td>
<td>Unidentified proteins</td>
</tr>
<tr>
<td>Aschelinthes</td>
<td>Egg envelope, capsule and cuticle</td>
<td>-</td>
<td>Upto 16.6</td>
<td>Unidentified proteins</td>
</tr>
<tr>
<td>Endoprocta</td>
<td>Cuticle</td>
<td>-</td>
<td>-</td>
<td>Tanned proteins</td>
</tr>
<tr>
<td>Ectoprocta</td>
<td>Ectocyst</td>
<td>Sometimes CaCO₃</td>
<td>13.5</td>
<td>Collagen</td>
</tr>
<tr>
<td>Phoronida</td>
<td>Tubes</td>
<td>-</td>
<td>3.8</td>
<td>-</td>
</tr>
<tr>
<td>Brachiopoda</td>
<td>Stalk cuticle, shell</td>
<td>CaCO₃</td>
<td>Upto 29.0</td>
<td>Collagen</td>
</tr>
<tr>
<td>Echiurdia</td>
<td>Hooked chaetae</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Annelida</td>
<td>Chaetae, jaws</td>
<td>Unidentified</td>
<td>0.28 to 30</td>
<td>Quinone-tanned proteins</td>
</tr>
</tbody>
</table>
### 1.1.1.3 Structure and function of chitin

Chemically, chitin may be termed as a derivative of cellulose, in which hydroxyl groups have been replaced by acetamido residues. In case of chitosan, the acetylated amino group of chitin is deacetylated.

Chitin contains 6–7% nitrogen while its deacetylated form, chitosan contains 7–9.5% nitrogen. In chitosan, between 60 to 80% of the acetyl groups available in chitin are removed (Mathur & Narang, 1990). There are three forms of chitin: α, β, and γ (Fig.1.3). The α-form, which is mainly obtained from crab, shrimp shells and fungal cell walls, is most widely distributed. Both α and β chitin/chitosan are

<table>
<thead>
<tr>
<th>Class</th>
<th>Structure</th>
<th>Calcium Carbonate</th>
<th>Protein(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mollusca</td>
<td>Jaws, radula, shell</td>
<td>CaCO$_3$, iron and silica</td>
<td>+ to 36.8 Conchiolin, tanned proteins</td>
</tr>
<tr>
<td>Onchophora</td>
<td>Cuticle</td>
<td>-</td>
<td>+ Proteins</td>
</tr>
<tr>
<td>Arthropoda</td>
<td>Cuticle</td>
<td>CaCO$_3$</td>
<td>20 to 85 Arthropodins, sclerotins, rosilins, mucins</td>
</tr>
<tr>
<td>Chaetogenatha</td>
<td>Grasping spines</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pogonophora</td>
<td>Tubes</td>
<td>-</td>
<td>33 Unidentified proteins</td>
</tr>
<tr>
<td>Tunicata</td>
<td>Peritrophic membrane</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

![Fig.1.2. Structure of chitin](http://www.statusseafood.com/wholesale/chitinchitosan/index.html)

Chitin contains 6–7% nitrogen while its deacetylated form, chitosan contains 7–9.5% nitrogen. In chitosan, between 60 to 80% of the acetyl groups available in chitin are removed (Mathur & Narang, 1990). There are three forms of chitin: α, β, and γ (Fig.1.3). The α-form, which is mainly obtained from crab, shrimp shells and fungal cell walls, is most widely distributed. Both α and β chitin/chitosan are

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commercially available. The α-chitin chains are aligned in anti-parallel fashion, which gives rise to strong hydrogen bonding and consequently makes it more stable (Sikorski et al., 2009). The β-form mainly obtained from molluscs such as squid, is arranged in parallel, whereas the γ-form may be predominantly found in squid and cuttlefish stomach lining and contains two parallel and one anti-parallel strands of chitin (Atkins, 1985). Conversion from the β-form to the α-form is possible, but not the reverse (Lavall et al., 2007; Mazeau et al., 2002; Schiffman & Schauer, 2009). The γ-chitin can be converted to α-chitin by treatment with lithium thiocyanate (Rudall & Kenchington, 1973). The distribution of the three different crystallographic forms of chitin may occur in different organs of the same animal, as in Loligo and Lingula, and may be associated with different functions. According to Rudall (1973), β-chitin (and probably γ-chitin) appears to be associated with collagen-type cuticles or with collagen-secreting neighbouring tissues, while α-chitin structure completely replaces collagen type cuticle.

Fig. 1.3. Polymorphic configurations of chitin: (a) α-chitin, (b) β-chitin, and (c) γ-chitin

1.1.1.4 Physical and chemical properties of chitin
Due to the insoluble nature of chitin, its isolation frequently requires use of drastic chemical treatments to remove the contaminants. Inorganic contaminants are generally removed by digestion with dilute mineral acids, whereas treatment with hot, dilute alkali helps to remove proteinaceous and other organic impurities. The most preferable and common source of chitin for experimental purposes is crustaceans shell and typical isolation procedure for the same has been described by Hackman (1954) using 2N HCl, which was further modified by Horwitz et al., (1957).
The chitin obtained by these methods is an amorphous solid, insoluble in water, dilute acids, dilute or concentrated alkalis, alcohol and other organic solvents (Jayme, 1971), but soluble in conc. HCl, H₂SO₄, 78-97% H₃PO₄ and anhydrous formic acid. There are substantial variations in the solubility, molecular weight, acetyl values, and specific rotation among chitin obtained from different origins or prepared by different methods (Jayme, 1971) (Table.1.1)

The acid hydrolysis (conc. HCl) of chitin offers D-glucosamine and acetic acid in 1:1 proportions. Acid hydrolysis under mild condition lead to 2 acetamido-2-deoxy-D-glucose, while partial hydrolysis, gives chitobiose, chitotriose, chitotetraose and higher oligosaccharides.

Chitin is insoluble in water due to its intermolecular hydrogen bonds (Minke & Blackwell, 1978). But water-soluble chitin-based derivatives such as chitosan or carboxymethyl chitin can be obtained. One of their most important features is their flexibility to be shaped into different forms such as fibers, hydrogels, beads, sponges, and membranes (Mano et al., 2007). The source of chitin affects its physical and chemical properties such as crystallinity, purity, polymer chain arrangement, and dictates its properties (Rinaudo, 2006; Khoushab & Yamabhai, 2010).

1.1.1.5 Chitosan
Chitosan, a non-crystalline fully deacetylated derivative of chitin, occurs in the walls of a limited but medically important group of fungi, the Zygomycetes. (Bartnicki, 1968; Kreger, 1954). These fungi are opportunistic invaders to human and can be major pathogens in burn wounds (Bruck et al., 1971). When chitin is treated with conc. alkali at high temperatures, it undergoes various degrees of deacetylation and degradation to give rise to chitosan (Fig.1.4). However, chitosan is a family name for a group of partially deacetylated products rather than a single substance. In Japan, huge quantities of chitin (million kg) from crab shells is processed annually to obtain chitosan for use as a flocculating agent in the clarification of sewage water (Imeri & Knorr, 1998). Chitosan is also being produced on commercial scale by fermentation of a Mucoralean fungus Absidia coerulea, which possess chitosan as its cell wall component.
Fig. 1.4. Steps involved in the preparation of chitin and chitosan from chitin bio-waste

(Visanarachchi et al., 2011)
1.1.1.6 Industrial and biomedical applications of chitin, chitosan and chitooligosaccharides

Chitin and chitosan have various characteristics that are not found in other natural polymers and biopolymers. However, for a long time, chitin and chitosan were unused bioresources. In the investigation of the importance of natural polymers, chitin and chitosan, as well as cellulose, are being considered functional polysaccharides, and are actively being studied and applied in various fields, such as medical treatment, medicine, food, chemical industries, fibers, and others (Uragami et al., 2001; Uragami & Tokura, 2006) (Fig.1.5). Chitin and chitosan have high organic solvent resistance, which is advantageous for preparation of separation membranes typically used with organic solvents e.g., gas permeation membranes, dialysis membrane, reverse osmosis.
membranes, ultrafiltration membranes, water/organic selective membranes, and career transport membranes etc. (Nakatsuka, & Andrady, 1992; Qurashi et al., 1992). Chitin and its derivatives have also been used in food and beverages industry, as wine clarification and stabilizing agent, de-acidifying and de-hazing agent in fruit juices, antimicrobial agent in food preservation and in agriculture like horticulture development and as plant growth promoters in orchid cultivation. Chitin and chitosan have also been explored for their unique biomedical applications as in chitosan-DNA nanoparticles delivery system for gene therapy, in tissue engineering and wound healing, antioxidant activity, use of D-glucosamine in dentistry and bone regeneration, in veterinary medicine, chitosan based materials as carriers for anticancer drugs, antidiabetic effect, Cholesterol lowering effect, in cosmetics etc. (Tian & Sun, 2008, Panos et al., 2008; Vyas et al., 2011) (Fig.1.6). These polysaccharides are also being used as complexing agents for heavy metals and in waste water treatment.

Energy Production: an emerging application

Insects are most dynamic widely distributed on earth, comprising 80% of all known species (Zhang et al., 2007). Robots with the ability to hunt and digest insects and obtain energy from them can serve humanity by performing missions in dangerous situations. A robot that contains a microbial fuel cell was created to digest chitin and metabolize it by bacteria. This process produces electrons that act as horsepower of the system (Ieropoulos et al., 2004). The system can take advantage of the wide distribution of arthropods and mollusks because chitin is available in both phyla. Since Arthropoda and Mollusca rank first and second in species diversity in all animal phyla (Giribet et al., 2006) and are a major source of chitin, this strategy can be highly applicable in both land and marine environments. Chitin has also been utilized by Clostridium paraputrificum M-21 to produce hydrogen gas. This gas is considered to be a potential source of alternative energy (Evvyerni et al., 2000; Morimoto et al., 2005). The advantage of using chitin in this way is that most chitin sources are waste materials, such as shrimp shells; therefore, it is a betterand there is no need to be concerned about pressure on food supplies.
1.1.2 Chitinase

The enzymatic hydrolysis of chitin to free N-acetyl D-glucosamine units is performed by a chitinolytic system, which is found in a variety of organisms such as actinomycetes, bacteria, fungi, yeasts, plants, protozoans, coelenterates, nematodes, mollusks, arthropods and also in human beings. In recent years, chitinases have received greater attention due to their wider range of biotechnological applications.
especially in the biocontrol of fungal phytopathogens (Mathivanan et al., 1998) and harmful insect pests (Mendosa et al., 1996; Pinto et al., 1997). Chitinases have also been used in the preparation of sphaeroplasts and protoplasts from yeast and fungal species (Pederby, 1985; Mizuno et al., 1997). Some other significant applications of chitinases include bio conversions of chitin waste to single cell protein and ethanol (Vyas & Deshpande, 1991) and fertilizers (Sakai et al., 1986).

Chitinase is the basic protein responsible to catalyze the hydrolysis of chitin. Relationship between mycolytic enzymes, chitinases and β-1, 3 glucanases produced by mycoparasitic fungi and their significance in fungal cell wall lysis and degradation has been well established (Elad et al., 1980, 1999). The complete enzymatic hydrolysis of chitin to free N-acetyl-D-glucosamine is performed by a chitinolytic system, the action of which is known to be synergistic and consecutive (Deshpande, 1986; Shaikh & Deshpande, 1993). Thus assay of chitinase could be used as a basis for screening potential bio-control agents (Elad et al., 1982).

1.1.2.1 Sources of chitinases

1.1.2.1.1 Plant chitinases

Chitinases are constitutively present in plants, seeds, stems, tubers and flowers, which are tissue-specific as well as developmentally regulated. Plant chitinases are induced by the attack of phytopathogens as pathogenesis-related proteins in plant self-defence or by contact with elicitors such as chitooligosaccharides or growth regulators such as ethylene (Koga et al., 1996; Gooday, 1996). The agricultural biotechnology offers new tools for the protection of plants from pathogens by using transgenic resistant plants. For instance, isolation of genes of chitinases from Trichoderma spp. were reported and transferred to plants in order to increase the resistance against phytopathogens (Lorito et al., 1996; Yedidia et al., 1999; Carsolio et al., 1999).

1.1.2.1.2 Insect chitinases

The chitinases present in the insects have been described from Bombyx mori, Manduca sexta. These enzymes play important roles as degradative enzymes during ecdysis, where endochitinases break randomly the cuticle to chitooligosaccharides that afterwards are hydrolysed by exoenzymes to N-acetyl D-glucosamine. The monomer is reused to synthesize a new cuticle. Insect chitinases also have a defensive
role against their own parasites. The production of enzymes in insects is regulated by hormones during the transformation of the larvae. (Arakane & Muthukrishnan, 2010).

1.1.2.1.3 Microbial chitinase
The microorganisms able to degrade chitin are widely distributed in nature (Deshpande, 1986). Due to its insolubility, size, molecular complexity and heterogeneous composition, the chitin is not degraded inside the cell, but the microorganisms secrete enzymes with different specificity, to transform or hydrolyse chitin (Cottrell et al., 1999). The microorganisms produce chitinases in higher amounts than animals and plants, generally as inducible extracellular that are of the two types, endochitinases and exochitinases. Among the bacteria producers are found the Serratia, Chromobacterium, Klebsiella, Bacillus, Streptomyces and fungi like Trichoderma, Penicillium, Lecanicillium, Neurospora, Mucor, Metarhizium, Beauveria, Lycoperdon and Aspergillus (van Aalten et al., 2000; Rojas-Avelizapa et al., 1999; Cruz et al., 1992; Leger et al., 1998; Matsumoto et al., 2006) (Table.1.2).

The microbial chitinases have grabbed wide attention in the bioconversion process to recycle crab and shellfish chitin waste (Wang & Chio, 1998; He et al., 2006). As in the bioconversion of cellulose to SCP the production of chitinase is thought to be one of the primary economic variables, estimated to account for 12% of the total production cost (Cosio et al., 1982). Though chitinases are present in a variety of living organisms such as snails, bean seeds, insects and microbes, last one being the most preferable source for industrial scale production (Cottrell, 1999).

Microbial chitinases have been produced by liquid batch fermentation, continuous fermentation, and fed-batch fermentation. In addition to these, solid-state fermentation and biphasic cell systems have also been used for the production of chitinase. Generally, chitinases produced from microorganisms are inducible in nature (Dahiya et al., 2006).

1.1.2.2 Classification of chitinase
Chitinase are classified in two different ways: 1) on their two innate properties, first, their mode of action on chitin and second, their amino acid sequence. Chitinolytic system, responsible for the enzymatic hydrolysis of chitin can be classified into two major categories. Endochitinases (EC 3.2.1.14) which cleave chitin randomly at internal sites, generating low molecular mass multimers of GlcNAc, such as
chitotetraose, chitotriose, and diacetylchitobiose. Exochitinases can be divided into two subcategories: chitobiosidases (EC 3.2.1.29), which catalyze the progressive release of diacetylchitobiose starting at the nonreducing end of chitin microfibril, and \(\beta(1, 4)\) N-acetyl glucosaminidases (EC 3.2.1.30), which cleave the oligomeric products of endochitinases and chitobiosidases, generating monomers of GlcNAc (Sahai & Manocha 1993). An alternative pathway involves the deacetylation of chitin to chitosan, which is finally converted to glucosamine residues by the action of chitosanase (EC 3.2.1.132) (Sahai & Manocha, 1993).

Secondly, based on amino acid sequence similarity, chitinolytic enzymes are grouped into families 18, 19, and 20 of glycosyl hydrolases (Henrissat & Bairoch, 1993). Family 18 is diverse in evolutionary terms and contains chitinases from bacteria, fungi, viruses, animals, and some plant chitinases. Family 19 consists of plant chitinases (classes I, II, and IV) and some Streptomyces chitinases (Hart et al., 1995). The chitinases of the two families, that is, 18 and 19, do not share amino acid sequence similarity. They have completely different 3-D structures and molecular mechanisms and are therefore likely to have evolved from different ancestors (Suzuki et al., 1999). Family 20 includes \(\beta\)-N-acetylhexosaminidases from bacteria, streptomycetes, and humans (Dahiya et al., 2006).

In fungi, chitinases are thought to have autolytic, nutritional, and morphogenetic roles (Adams, 2004). Chitinases in mycoparasitic fungi are most commonly suggested to be involved in mycoparasitism (Haran et al., 1996). Chitinases in bacteria are shown to play a role in the digestion of chitin for utilization as a carbon and energy source and recycling chitin in nature (Park et al., 1997; Tsujibo et al., 1993; Svitil et al., 1997). In insects, chitinases are associated with post embryonic development and degradation of old cuticle (Merzendorfer & Zimoch, 2003). Plant chitinases are mainly involved in defence and development (Graham & Sticklen, 1994). Chitinases encoded by viruses have roles in pathogenesis (Patil et al., 2000). Human chitinases are suggested to play a role in defence against chitinous human pathogens (Boot et al., 2001; Van Eijk et al., 2005; Boot et al., 2005). On the other hand, chitinases have shown immense potential applications in agricultural, biological and environmental fields. Due to important biophysiological functions and applications of chitinase, a considerable amount of research on fungal chitinases has been carried out in recent years (Chuan, 2006) (Table 1.2).
### Table 1.2. Role of chitinases in different phyla

*(Reproduced from Gohel et al., 2006)*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Role of chitinases</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Mineralization of chitin, also in nutrition and parasitism.</td>
<td>Flach <em>et al.</em>, 1992; Connell <em>et al.</em>, 1998</td>
</tr>
<tr>
<td>Fungi</td>
<td>Physiological role in cell division, differentiation and nutritional role related to mycoparasitic activity (e.g. in <em>Trichoderma</em> sp.)</td>
<td>Kuranda &amp; Robins, 1991; Gooday <em>et al.</em>, 1992; Mellor <em>et al.</em>, 1994; Alalam <em>et al.</em>, 1995</td>
</tr>
<tr>
<td>Plants</td>
<td>Defence against fungal and bacterial pathogens by degradation of their cell walls. Specific isoforms may play a role in embryo development, pollination and sexual reproduction.</td>
<td>Schlumbaum <em>et al.</em>, 1986; De Jong <em>et al.</em>, 1992; Leung, 1992; Kim &amp; Chung, 2002</td>
</tr>
<tr>
<td>Insects</td>
<td>Developmental process of cuticle degradation at different larval stages.</td>
<td>Kramer &amp; Fukamiso, 1985; Merzendorfer &amp; Zimoch, 2003</td>
</tr>
<tr>
<td>Protozoa</td>
<td>Malarial parasites produce sufficient quantities of chitinase to penetrate the chitin containing peritropic matrix of the mosquito midgut.</td>
<td>Huber <em>et al.</em>, 1991; Langer <em>et al.</em>, 2002</td>
</tr>
<tr>
<td>Human</td>
<td>Chitotriosidase activity helps in defence against nematodal infections. Moreover, its enzymatic activity is markedly elevated in serum of patients suffering from lysosomal lipid storage disorders, sarcoidosis and thalassemia.</td>
<td>Escott <em>et al.</em>, 1996; Choi <em>et al.</em>, 2001; Aguilera <em>et al.</em>, 2003; Gianfrancesco &amp; Musumeci, 2004</td>
</tr>
<tr>
<td>Animal</td>
<td>The high chitinase level in goat and bovine blood (serum) might be a function of slow renal secretion which keeps the enzyme level comparatively low in case of abnormal lysozyme production (monocytic-myelomonocytic leukemias)</td>
<td>Lundblad <em>et al.</em>, 1974</td>
</tr>
<tr>
<td>Yeast</td>
<td>A-subunit of toxin secreted by <em>Kluyveromyces lactis</em> has chitinase activity which is most likely required for the γ subunit to gain entry to the sensitive cell. Chitinases has an essential role in cell separation during budding of the chitinous yeast <em>Saccharomyces cerevisiae</em>. <em>Saccharomyces cerevisiae</em> chitinase also used as antifungal.</td>
<td>Butler <em>et al</em>., 1991; Kuranda &amp; Robins, 1991; Smit <em>et al</em>., 2001; Carstens <em>et al</em>., 2003; David, 2004</td>
</tr>
</tbody>
</table>

1.1.2.4 Binding mode of chitinase

The chitooligosaccharides and their reduced or methylated derivatives have been used to elucidate the mode of binding of chitinase and their action pattern, for instance, from the analysis of the hydrolysis products of the oligosaccharides, it has been suggested that the chitinase from *Pycnoporus cinnabarinus* had an exo-type action, predominantly hydrolysing the second β-N-acetylglucosaminide linkage from the non-reducing end (Ohtakara & Mitsutomi, 1988). Most of the carbohydrases show the similar protein-saccharide interactions, although they show widely diverse three-dimensional structures and binding site topologies.

1.1.2.5 Chitinase inhibitors

In addition to general enzyme inhibitors, such as organic compounds and oxidizing/reducing agents, a number of reports are available on the natural chitinase inhibitors. Allosamidin, an antibiotic produced by *Streptomyces* sp., is a known specific inhibitor of chitinases from insects, yeast, fungi, and human serum. It is similar to GlcNAc but lacks a pyranose ring oxygen and contains an oxazoline ring in which the methyl group is substituted by dimethylamine. Allosamidin is a competitive inhibitor. Allosamidin exerts its inhibitory effect by acting as a nonhydrolyzable analog of the oxazolinium ion intermediate (Tews *et al*., 1997). It was reported that allosamidin inhibited the *Bacillus* sp. BG-11 chitinase by 50 and 70% at a concentration of 30 and 50 µgml⁻¹ of enzyme solution, respectively, with an IC50 value of 40 µM (Bhushan & Hoondal, 1998).
1.1.2.6 Molecular genetics for enhanced production of chitinase

A number of naturally occurring organisms serve as a major source of chitinolytic enzymes but genetic improvement plays a significant role in their biotechnological applications. The conventional procedures for the strain improvement are chemical, physical mutation and protoplast fusion. In addition to mutation and protoplast fusion, molecular cloning is being effectively used to achieve overproduction of chitinases lately, to change in their localization, viz., periplasmic or extracellular and to understand the organism itself. Molecular cloning of chitinase genes has been reviewed earlier by different workers world over (Shaikh & Deshpande, 1993; Flach et al., 1992; Sahai & Manocha, 1993). Recently, for the constitutive, extracellular activity, the co-transformation of Trichoderma reesei protoplasts with Aphanocladium album chitinase was reported (Deane et al., 1999). The 6.5-fold higher activity expressed in transformant was found to be useful in bioremediation and biocontrol activities (Deane et al., 1999). Number of reports on molecular cloning for chitinases, either to increase biocontrol efficiency of B. thuringiensis, to prepare highly active chitinase preparation or to develop transgenic plants for the increased resistance have been described (Wiwat et al., 1996; Tantimavanich et al., 1997). The B. thuringiensis strains are known to produce d-toxin that kills number of insect pests however a combination of d-toxin and chitinase has been reported to be more effective. Some transgenic plants bearing chitinase gene with develop resistance against fungal invasion are summarized in Table 1.3.

Studies related to molecular aspects of chitinolytic enzymes are becoming indispensible for designing a more efficient chitinase producer and production of transgenic plants that can be used for the control of fungal and insect pathogens. Furthermore, biochemical and molecular studies could lead to a better understanding of the chitinase secretory process and the development of cloning strategies suitable for secretion of desired products.

Table 1.3: Transgenic plants transformed using chitinase gene

(Reproduced from Gohel et al., 2006)

<table>
<thead>
<tr>
<th>Transgenic plant</th>
<th>Gene</th>
<th>Source</th>
<th>Pathogen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broccoli</td>
<td>Endochitinase</td>
<td>Trichoderma</td>
<td>Alternaria sp.</td>
<td>Mora &amp;</td>
</tr>
</tbody>
</table>
1.1.2.7 Applications of chitinases

In recent times, chitinases have gained interest in different biotechnological applications due to their ability to degrade chitin in the fungal cell wall and insect exoskeleton, leading to their use as antimicrobial or insecticidal agents. (Karasuda et
Another interesting application of chitinase is for bioconversion of chitin, a cheap biomaterial, into pharmacological active products, namely N-acetylglucosamine and chito-oligosaccharides (Bhattacharya et al., 2007). Production of chitin derivatives with suitable enzymes is more appropriate for sustaining the environment than using chemical reactions (Songsiriritthigul et al., 2009). Other interesting applications include the preparation of protoplasts from filamentous fungi, bio-control of insects and mosquitoes as well as the production of single cell protein (Dahiya et al., 2006; Hayes et al., 2006). Thus, there have been many reports on cloning, expression and characterization of chitinases from various organisms, including bacteria, fungi, plant and animals (Deshpande, 1986). The major applications of chitinases are discussed below:

1.1.2.7.1 Cytochemical localization of chitin/chitosan using chitinase/chitosanase gold complexes
Chitin and chitosan are the most ubiquitous polymers of fungal cell walls. Wheat germ agglutinin–gold complex and chitinase gold complex have been used as probes for the detection of GlcNAc residues in the secondary cell walls of plants and in pathogenic fungi (Benhamou & Asselin, 1989). Grenier et al. (1991) report the tagging of a barley chitosanase with colloidal gold particles for the localization of chitosan in spore and hyphal cell walls of fungi. This technique was used for the detection of chitosan in the cell walls of Ophiostoma ulmi and Aspergillus niger. Chitinase gold-labelled complexes have also been used for the immunocytochemical and cytochemical localization of chitin and N-acetyl-D-glucosamine residues in a biotrophic mycoparasite, Piptocephalis virginiana (Manocha & Zhonghua, 1997).

1.1.2.7.2 Production of single-cell protein
The solid waste from shellfish processing is mainly composed of chitin, CaCO₃, and protein. Revah-Moiseev & Carrod (1981) suggested the use of shellfish waste for the bioconversion of chitin to yeast single-cell protein (SCP) using chitinolytic enzymes. They used the Serratia marcescens chitinase system to hydrolyze the chitin and Pichia kudriavazevii to yield SCP (with 45% protein and 8–11% nucleic acids). The commonly used fungi as the source of SCP are Hansenula polymorpha, Candida tropicalis, Saccharomyces cerevisiae, and Myrothecium verrucaria. Vyas &
Deshpande (1991) utilized the chitinolytic enzymes of *M. verrucaria* and *S. cerevisiae* for the production of SCP from chitinous waste.

1.1.2.7.3 Production of protoplasts

Fungal protoplasts have been used as an effective experimental tool in studying cell wall synthesis, enzyme synthesis, and secretion, as well as in strain improvement for biotechnological applications. Since fungi have chitin in their cell walls, the chitinolytic enzyme seems to be essential along with other wall-degrading enzymes for protoplast formation from fungi. Dahiya *et al*. (2005a) reported the effectiveness of *Enterobacter* sp. NRG4 chitinase in the generation of protoplasts from *Trichoderma reesei*, *Pleurotus florida*, *Agaricus bisporus*, and *Aspergillus niger*. Mizuno *et al*. (1997) isolated protoplast from *Schizophyllum commune* using the culture filtrate of *B. circulans* KA-304. An enzyme complex from *B. circulans* WL-12 with high chitinase activity was effective in generating protoplasts from *Phaffia rhodozyme* (Johnson *et al*., 1979). Gautam *et al*., 1996 reported protoplast formation from *Malbranchea sulfurea* using chitinase from *Paecilomyces variotii*. Similarly, Kitomoto (1988) showed the protoplast generation from various fungi using chitinase from *Trichoderma harzianum*.

1.1.2.7.4 Production of chitooligosaccharides, glucosamine, and GlcNAc

Chitooligosaccharides, glucosamines, and GlcNAc have an immense pharmaceutical potential. Chitooligosaccharides are potentially useful in medicines for mankind. For example, chitoheptose and chitohexose showed antitumor activity. A chitinase from *Vibrio alginolyticus* was used to prepare chitopentose and chitotriose from colloidal chitin (Murao *et al*., 1992). The chitobiose produced was subjected to chemical modifications to give novel disaccharide derivatives of 2-acetamido 2-deoxy D-allopyranose moieties that are potential intermediates for the synthesis of an enzyme inhibitor, that is, N, N′-diacetyl-β- chitobiosyl allosamizoline (Terayama *et al*., 1993).

Specific combinations of chitinolytic enzymes would be necessary to obtain the desired chain length of the oligomer. For example, the production of chitooligosaccharides requires high levels of endochitinase and low levels of N-acetylglucosaminidase and exochitinase, whereas the production of GlcNAc requires higher proportion of exochitinase and N-acetylglucosaminidase (Aloise *et al*., 1996).
1.1.2.7.5 Detection of fungal biomass

A variety of methods have been described to quantify fungi in soil. The techniques include direct microscopic observation and extraction of fungus-specific indicator molecules such as glucosamine ergosterol. A strong correlation has been reported between chitinase activity and fungal population in soils. Such correlation was not found for bacteria and actinomycetes. Thus, chitinase activity appears to be a suitable indicator of actively growing fungi in soil. Miller et al., (1998) reported the correlation of chitinase activity with the content of fungus-specific indicator molecules 18:2ω6 phospholipid fatty acid and ergosterol using specific methylumbelliferyl substrates.

Similarly, chitinase and chitin-binding proteins can be used for the detection of fungal infections in humans (Laine & Lo, 1996).

1.1.2.7.6 Control of mosquito

The worldwide socioeconomic aspects of diseases spread by mosquitoes made them potential targets for various pest control agents. In case of mosquitoes, entomopathogenic fungus such as Beauveria bassiana could not infect the eggs of Aedes aegypti, a vector of yellow fever and dengue, and other related species due to the aquatic environment. The scarabaeid eggs laid in the soil were found to be susceptible to B. bassiana (Ferron, 1985). Myrothecium verrucaria, a saprophytic fungus, produces a total complex of an insect cuticle-degrading enzyme (Shaikh & Desphande, 1993). It has been seen that both first and fourth instar larvae of mosquito A. aegypti can be killed within 48 h with the help of the crude preparation from M. verrucaria (Mendonsa et al., 1996). Though 100% mortality was observed within 48 h, purified endochitinase lethal times (LT50) were 48 and 120 h for first and fourth instar larvae, respectively. However, the time period was found to be decreased, corresponding to 24 h and 48 h, when the purified chitinase was supplemented with lipolytic activity.

1.1.2.7.7 Fungal and insect morphogenesis

Chitinases play an important role in yeast and insect morphogenesis. Kuranda & Robbins (1991) reported the role of chitinases in cell separation during growth in S. cerevisiae, and Shimono et al., (2002) studied the functional expression of chitinase and chitosanase and their effects on morphogenesis in the yeast S. pombe. When the
chiA gene was expressed in *S. pombe*, yeast cells grow slowly and cells become elongated, but when the *choA* gene was expressed, cells become swollen. Simultaneous expression of both *chiA* and *choA* genes resulted in elongated and swollen cells.

1.1.2.7.9 Control of phytopathogenic fungi

A biological control agent of fungal root pathogen should exhibit a sufficient amount of antagonistic activity. The chitinase produced by *Enterobacter* sp. was highly active toward *Fusarium moniliforme*, *A. niger*, *Mucor rouxi*, and *Rhizopus nigricans* (Dahiya *et al.*, 2005). The chitinase from *Alcaligenes xylosoxydans* inhibited the growth of *Fusarium udum* and *Rhizoctonia bataticola* (Vaidya *et al.*, 2001). Mahadevan & Crawford (1997) reported the antagonistic action of *Streptomyces lydicus* WXEC108 against *Pythium ultimum* and *Rhizoctonia solani*, which cause disease in cotton and pea respectively.

Chitinases can be added as a supplement to the commonly used fungicides and insecticides not only to make them more potent, but also to minimize the concentration of chemically synthesized active ingredients of the fungicides and insecticides that are otherwise harmful to the environment and human health.

1.1.2.7.8 Biomedical applications

Chitinases can be employed in human health care, such as making ophthalmic preparations with chitinases and microbiocides. A direct medical use has been suggested for chitinases in the therapy for fungal diseases in potentiating the activity of antifungal drugs (Pope & Davis, 1979; Orunsi & Trinci, 1985). They can also be used as potential additives in antifungal creams and ointments due to their topical applications.

1.1.2.7.10 In the recycling of chitinous bio-waste

Worldwide production of chitin has been estimated to be approximately $10^{11}$ tons per annum making it one of the most abundant natural compounds on earth (Kurita, 2006). Shrimps, crabs, lobsters, krill, and squid wastes from the marine processing industry have become the major resource used today (Teng *et al.*, 2001). In the last two decades there has been a lot of focus on the production of N-acetyl glucosamine by enzymatic hydrolysis of chitin. Tom & Carroad (1981) have described a process
for the bioconversion of shell-fish waste to N-acetyl D-glucosamine and other value added products.

1.1.3 Scope of the work

The exploration of fungal diversity for novel molecule has been a topic of intense research in recent years. Globally, over the years emphasis has been on screening the rapidly growing and easily cultured species of fungi. Recent trends however have shifted towards realizing that the lesser investigated taxa belonging to diverse taxonomic groups which may also be a potential source of hitherto unrecognized bioactive potential. The bio prospecting of fungi can play an important role in generating bio-economy by exploring the genetic and biochemical diversity captured in natural resources. With these backgrounds exploration of rare/interesting fungal strains from unusual substrates/ ecological niches were undertaken for their chitinolytic potential

Saprophytic Entomophthorales group was focused mainly because members of this group have extensively been studied for characterization of different enzymes, e.g., proteases and lipases but only a few reports are available on chitinase. Being a group of entomopathogenic fungi, saprophytic Entomophthorales needed more attention for chitinase production in particular the genus Basidiobolus which helps to establish the pathogenesis of the fungi of this group.

Basidiobolus ranarum Eidam. (1886) is a member of saprophytic Entomophthorales group of fungi. This genus has been explored by different workers for the production of various enzymes as urease, trypsin, lipase, lecithinase, gelatinase and protease, but studies on chitinase are rarely done in a systematic way (Echetebu & Ononogbu 1982; Feio et al., 1999).

Considering these facts, the study was planned under the following objectives:

1. To isolate, identify and screen fungi from different substrates using selective media for chitinase producing fungal isolates.
2. To optimize chitinase production with respect to various fermentation parameters.
3. To study biotechnological potential.
4. To purify and characterize the chitinase.