I. INTRODUCTION
Chitin is the most widespread amino polysaccharide in nature and is estimated annually to be produced almost as much as cellulose. It is a cationic aminopolysaccharide, composed of β(1-4) linked N-acetyl-D-glucosamine (NAG) residues. It is present in the exoskeleton of invertebrates e.g. crustaceans, molluscs, marine diatoms and insects and in algae and fungi among microorganisms. Derivatives of chitin oligomers have also been implicated as morphogenic factors in the communication between leguminous plants and *Rhizobium* and even in vertebrates, where they may be important during early stages of embryogenesis (Bakers et al., 1999). Annual synthesis of this polysaccharide in fresh water and marine ecosystems is estimated to be 600 and 1600 million tons, respectively (Cauchie, 1997). The best characterized sources of chitin are shellfish (including shrimp, crab, lobster and krill), oyster and squid, harvested in quantities of about 29.9, 1.4 and 0.7 million tons per annum (Synowiecki and Al-Khateeb, 2000). In India alone 60,000 to 80,000 tonnes of chitinous wastes are produced annually, from which a lot of chitin can be recovered (Suresh and Chandrasekaran, 1998). Chitin is the most underexploited biomass resource available on Earth. At present only a small quantity of shell waste is utilized for animal feed or chitin isolation (Synowiecki and Al-Khateeb, 2003).

Conventionally these wastes are disposed off either by burning or land filling but these methods are harmful to the environment since burning releases carbon dioxide and carbon monoxide to the environment, which adds to global warming while land filling is harmful due to slow rate of degradation and concomitant release of a potent pollutant of ground water, namely, ammonia (Muzzarelli, 1997; Das et al., 2012). The cost of transporting such waste, environmental pollution concern and ethical questions as to the morality of ignoring 70-80% of the dry weight of the catch have highlighted the necessity of finding alternative method (Simpson and Haard, 1985; Nicol, 1991; Vyas and Deshpande, 1991). Utilization of such chitinous wastes for the production of some useful products is being considered lately, and two different approaches are being investigated:

(a) The formation of a useful product such as chitin and chitosan through biological (Gagne and Simpson, 1993) and chemical treatment (Brine et al.,
1981), for use in sewage treatment, animal feed, food preservation, and formulations of biofungicides (Muzzarelli, 1997; Gohel et al., 2005)

(b) Using the waste as a carbon source in fermentation processes for the production of useful products such as chitinolytic enzymes by microorganisms (Wang et al. 2001; Gohel et al., 2007).

Recently the commercial value of chitin has increased because of the beneficial properties of its soluble derivatives, which are suitable in chemistry, biotechnology, agriculture, food processing, cosmetics, veterinary, medicine, dentistry, environment protection and paper or textile production (Synowiecki and Al-Khateeb, 2003; Tharanathan and Kittur, 2003; Das et al., 2012). Generation of this enormous amount of waste and more importantly the increasing commercial value of the soluble derivatives of chitin necessitates the development of a suitable process for solubilization of chitinous waste and its conversion into useful oligomers. The chemical methods by which these polymers and their oligomers are produced commercially involve treatment with harsh chemicals like hydrochloric acid and sodium hydroxide. Besides being environmentally unsafe, the use of these chemicals leads to products that lack uniformity. The enzymatic methods, which employ enzymes such as chitinases, are mild and eco-friendly, and thus preferred over chemical methods.

CHITIN: OCCURRENCE, STRUCTURAL ORGANIZATION, BIOSYNTHESIS, ASSOCIATIONS AND HYDROLYSIS

Chitin, a Greek word for ‘envelop’, was discovered in 1811 as a substance occurring in mushrooms. Chitin is very widely distributed especially in animals, and it also exists in less evolved taxonomic groups such as protozoa (Ruiz-Herrera, 1978). In plants, chitinous cell walls are only found in those forms, such as fungi and moulds that like animals find considerable nitrogen in their food. Chitin is also believed to constitute the cell wall of some lower green plants such as chlorophyceae (Tharanathan and Kittur, 2003). Chitinous structures are mainly of ectodermal origin in multicellular animals and form the characteristic exoskeleton of most of the invertebrates (Jeuniaux, 1971). Arthropods are particularly able to synthesize chitin, and concentrations of up to 85% are found in their shells. The chitin content in various organisms is given in Table 1.1.
In insects, chitin forms much of the animal’s cuticular exoskeleton that is regularly shed and replaced by a new cuticle (Merzendorfer and Zimoch 2003). Chitin is also found in internal structures of insects. Besides its occurrence in tracheal cuticles, it is also a constituent part of the peritrophic matrices that line the inner surface of the gut in many insects and other invertebrates, protecting the intestinal epithelium from mechanical disruption, radical oxygen species and invasion by microorganisms (Peters 1992; Lehane 1997; Barbehenn and Stannard 2004). All three crystalline modifications are actually found in chitinous structures of insects. The α-form is most prevalent in chitinous cuticles, whereas

<table>
<thead>
<tr>
<th>Organism</th>
<th>chitin content (%)</th>
<th>Organism</th>
<th>chitin content (%)</th>
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</thead>
<tbody>
<tr>
<td><strong>Crustaceans</strong></td>
<td></td>
<td><strong>Molluscan Organs</strong></td>
<td></td>
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<tr>
<td>Cancer (crab)</td>
<td>72.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Ascaris lumbricoides</td>
<td>16.3&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>Carcinus (crab)</td>
<td>64.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Meloidogyne incognita</td>
<td>30&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td>Paralithodes (King crab)</td>
<td>35&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
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<tr>
<td>Callinectes (blue crab)</td>
<td>14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Clamshell</td>
<td>6.1</td>
</tr>
<tr>
<td>Crangon (Shrimp)</td>
<td>69.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Squid, Skeletal pen</td>
<td>41</td>
</tr>
<tr>
<td>Alaskan shrimp</td>
<td>28&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Krill, deproteinized shell</td>
<td>40.2</td>
</tr>
<tr>
<td>Nephrops (lobster)</td>
<td>69.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Oyster shell</td>
<td>3.6</td>
</tr>
<tr>
<td>Homarus (lobster)</td>
<td>60-75&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
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<tr>
<td>Lepas (barnacles)</td>
<td>58.3&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td><strong>Fungi</strong></td>
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<tr>
<td>Penicillium notatum</td>
<td>18.5&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>P. chrysogenum</td>
<td>20.1&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>Saccharomyces cerevisae</td>
<td>2.9&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>Mucor rouxii</td>
<td>44.5</td>
<td></td>
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<tr>
<td>Lactarius vellereus</td>
<td>19</td>
<td></td>
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<tr>
<td>Neurospora crassa</td>
<td>8-</td>
<td></td>
<td></td>
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<tr>
<td>Trichoderma viridis</td>
<td>12-22&lt;sup&gt;e&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Histoplasma capsulatum</td>
<td>25-</td>
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<tr>
<td>Paracoccidioides brasiliensis</td>
<td>11&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>Aspergillus niger</td>
<td>42&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>Tremeliamesenterica</td>
<td>3.7&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td>Aspergillus phoenicis</td>
<td>23.7&lt;sup&gt;e&lt;/sup&gt;</td>
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</table>

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the β and γ forms are frequently found in cocoons (Kenchington, 1976; Peters, 1992). Peritrophic matrices usually comprise α- and β-chitins.

Among microorganisms, chitin is widely distributed in fungi, occurring in Basidiomycetes, Ascomycetes, and Phycomycetes, where it is a component of the cell walls and structural membranes of mycelia, stalks, and spores. The amounts vary between traces and up to 45% of the organic fraction, the rest being mostly proteins, glucans and mannans (Roberts, 1992). Variations in the amounts of chitin may depend on physiological parameters in natural environments as well as on the fermentation conditions in biotechnological processing or in cultures of fungi. Hyphal walls of the Oomycete Pythium ultimum contain cellulose and chitin, whereas the Ascomycete Fusarium oxysporum and the Basidiomycete Rhizoctonia solani contain only chitin (Cherif et al., 1993). The zoopathogenic fungi Cryptococcus neoformans, Pityrosporum canis and Rhizopus oryzae contain chitin, but not β-(1,3)-glucan (Nicholas et al., 1994). The mycelia, and the caps and stalks of fruiting bodies of four edible mushrooms Lentinus edodes, Lycophyllum shimeji, Pleurotussajor-caju, and Volvariella volvacea contain chitin as a minor component (Cheung, 1996). Slime moulds (Myxomycetes) and bacteria (Schizomycetes) are devoid of chitin.

**Structural organization of chitin**

Chitin is a homopolymer consisting of N-acetylglucosamine residues linked by β-(1-4)-glycosidic bonds. In the linear chitin chain, every single sugar is rotated by 180° with respect to its neighbouring sugars. Thus, the repeating unit in the chitin chain is chitobiose. Chitin polymers tend to form microfibrils (also referred to as rods or crystallites) that are stabilized by hydrogen bonds formed between the amine and carbonyl groups (Fig. 1.1). X-ray diffraction analysis suggested that chitin is a polymorphic substance that depends upon the source, and occurs in two crystalline allomorphs, a more abundant α- and less common β- form. In both forms chitin chains are arranged in sheets, the chains in any one sheet have the same direction. They mainly differ in the degree of hydration, in the size of the unit cell and in the number of chitin chains per unit cell (Rudall and Kenchington, 1973; Kramer and Koga, 1986). While α-chitin has no water molecule within its unit cell, the unit cell of β-chitin expands along its
β parameter when the samples are immersed in water, yielding the β-chitin dihydrate. β-chitin can also accept other small molecules such as a series of linear alcohols that intercalates into its lattice to yield crystallosolvates. These crystallosolvates keep the fibrillar morphology of the parent chitin, and on drying, they revert normally to the anhydrous structure without any apparent modification of morphology and crystallinity (Saito et al., 2000).

In α-chitin adjacent sheets have the chains oriented in opposite direction i.e. they are antiparallel, while in β-chitin, the adjacent sheets are parallel or have the same direction (Fig. 1.1) [Aranaz et. al., 2009]. In both structures, the sheets are tightly held by a number of inter-sheet H-bonds dominated by CO-NH hydrogen bonds. In α-chitin there are also some intra-sheet H-bonds involving the association of hydroxymethyl groups of adjacent chains. A third form, called gamma-chitin, is considered to be a distorted version of either α- or β- forms, where every third sheet has the opposite direction (Tharanathan and Kittur, 2003). In addition, non-crystalline, transient states have also been reported in fungi (Vermeulen and Wessels, 1986).

The anti-parallel arrangement of chitin molecules in the α form allows tight packaging into chitin microfibrils, consisting of many single chitin chains that are stabilized by a high number of hydrogen bonds formed within and between the molecules. This arrangement may contribute significantly to the physicochemical properties of the chitinous structure such as mechanical strength and stability (Giraud-Guille and Bouligand, 1986). By contrast, in the β- and γ-chains, packing tightness and number of inter-chain hydrogen bonds are reduced, resulting in an increased number of hydrogen bonds with water. The high degree of hydration and reduced packaging tightness result in more flexible and soft chitinous structures as found in insects’ peritrophic matrices or cocoons (Merzendorfer and Zimoch, 2003).

α-chitin occurs in fungal and yeast cell walls, in krills, in lobsters and crabs tendons and shells, in shrimp shells and in insect cuticles. The chitin formed from recrystallization (Saito et al 2000), in vitro synthesis or enzymatic polymerization is also α-chitin. The commercial source of β-chitin is squid pens. It also occurs in the spines of some diatoms, in tubes of pagonophoran and
vestimentiferan worms and in aphrodite chaetae. Fully acetylated/particularly pure form of $\beta$-chitin is found in monocrystalline spines secreted by the diatom *Thalassiosira fluviatilis* (Bartnicki-Garcia and Lippman, 1982).

**Fig. 1.1.** (a) Chemical structure of chitin. The grey box indicates one N-acetylg glucosamine subunit of the chitin chain. (b) The two major types of chitin are characterized by an antiparallel ($\alpha$-chitin) or parallel ($\beta$-chitin) arrangement of the chains (Seidl, 2008).

### Synthesis of chitin

Synthesis of chitin in all organisms occurs as a result of transglycosylation reaction catalyzed by membrane bound enzymes collectively called as chitin synthetases. These utilize the nucleotide uridine diphosphate N-acetylg glucosamine (UDP-NAG) as sugar donor and add UDP-NAG units to the non-reducing end of the growing polymer. The transfer of the NAG residues is thought to occur by inversion of the configuration at the anomeric center resulting in the formation of a $\beta$-glycosidic bond. The chains are believed to be produced at the cytoplasmic side of the synthase, translocated to the extracellular space and assembled as microfibrils. In fungi chitin microfibrils are usually made of 20-400 sugar chains associated through H-bonding. In other organisms the size and packing of chain is more variable. Also variable is the association of chitin with other components.

### Chitin associations

Once in the extracellular space, due to availability of reactive free amino groups, chitin may associate chemically with other components of the cell walls or...
exoskeletons, thus acquiring different properties. In invertebrates, protein forms covalent links with chitin, giving rise to pliable and flexible structures with higher strength. Whether in its $\alpha$-or $\beta$-form, chitin is covalently linked to arthropodins, resilins, and sclerotins to form more or less stable glycoproteins through aspartyl and histidyl residues (Tharanathan and Kittur, 2003). The resulting chitin-protein complexes gain stability, providing hardness and rigidity and can associate with additional substances such as lipoproteins and waxes which provide impermeability properties to exoskeletons. Chitin protein aggregates provides a substrate for calcium and silica deposition. This mineralization process seems to be common in crustaceans and molluscs, where it provides rigidity and ensures stability to the exoskeletons. Chitin also forms conjugates with carotenoids, giving color to the tissues in insects and crustaceans (Fox, 1973).

In fungi, chitin associates with glycoproteins and polysaccharides such as galactomannan and glucans (Muzzarelli et al., 1980). In the macromolecular network of the cell wall, glucan chains are linked to chitin through their reducing ends via amino acids, particularly lysine (Sietsma and Wessels, 1981). Apart from the linkages of the two polymers to each other, both chitin and $\beta$-glucan chains are also hydrogen bonded among themselves (Tharanathan and Kittur, 2003).

**Chitin hydrolysis**

As an important source of carbon and nitrogen, chitin is recycled by many saprophytic microorganisms including bacteria and fungi. Many saprophytic chitinolytic microbes produce a whole chitinolytic system comprising an endochitinase, chitobiase and an exochitinase whose synergistic or consecutive actions degrade chitin to free sugar NAG (N-acetylglucosamine).

The possibility of exploiting these organisms to economically generate commercially useful products has been explored. Wang et al. (2001) reported microbial reclamation of shellfish wastes for the production of chitinases where they prepared shrimp and crab shell powder by treating shellfish processing waste with boiling and crushing and it was used as a substrate for chitinolytic microorganisms. Rattanakit et al. (2002) reported a chitinase formulation by
using shrimp shellfish waste as a substrate for solid state cultivation of *Aspergillus* sp. SI-13. Besides enzymes, examples of SCP, ethanol and biodiesel production from chitinous waste have been cited in literature (Ferrer et al., 1996; Cody et al., 1990; Zang et al., 2011). An economically viable biotechnological process for such direct utilization of chitinous residues is, however, still awaited.

Alternatively, chitin can also be extracted from shellfish waste and digested to yield oligomers and monomers that find growing market. Chitin is inert to most commercially available solvents but is fairly stable under mild acidic and basic conditions and thus obtained as the residue remaining after decomposition of the other components with acid and alkali (Bade, 1997; No and Meyers, 1997).

Presently, shells are first treated with dilute hydrochloric acid at room temperature to remove metal salts, primarily calcium carbonate. The decalcified shells are ground and heated at about 100°C in 1-2 M sodium hydroxide to decompose proteins and pigments. Sometimes repetition of the treatments may be required. α-chitin is obtained on drying as almost colorless to off-white flakes or powdery materials in 30–35% yield based on dried shrimp shells (Sannan et al., 1976). Starting from crab shells, the yield is usually lower than that from shrimp shells because of the higher content of calcium carbonate. β-chitin from squid pens are associated with proteins and only a small amount of calcium carbonate and thus can be isolated under similar, but milder conditions (Kurita et al., 2006).

Thus extracted chitin is used almost solely as a raw material for the production of glucosamine along with oligosaccharides and chitosan (Kurita et al., 2006). The generation of these soluble and industrially important products require more rigorous acid and base treatment (Einbu and Varun et al., 2008). These chemical methods are associated with the problems of low yield, high cost of purification and environmental pollution (Sakai, 1995; Sukwattanasinitt et al., 2002; Wang et al., 2010). Alternatively, with its advantages in environmental compatibility, low cost and reproducibility, enzymatic hydrolysis has attracted attention of scientists in recent years (Yang et al., 2000; Kadokura et al., 2007). Especially for the production of chitooligosaccharides, enzymatic hydrolysis has
distinct advantage over acid hydrolysis because the composition of oligosaccharides can be adjusted readily by changing either the amount or type of chitinolytic enzymes or controlling reaction conditions (Lee et al., 1996)

CHITIN HYDROLYZING ENZYMES

Classification

Chitinases catalyze the hydrolysis of chitin to its oligosaccharides and can be classified into two major categories (Graham and Sticklen, 1994). Endochitinases (EC 3.2.1.14) cleave chitin randomly at internal sites, generating soluble, low molecular mass multimers of NAG such as chitotetraose, chitotriose and diacetylchitobiose. While exochitinases are further divided into two subcategories: chitobiosidases and β-(1,4) N-acetyl glucosaminidases. Chitobiosidases (EC 3.2.1.29) catalyze the progressive release of diacetylchitobiose starting at the non-reducing end of chitin microfibril. They form diacetylchitobioses and no monocaccharide or oligosaccharides are formed. β-(1,4) N-acetyl glucosaminidases (GlcNAcase, EC 3.2.1.30) or chitobiases catalyze the release of terminal, non-reducing N-acetylglucosamine residues in an exo-type fashion from chitin and oligomers of chitin, but in general they have the highest affinity for the dimer N,N’-diacetylchitobiose (GlcNAc)₂ and convert it into two monomers (Horsch et al. 1997). As the enzyme has broad substrate specificity, it can also be called β-(1,4) N-acetyl hexosaminidase (HexNAcase, EC 3.2.1.52) [Cannon et al 1994].

However, the enzymatic properties of chitinases are more complex and versatile than reflected in the exo-/endo classification. Detailed studies of the chitinolytic system of the bacterium Serratia marcescens demonstrated another way to classify the enzymatic properties of chitinases by grouping them into processive and non-processive enzymes (Horn et al. 2006; Sorbotten et al. 2005; Uchiyama et al. 2001). Processive chitinases do not release the substrate after hydrolytic cleavage but slide it through the active site-tunnel for the next cleavage step to occur. The presence of a carbohydrate binding domain can enhance processivity, but is not essential for it.
Introduction

Non-processive chitinases dissociate completely from the substrate after hydrolysis. This leads for non-processive enzymes to substrate degradation patterns with a homogenous distribution of medium chain (6–30mer) products and for processive enzymes to remnants of the polymeric substrate and only 2–8mer short chain degradation products (Horn et al. 2006). Chitinases can contain various carbohydrate-binding modules (CBMs). These CBMs include different families classically defined as chitin- and cellulose-binding domains due to their preferred affinity for these carbohydrates (Henrissat 1999).

Chitinases are classified as glycosyl hydrolases. The glycosyl hydrolase classification system is based on the amino acid sequence similarity of the catalytic domains and chitinases are placed in families 18, 19, and 20 (Henrissat and Bairoch, 1993; Carbohydrate-active enzymes family server, http://www.cazy.org/; http://afmb.cnrs.mrs.fr/*pedro/CAZY/db.html). The family 18 chitinases are diverse in evolutionary terms and includes chitinases from bacteria, fungi, viruses, animals, and some plants. Family 19 members are almost exclusively found in plants with the exception of some Streptomyces chitinases. Family 18 and family 19 chitinases have completely different 3-D structures and molecular mechanisms (Table 1), suggesting that they have

![Fig. 1.2. Depiction of mechanism of action of the three types of chitinases](image-url)
arisen from different ancestors (Hamel et al 1997; Suzuki et al 1999). The family 20 includes the β-N-acetylhexosaminidases from bacteria, fungi, and mammals. As chitin in most of the organisms occur as a heteropolymer of acetylated and deacetylated glucosamine residues, thus chitin deacetylases and chitosanases too form a part of the repertoire of chitin hydrolyzing enzymes. Chitin/chitooligosaccharide deacetylases cleaves off the acetyl group from NAG residues in chitin/ chitooligosaccharide and chitosanase hydrolyze the β-glycosidic bond between deacetylated chitin residues. Chitin deacetylases are included in the carbohydrate esterase family 4 of glycoside hydrolases (Caufrier et al., 2003), while chitosanases have been classified into family 5, 8, 46, 75 and 80 of glycoside hydrolases (Cheng et al., 2006).

Table 1.2. Differences between family 18 and 19 of chitinases.

<table>
<thead>
<tr>
<th></th>
<th>Family 18</th>
<th>Family 19</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Occurrence</strong></td>
<td>Bacteria, fungi, viruses and animals</td>
<td>Plants and <em>Streptomyces griseus</em> chitinase C</td>
</tr>
<tr>
<td><strong>Mode of action</strong></td>
<td>Retention of the anomeric configuration</td>
<td>Inversion</td>
</tr>
<tr>
<td><strong>Catalytic mechanism</strong></td>
<td>Substrate assisted catalysis</td>
<td>Acid-base catalysis</td>
</tr>
<tr>
<td><strong>Sensitivity to allosamidin</strong></td>
<td>Sensitive</td>
<td>Insensitive</td>
</tr>
<tr>
<td><strong>3D Structure</strong></td>
<td>(β/α)8 barrel fold</td>
<td>Bilobal structure with high α-helical content</td>
</tr>
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</table>

**Enzyme assays**

Techniques and methods have been developed for the estimation of randomly hydrolyzing endo-chitinases and exo-hydrolytic N-acetylglucosaminidase activities employing various soluble and insoluble substrates. Chitinase activity is often determined by measuring the amount of reducing sugars liberated from colloidal chitin by enzyme activity (Reissig et al., 1955). Endochitinase activity is also measured by the reduction of turbidity of a suspension of colloidal chitin (Tronsmo and Harman, 1993).

Chromogenic substrates such as dye-labelled chitin and p-nitrophenyl (pNP) labelled substrates are also used to measure chitinase activity by estimating released dye/pNP spectrophotometrically. A carboxymethyl-substituted soluble chitin covalently linked with Remazol Brilliant Violet 5R is suitable for the
screening of chitinolytic microorganisms and for detection of chitinase activity by plate-clearing assay (Wirth and Wolf, 1990). The method is based on the precipitability of the non-hydrolyzed chitin by HCl. The absorbance in the supernatant (containing lower oligomers) is used to measure the enzyme activity. The use of p-nitrophenyl- labelled substrates allow for detection of three different chitinase types by acting as dimeric, trimeric, and tetrameric substrates, respectively. Glucosaminidase, chitobiosidase and endochitinase activities are determined by measuring the release of p-nitrophenyl from pNP-GlcNAc, pNP-(GlcNAc)₂ and pNP-(Glc-NAc)₃, respectively (Harman et al., 1993; Robert and Selitrennikoff, 1988; Patil et al., 2000).

Trudel and Asselin (1989) developed a technique for the detection of chitinase activity after native or denaturing polyacrylamide gel electrophoresis (PAGE) by incorporating glycol chitin into the gel. As glycol chitin exhibits high affinity towards Calcofluor white M2R, the lysis zones can be visualized by UV illumination as non-fluorescent dark bands in contrast to the fluorescent intact glycol chitin.

In addition, enzyme activity can also be detected on gels by using fluorescent substrates (Tronsmo and Harman, 1993). The chitinases appear as fluorescent bands under UV light because of enzymatic hydrolysis of fluorescent 4-methylumbelliferone from the GlcNAc mono- and oligosaccharides, such as 4-MU-GlcNAc, 4-MU-(GlcNAc)₂ and 4-MU- (GlcNAc)₃. The dimer is the preferred substrate for glucosaminidases. Chitobiosidases release fluorescent product from only the trimeric substrate and endochitinases are identified by digestion of the tetrameric substrate (Haran et al., 1995).

**Sources of chitinases/Chitinolytic organisms**

Chitinases have broad spectrum of distribution in nature including bacteria, fungi, nematodes, plants, insects, fish and human (Jeuniaux, 1966). The physiological functions of chitinases depend on their source.
Fungi

Chitinases and glucanases are important class of hydrolytic enzymes that are essential for the fungal kingdom and are required for the maintenance of wall plasticity, during cell expansion and division in yeasts, and during spore germination, hyphal branching and septum formation in filamentous fungi. They may contribute to breakage and re-forming of bonds within and between polymers, leading to re-modelling of the cell wall during growth and morphogenesis. For example, during budding in *S. cerevisiae* a division septum is laid down between the cells and within a ring of chitin deposited at the bud site. Degradation of this material leads to cell separation and involves an extensively glycosylated endochitinase with an apparent molecular mass of approximately 130 kDa. Disruption of the gene encoding this enzyme or a potent chitinase inhibitor led to cell separation defect and pseudohyphal growth (Kuranda & Robbins, 1991; Sakuda et al., 1990; King & Butler, 1998).

When the dimorphic pathogen of humans, *Candida albicans* was grown in a yeast phase, the transcripts of chitinase genes, CHT2 and CHT3 were greater as compared to a mycelial phase and gene disruption experiments suggested a role for the gene product in cell separation (Kuranda & Robbins, 1991; McCreath et al., 1995).

Plasmids of some strains of yeast encode toxins that kill other strains. Killer toxins of some strains of *Kluyveromyces lactis* and *Pichia acaciae* have chitinase activity. In *K. lactis* the toxin is a trimeric protein, with the intracellular gamma subunit responsible for killing a susceptible cell of *Saccharomyces cerevisiae*, while the alpha subunit has exochitinase activity essential for the action of toxin, shown by the inhibition of activity by allosamidin (Magliani et al., 1997).

Fungal cell wall chitinases also have roles during sporulation in filamentous fungi, as the specific chitinase inhibitors allosamidin or demethylallosamidin inhibited fragmentation of hyphae into arthroconidia (Yamanaka et al., 1994; Sandor et al., 1998). Disruption of the gene encoding the *A. nidulans* chitinase, ChiA, led to a decrease in the frequency of spore germination and a lower hyphal growth rate (Takaya et al., 1998). However in *A. fumigatus*, disruption of
gene encoding chitinase, ChiB1p had no effect on growth and morphogenesis and may contribute to the digestion and utilization of exogenous chitin (Reichard et al., 2000; Jaques et al., 2003). A single organism may produce several chitinases for various purposes. There are 15 potential chitinase open reading frames (ORFs) in the genome of *A. nidulans*. Among these, the class V endochitinase ChiB was shown to play an important role in autolysis. Levels of ChiB significantly increased when the fungal cells were starved for carbon sources, an induced condition for hyphal autolysis of *A. nidulans* (Yamazaki et al., 2007).

Chitinases play an important role in the mycoparasitic progress, especially in the cell wall penetration and nutrient utilization by degrading the cell walls in fungi (Inbar and Chet, 1995; Elad et al., 1983; Chet et al., 1993). It has been reported that disruption of ech42 gene affects mycoparasitism in *T. harzianum* (Woo et al., 1998). Chitinases from other parasitic fungi have also been reported, such as *Aphanocladium album* (Kunz et al., 1992), *Gliocladium virens* (Di Pietro et al., 1993), *Fusarium chlamydosporum* (Mathivanan et al., 1998), *Trichothecium roseum* (Li et al., 2004), *Stachybotrya elegans* (Taylor et al., 2002), *Talaromyces flavus* (Li et al., 2005), *Beauveria bassiana* (Bidochka et al., 1993; Peng et al., 1996; Fang et al., 2005), *Metarhizium anisopliae* (Pinto et al., 1997; St. Leger et al., 1996; Kang et al., 1999) *Verticillium chlamydosporium* and *V. suchlasporium* (Tikhonov et al., 2002). Similarly, chitinases are involved in the association of plant roots with mycorrhizal or endophytic fungi. Examples include *Hebeloma syrjense* (Tibbett and Sanders, 2002), *Neotyphodium* and *Epichloe* (Li et al., 2004).

**Bacterial Sources**

Many prokaryotes efficiently digest the crystalline and complexed chitin and utilize it as a carbon source, thereby playing an important role in recycling this compound. The major producers of chitin degrading enzymes are in the genera *Aeromonas* (Chen et al., 1991; Lin et al., 1997; Kojima et al., 2005; Lan et al., 2006), *Serratia* (Xia et al., 2011), *Enterobacter* (Dahiya et al., 2005; Chernin et al., 1995; Velusamy and Kim, 2011), *Vibrio* (Svital et al., 1997; Li and Roseman, 2003; Sugintal et al., 2010), *Streptomyces* (Ohno et al., 1996;
Kim et al., 2003; Tsujibo et al., 1993), and *Bacillus* (Battacharya et al., 2007; Wang et al., 2001; Wang et al., 2002; Cho et al., 2011).

Prokaryotes are generally the only chitin degraders/responsible for chitin recycling in extreme environments. One such environment is the rumen of herbivores where *Clostridium* sp. is involved in the anaerobic degradation of chitin (Simunek et al., 2004; Tishchenko et al., 2010). *Clostridium thermocellum* is a thermophilic bacterium that degrades/hydrolyze chitin in hot springs and self-heated, rotting biomass (Zverlov et al., 2002). Other aerobic thermophilic bacteria that produce chitinase are *Thermococcus kodakaraensis* KOD1 (Imanaka et al., 2001), *Pyrococcus furiosus* (Oku and Ishikawa, 2006) and *Thermococcus chitonophagus* (Andronopoulou et al., 2004).

The chitinolytic systems of bacteria involve multiple isomeric forms of chitinases that include endochitinases, exochitinases and N-acetylglucosaminidases. Chitinolytic system of *Bacillus circulans* WL-12 is one of the most extensively studied systems so far. Six chitinases with differing enzyme activities have been detected in the culture supernatant of this bacterium grown in the presence of chitin (Watanabe et al., 1990; Watanabe et al., 1992). The chitinolytic machinery of *Serratia marcescens* comprises three chitinases ChiA, ChiB and ChiC which preferentially produce dimers, a chitin binding protein CBP21 (Kolstad et al., 2005) and a hexosaminidase which further degrades the major end product of chitinases i.e. chitobiose. ChiA and ChiB are processive exochitinases which degrade chitin chains in opposite directions, while ChiC is a nonprocessive endochitinase. Three separate chitinase genes have been identified in *Streptomyces lividans* (Miyashita et al., 1991). Chitinases with diverse enzymatic properties may be synthesized from separate genes as in *Streptomyces lividans*, or result from proteolytic processing in an *Alteromonas* sp. (Tsujibo et al., 1993) and *Streptomyces olivaceoviridis* (Romaguera et al., 1992).

**Animals**

Chitinases or chitinase-like proteins have been found in all insect species studied belonging to different orders including dipterans, lepidopterans, coleopterans, hemipterans and hymenopterans (Koga et al., 1997; Souza-Neto...
et al., 2003). Insect chitinases are almost exclusively endochitinases and have little or no exochitinase activity as shown by their inability to hydrolyze methylumbelliferyl-N-acetylglucosamine or p-nitrophenyl-N-acetylglucosamine or chitobiose. They often act in concert with N-acetylglucosaminidase. The combined action of endochitinase and N-acetylglucosaminidase is synergistic and leads to rapid depolymerization of chitin in insects (Fukamizo and Kramer, 1985). Only baculoviral chitinases, which may have a bacterial origin, have been reported to have both endo- and exo-chitinolytic activity (Hawtin et al., 1997). In some cases, additional proteins with chitin-binding domains (CBD) but devoid of catalytic activity help in the degradation of chitin (Vaaje-Kolstad et al., 2005).

The main function of insect chitinases is in the turnover of chitin-containing extracellular matrices such as the insect cuticle and the peritrophic matrix (PM) during molting. Insects periodically shed their old exoskeletons and either continuously or periodically shed their peritrophic membranes and resynthesize new ones (Lehane et al., 1997). This process is mediated by the elaboration of chitinases in the moulting fluid that accumulates in the space between the old cuticle and the epidermis and in gut tissue. The NAG-containing products of hydrolysis are ultimately recycled for the synthesis of a new cuticle (Kramer and Muthukrishnan, 1997). In addition, chitinases may have a digestive function in insects, if their diet contains chitin. Chitinase-like proteins that lack enzymatic activity may have roles in immunity or as growth factors (Arakrane and Muthukrishnan, 2010).

Chitinases have been generally considered to lack in mammalian bodies due to the absence of chitin. However, recent studies have identified chitinases and chitinase-like proteins (CLPs) belonging to the glycohydrolase family 18 in mice and human. Chitotriosidase from human macrophages (Renkema et al., 1995) and acidic mammalian chitinase AMCase the mouse stomach (Boot et al., 2001) possess chitinase enzymatic activity, whereas other mammalian chitinases, including CLPs, do not possess this activity as a result of mutations in their highly conserved putative active sites (Chang et al., 2001). Mammalian chitinases with enzymatic activity have a chitin-binding domain that contains six cysteine residues responsible for their binding to chitin (Tjoelker et al., 2000).
These mammalian chitinases possess a conserved sequence motif (DXXDXDXE) on strand β4, and catalytic activity in these chitinases is mediated by the glutamic acid (E), which protonates the glycosidic bond with chitin (Aalten et al., 2001). CLPs (also termed chitolectins) do not contain typical chitin-binding domains, but still can bind to chitin with high affinity.

Mammalian chitinases and chitinase like proteins are induced at sites of inflammation (such as parasitic infections) (Chang et al., 2001) and remodelling (Ostergaard et al., 2002). This raises the possibility that these molecules play active roles in human anti-parasite and anti-infective defense and repair responses.

**Plants**
Chitinases have been reported from many monocotyledonous and dicotyledonous plant species and occur in widely different tissues, including embryos, seeds, cotyledons, stems, leaves, roots and flowers. Many plant chitinases are expressed constitutively, generally at a low level (Punja and Zhang, 1993). No substrate for this enzyme is identified in plants, whereas chitin is commonly a component of fungal cell walls and insect exoskeletons, organisms which include many important pathogens and pests. Besides, the dramatic increase in chitinase levels by various biotic (fungi, bacteria, viruses and viroids) and abiotic factors (ethylene, salicylic acid, salt solutions, ozone and UV light) suggest their role in plant defence response (Collinge et al., 1993). Furthermore, chitinase has been shown to accumulate around fungal hyphal material in *Planta* (Benhamou et al., 1990; Wubben et al., 1992) and it has now been demonstrated that enhanced chitinase levels in transgenic plants can indeed reduce the damage caused by pathogens (Broglie et al., 1991).

Chitinase expression is also under developmental control in certain organs and tissues and seems to have an important function in early embryo development, indicating additional non-defensive roles (de Jong et al., 1992; Lotan et al., 1989; Neale et al., 1990). Chitinase can inactivate the lipo-oligosaccharide signal molecules produced by certain *Rhizobium* strains (Roche et al., 1991) which are responsible for the induction of root hair deformations, cortical cell divisions and nodule development in the roots of legume hosts (Truchet et al.,
Similar relations have been observed during symbiosis formation between plants and mycorrhizal fungi (Xie et al., 1999). When the fungus which infects the roots is compatible, its chitooligosaccharides are hydrolysed by plant chitinases, and the plant defence reaction is reduced. However, if the fungus is not symbiotic, then the chitinases present in the roots do not cleave the fungal elicitors, which subsequently bind to plasmalemma receptors and trigger a hypersensitive reaction (Salzer et al., 1997). Many purified plant endochitinases also show some degree of lysozyme (EC 3.2.1.17) activity, i.e. they can hydrolyse \( \beta \)-1,4-linkages between N-acetylmuramic acid and GlcNAc residues in bacterial cell wall peptidoglycan (Boller, 1988; Majeau et al., 1990; Roberts and Selitrennikoff, 1988).

Some apoplastic chitinases from monocotyledonous plants also have antifreeze activity (Kasprzewska, 2003). Recently a chitinase with antifreeze activity is also purified from the corolla of a dicot, wintersweet (Zhang et al., 2010). It is also suggested that chitinase take part in programmed cell death (PCD) (Hangel et al., 1998, Passarinho et al., 2001). This conclusion is supported by the observation that the chitinase (EP3) gene is activated earlier in *Daucus carota* cells which are in an apoptosis preceding stage (Hangel et al., 1998). It seems that in *Arabidopsis thaliana*, class IV chitinase, similarly to *Daucus carota* EP3, is involved in regulation of processes leading to PCD (Passarinho et al., 2001).

**Thermophilic microbes**

Thermophilic organisms are particularly important in biotechnology as a source of thermostable enzymes. Though thermostable enzymes have been reported from mesophiles, the productivity is high in thermophiles and the expectancy of finding more thermostable and more chemoresistant enzyme is higher in thermophiles than in mesophiles (Maheshwari et al., 2000; Vieille and Zeikus, 2001). Exploration of chitinases from thermophilic bacteria and fungi is also interesting from the point of view of understanding the mechanism of thermostolerance of such enzymes.

The thermophilic organisms *Bacillus licheniformis* X-7u (Takayanagi et al., 1991), *Bacillus* sp. BG-11 (Bharat and Hoondal, 1998) and *Streptomyces thermoviolaceus* OPC-520 were reported to be the major sources of chitinases
A thermophilic Bacillus strain isolated from chitin-containing compost produces three different endochitinases in its culture fluid showing temperature optima of 75, 65 and 75 °C (Kenji et al., 1994). Thermostable exochitinases were also isolated from Bacillus stearothermophilus CH-4, isolated from a compost of organic solid wastes (Kenji et al., 1998).

Clostridium thermocellum, an anaerobic, saccharolytic, thermophilic bacterium that occurs in hot springs and self-heated, rotting biomass, produces an endochitinase as part of its cellulosome (Zverlov et al., 2002). Thermostable chitinases are also reported from the extreme thermophilic anaerobic archaeon Thermococcus chitinophagus (Huber et al., 1995; Andronopoulou and Vorgias, 2003), Thermococcus kodakaraensis (Tanaka et al., 2003) and Pyrococcus furiosus (Gao et al., 2003).

Thermophily in fungi is not as extreme as in eubacteria or archaean, however, 0.06% of recorded fungal species are able to breach the upper temperature limit of eukaryotes and can thrive at temperatures between 45 and 55°C (Cooney and Emerson, 1964; Maheshwari et al., 2000). A thermophilic fungus is one that grows optimally at or above 40°C (Crisan, 1973). Thermophilic fungi have been reported from decomposing organic materials like wood chip, animal dung, plant straw, municipal refuge etc. in which the activity of mesophiles results in thermogenic conditions or man-made habitats such as cooling towers, effluent of nuclear power reactors and ducts employed for thermal insulation (Johri et al., 1999).

Chitinolytic enzymes have been described from a few thermophilic fungi; Mucor miehei, Talaromyces emersonii, T. leycettanus and Thermomyces lanuginosus (Jensen and Olsen, 1999). Talaromyces emersonii produces an inducible chitinolytic system consisting of a chitinase and an N-acetylglusamidase with optimal activities in the range of 65 to 75°C (Hendy et al., 1990; McCormack et al., 1991). A 48 kD chitinase is reported from Thermomyces lanuginosus and is stable for 20 min at 70°C and for 25 min at 65°C (Guo et al., 2005). Recently, a chitinase encoding 1326 bp cDNA was sequenced and its alignment with other family 18 chitinases showed low overall homology except
for highly conserved regions among microbial chitinases (Guo et al., 2006; Guo et al., 2008). Likewise the catalytic domains of two chitinases from *Thermoascus aurantiacus* var. *levisporus* and *Chaetomium thermophilum* are similar with other family 18 glycosyl hydrolases. Both these enzymes have high thermostability (Li et al., 2010). Chitin deacetylase has also been reported in the cell extracts of the thermophile *Mucor miehei* (Kauss et al., 1983).

**PRODUCTION**

Microbial chitinase has 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, chitinase produced from microorganisms is inducible in nature.

**Submerged fermentation**

Extracellular chitinase production is reported to be influenced by media components such as carbon sources, nitrogen sources, and agricultural residues such as rice bran, wheat bran, etc. (Bhushan 1998; Dahiya et al. 2005b). Several other physical factors such as aeration, pH, and incubation temperature also affect chitinase production. Chitinase production levels from different organisms and process variables are listed in **table 1.3**.

**Table 1.3. Chitinase production in submerged fermentation**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Production levels</th>
<th>Medium</th>
<th>pH</th>
<th>Temp.</th>
<th>Fermentation time</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichoderma harzianum</em> TUBF 966</td>
<td>14.7 U/ml</td>
<td>1.5% colloidal chitin, 0.42% peptone</td>
<td>5.5</td>
<td>30ºC</td>
<td>96h</td>
<td>Sandhya et al. 2004</td>
</tr>
<tr>
<td><em>Verticillium lecanii</em> F091</td>
<td>19.9 mU/ml</td>
<td>4.52% (w/v) maltose, 1.79% marine peptone extract, 0.41% shrimp powder and 0.3% soy protein</td>
<td>4</td>
<td>24ºC</td>
<td>144h</td>
<td>Liu et al. 2003</td>
</tr>
<tr>
<td><strong>Bacillus sp. BG-11</strong></td>
<td>76 U/ml</td>
<td>1% swollen chitin and 0.5% glucose</td>
<td>8.5</td>
<td>50°C</td>
<td>72h</td>
<td>Bhushan 2000</td>
</tr>
<tr>
<td>------------------------</td>
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<td>----------------------------------</td>
<td>------</td>
<td>-------</td>
<td>-----</td>
<td>--------------</td>
</tr>
<tr>
<td><strong>Alcaligens xylosoxydans</strong></td>
<td>29 U/ml</td>
<td>1.5% chitin, 0.03% yeast extract and 0.012% tween-20</td>
<td>8.5</td>
<td>30°C</td>
<td>72h</td>
<td>Vaidya et al. 2003</td>
</tr>
<tr>
<td><strong>Pantoea dispersa</strong></td>
<td>452.56 U/ml</td>
<td>0.5% chitin, 0.05% peptone, 0.005% yeast extract and 0.05% urea</td>
<td>7.2</td>
<td>30</td>
<td>144h</td>
<td>Gohel et al., 2006</td>
</tr>
<tr>
<td><strong>Colletotrichum gloeosporioides</strong></td>
<td>2.4 U</td>
<td>0.5% colloidal chitin</td>
<td>7</td>
<td>28</td>
<td>5d</td>
<td>Souza et al., 2005</td>
</tr>
<tr>
<td><strong>Talaromyces emersonii</strong></td>
<td>0.45 µM/h/ml</td>
<td>1-2% Chitin, mineral salt medium</td>
<td>5</td>
<td>45</td>
<td>240h</td>
<td>McComark et al., 1991</td>
</tr>
</tbody>
</table>

**Physical factors affecting chitinase production**

**Temperature**

Temperature is an important factor that influences growth and metabolism of all microorganisms. Mesophilic as well as thermophilic microorganisms have been reported to produce chitinases. The optimum temperature for chitinase production from most mesophiles lies in the range between 24-37°C. For example, *Vibrio alginolyticus* produces chitinase optimally at 37°C (Ohishi et al., 1996), *Bacillus pabuli* K1 (Frangnberg and Schnurer, 1994) and *Serratia marcescens* at 30°C (Khoury et al., 1997), *Streptomyces lividans* at 25-30°C, *Stachybotrys elegans* at 24°C (Tweddell et al., 1994), *Myrothecium verucaria* (Vyas and Deshpande, 1989), *Tricoderma harzianum* (Kapat et al., 1996) and *Fusarium chlamydosporum* (Mathivanan et al., 1997) at 28°C.

Thermophilic microorganisms produce chitinases at relatively higher temperatures. *Bacillus licheniformis* secretes four chitinases (I-IV) when grown at 50°C (Takayanagi et al., 1991). *Thermococcus chitinophagus* produces an extracellular, a membrane associated and an periplasmic chitinase at 85°C (Andronopoulou et al., 2004). A thermophilic fungi, *Talaromyces emersonii*
CBS8 1470 produces a thermostable chitinase at 45°C (McComack et al., 1991) whereas *Thermomyces lanuginosus* SY-2 has been reported to produce chitinase at 50°C (Guo et al., 2008).

**pH**

Microorganisms generally produce chitinase in the pH range of 5-8. For example, *Bacillus licheniformis* (Takayanagi et al., 1991), *Vibrio alginolyticus* (Ohishi et al., 1996), *Colletotrichum gloeosporioides* (Souza et al., 2005), *Trichoderma viridae* F-19 (Rogalski et al., 1997) and *Streptomyces cinereoruber* (Tagawa and Okazaki et al., 1991) produce chitinase in batch culture at pH 7.0. While *Trichoderma harzianum* (Kapat et al., 1996), *Talaromyces emersonii* (McComack et al., 1991) and *Nocardia orientalis* (Usui et al., 1984) produce chitinase optimally when the medium pH is 5. Organisms that produce chitinase in an alkaline (pH 8) production medium are *Alcaligenes xylosoxydans* (Macmil et al., 2005) and *Serratia marcescens* (Khoury et al., 1997).

**Agitation**

In submerged fermentation, agitation influencing dissolved oxygen tension is an important parameter that affects the productivity of a process. Although agitation improves the mixing and mass and heat transfer in a fermentor, it may also have many negative effects on morphological state of the organism such as rupture of cells, vacuolation and autolysis that can cause a decrease in productivity (Cui et al., 1997). Agitation rate is also one of the most critical parameter used for scale up (Felse and Panda, 2000). Felse and Panda (2000) optimized the agitator speed for maximal chitinase production in a bioreactor. They observed a consistent increase in cell growth, chitinase production, and chitin conversion up to agitator speed of 224 rpm and thereafter it declined. At lower agitator speed, the uptake of substrate was very low due to incomplete mixing and/or mass transfer resistance. At higher agitation speeds, chitinase production rapidly declined. This may be due to shear inactivation of the enzyme. *Paenibacillus* sp. CHE-N1 yielded optimal chitinase activity at an aeration rate of 3 vvm and an agitation rate of 200 rpm. At higher and lower agitation speeds, shear stress and mass transfer limitation come into play. Liu et al. (2003) studied the effects of submerged cultivation parameters on chitinase
production by *Verticillium lecanii* and found that the highest enzyme production was attained at 150 rpm and pH 4.

**Nutritional parameters**
Carbon sources are important effectors for biomass and enzyme production. The chitinase production is generally inducible by chitin and catabolically repressed by glucose. Chitinase synthesis is regulated by products of chitin degradation through an inducer-repressor mechanism. High chitinase activity was found only in cultures supplied with chitin, but not with other polymers such as pectin, xylan and cellulose, which is indicative of induction (St Leger, 1986); a repressible constitutive enzyme should have appeared in high levels on polysaccharides which, especially in insoluble forms, may be insufficient to produce catabolite repression (Cooper, 1977). Vaidya et al. (2001), while studying the effect of different carbon sources on endochitinase production by *Alcaligenes xylosoxydans* and Souza et al. (2005) in *Colletotrichum gloesporioides*, found that glucose, lactose, glucosamine, xylose, and sucrose induced lower titres of the enzyme when compared to chitin. The low production levels on carbon sources other than chitin may be due to constitutive production, since this enzyme is involved in some stages of fungal development. Vaidya et al. (2001) observed a synergistic effect of arabinose and chitin on chitinase production in *Alcaligenes xylosoxydans*. Gupta et al. (1995) had also reported a similar effect in *Streptomyces viridificans* and suggested a possible relationship with the arabinose operon or its product in the induction of the chitinolytic system.

Chitinase synthesis was repressed by glucose and N-acetylglucosamine (NAG) in *Trichoderma harzianum* and by glucose in *Streptomyces thermoviolaceus*. A suppressing effect of glucose was also reported on chitinase production by *Streptomyces lividans* by Miyashita et al. (1991). Chitinase production by *Streptomyces lydicus* WYEC108 was induced by colloidal chitin, N-acetylglucosamine and diacetyl-chitooligosaccharides, and repressed by various pentoses, hexoses and high levels of glucose. Generally organisms possess a chitinolytic assembly with different chitinases responding to different
regulators, thus the effect of glucose and hydrolysis products such as NAG on chitinase synthesis is complex and varies with the organism.

Among all carbon sources studied for all organisms, colloidal chitin was found to be the best carbon source. A cell wall fragment was the best carbon source next to chitin for fungal chitinase production, while no reports are available on the use of cell wall fragments for bacterial chitinase production (Felse and Panda, 2000). Chitinase production by B. pabuli K1 was induced by chitin as well as by chito-oligosaccharides. The induction seemed to be most efficient with chitin as inducer. The explanation given for the lower activities obtained with (GlcNAc)₂, (GlcNAc)₃, and (GlcNAc)₄ was catabolite repression because of the initially high concentrations of soluble substrate. When the insoluble chitin is hydrolysed, chitooligosaccharides may be released so slowly that concentrations remain below catabolite repression threshold (Frandberg and Schnurer, 1994).

Monreal and Reese (1969) found that the production of chitinases from Serratia marcescens was repressed by GlcNAc. Chitinase production in Streptomyces lividans was induced by chitobiose and chitin but not by GlcNAc (Neugebauer et al. 1991). However, slow-feeding of M. anisopliae cultures with sugars or alanine (about 20 pg ml⁻¹ h⁻¹) in a carbon deficient medium to prevent catabolite repression demonstrated that the most effective inducer of chitinase was N-acetylglucosamine. Increasing the rate of release of N-acetylglucosamine decreased chitinase synthesis by about 87 % while causing a sevenfold increase in growth. Reducing group determination showed that GlcNAc, when supplied at 20 pg ml⁻¹ h⁻¹, never exceeded 30 pg ml⁻¹ in culture media. At the higher supply rate, however, GlcNAc accumulated in cultures to as much as 1 mg ml⁻¹. These values suggest that GlcNAc may cause catabolite repression of the chitinase when in excess of the immediate growth requirements of the organism (St Leger et al., 1986). Induction of chitinase synthesis is apparently not specific to GlcNAc as glucosamine also allowed production of the enzyme. This may be an adaptation by the fungus to the fact that chitin from natural sources appears to be partially deacetylated (Hackman & Goldberg, 1965, 1974).
Introduction

Polymers like starch, laminarin and β-glucan repress chitinase production in bacterial strains. It is suggested that other polymer hydrolysing enzymes, such as amylases and β-glucanases, were synthesized, which in turn produced glucose at concentrations high enough to cause catabolite repression (Frandberg and Schnurer, 1994). In fungi addition of pectin, laminarin, starch and glucan increase chitinase production (Felse and Panda, 2000). High concentrations of CMcellulose and pectin appeared to stimulate chitinase production in B. pabuli K1 and it appears that it could grow to a limited extent on these polymers, thereby producing more chitinase synthesizing cells without any repression of activity (Frandberg and Schnurer, 1994).

Nitrogen sources

As far as nitrogen sources are concerned organic and complex sources support more chitinase production than the inorganic and simple ones (Souza et. al., 2005). Vaidya et al., (2001) reported a significant increase in chitinase production by Alcaligenes xylosoxydans only when organic nitrogen sources such as yeast extract and peptone were used, the inorganic ones had either no or inhibitory effect on enzyme production. Organic nitrogen sources such as peptone and tryptone increased chitinase production, while inorganic N-sources such as sodium nitrate and ammonium nitrate reduced the enzyme yield in T. harzianum TUBF 966 (Sandhya et al. 2004). In wheat bran and colloidal chitin medium, yeast extract (1%) enhanced chitinase yield by 127.5%, while ammonium chloride adversely affected its yield (Suresh and Chandrasekaran, 1999).

Organic nitrogen sources do not necessarily increase chitinase production and yeast extract (Suresh and Chandrasekaran, 1998), peptone (Kapat et al., 1996), beef extract and malt extract (Huang et al., 1996) have also been reported to reduce the enzyme yield. Addition of urea into the medium was found to reduce chitinase yield by 39.7% for T. harzianum (Sandhya et al., 2004). Kapat et al. (1996) also observed that exclusion of urea form the medium increased chitinase production.

All microorganisms require certain mineral elements for growth and metabolism and some also require certain growth factors such as vitamins. In many media,
magnesium, phosphorus, potassium, sulphur, calcium and chlorine are essential and must be added as distinct components (Stanbury et al., 1997). Dipotassium hydrogen phosphate and magnesium sulphate are most commonly added mineral elements/salts in chitinase production media (Vaidya et al., 2003; Souza et al., 2005; Gohel et al., 2006; Sandhya et al., 2004). Other minor mineral elements such as cobalt, copper, iron, manganese, molybdenum and zinc are also essential and are either present in other major medium components such as yeast extract or can be added as trace elements (Felse and Panda, 2000; Kao et al., 2007).

**Solid state fermentation**

Solid state fermentation (SSF) is defined as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can, in addition, be used as carbon and energy source. The fermentation takes place in the absence or near absence of free water, thus being close to the natural environment to which microorganisms are adapted (Pandey et al. 2000; Holker et al., 2004). Direct comparison of various parameters such as growth rate, productivity or volume activity favoured solid state fermentation over submerged fermentation (SmF) in the majority of cases. The cost-factor for the production of “bulk-ware” enzymes in most cases also favours SSF over SmF (Tengerdy et al., 1996). Biological parameters, such as the stability of the produced enzymes at high temperature or extreme pH, have also been reported to be better in SSF (Deschamps and Huet 1985; Acuna-Arguelles et al. 1995). Catabolite repression or protein degradation by proteases, the severe problems in SmF, were often reduced or absent in SSF (Solis-Pereira et al. 1993; Aguilar et al. 2001). **Table 1.4** lists some advantages of SSF over SmF.

A large number of microorganisms, including bacteria, yeast and fungi produce different groups of enzymes. *Trichoderma* spp. and *Aspergillus* spp. have most widely been used for hydrolytic enzymes, e.g. chitinases, cellulases, xylanases. Besides *Beauveria* spp., *Penicillium* spp., *Verticillium lecanii* and *Fusarium oxysporum* are also reported to produce chitinase on solid substrates (**Table 1.5**). Among prokaryotes *Serratia marcescens*, *Enterobacter sp. NRG4* and *Oerskivia xanthineolytica* NCIM 2839 have been used for chitinase
production under solid state fermentation (Sudhakar and Nagarajan, 2010; Dahiya et al., 2005; Waghmare et al., 2011).

Table 1.4. Advantages of SSF over SmF

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Consequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological advantages</td>
<td></td>
</tr>
<tr>
<td>Low water demand</td>
<td>Less waste water</td>
</tr>
<tr>
<td>High concentration of end product</td>
<td>Lower downstream costs</td>
</tr>
<tr>
<td>Catabolite repression lower/missing</td>
<td>Fermentation in presence of glucose</td>
</tr>
<tr>
<td>Utilization of solid substrates</td>
<td>High concentration of growth substrates</td>
</tr>
<tr>
<td>Lower sterility demands</td>
<td>Mixed cultures of fermenting microorganisms</td>
</tr>
<tr>
<td>Solid support for microorganism</td>
<td>Better performance of cultivated microorganisms</td>
</tr>
<tr>
<td>Simulation of the natural environment</td>
<td>Synergism of metabolic performance</td>
</tr>
<tr>
<td>Fermentation of water-insoluble solid substrates</td>
<td></td>
</tr>
<tr>
<td>Mixed culture of microorganisms</td>
<td></td>
</tr>
<tr>
<td>Processing advantages</td>
<td></td>
</tr>
<tr>
<td>High-volume productivity</td>
<td>Smaller fermentor volumes</td>
</tr>
<tr>
<td>Low energy demand for heating</td>
<td>Cheap and abundant carbon sources</td>
</tr>
<tr>
<td>Easy aeration</td>
<td>No loss of microorganisms during fermentation</td>
</tr>
<tr>
<td>Utilisation of otherwise unusable carbon sources</td>
<td></td>
</tr>
<tr>
<td>No anti-foam chemicals</td>
<td></td>
</tr>
</tbody>
</table>

Factors affecting enzyme production in solid state fermentation systems

The major factors that affect microbial synthesis of enzymes in a SSF system include: selection of a suitable substrate and microorganism; pre-treatment of the substrate; particle size (inter-particle space and surface area) of the substrate; water content and $a_w$ of the substrate; relative humidity; type and size of the inoculum; control of temperature of fermenting matter/removal of metabolic heat; period of cultivation; maintenance of uniformity in the environment of SSF system, and the gaseous atmosphere, i.e. oxygen consumption rate and carbon dioxide evolution rate.
Table 1.5. Chitinase production in Solid state fermentation

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Chitinase production</th>
<th>Substrate</th>
<th>Temp.</th>
<th>Initial pH</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trichoderma harzianum</em></td>
<td>3.18 U/gds</td>
<td>Wheat bran moistened with 65.7% salt solution, 1% (w/w) colloidal chitin, 2% (w/w) yeast extract</td>
<td>30ºC</td>
<td>4.5</td>
<td>Nampoothiri et al. 2004</td>
</tr>
<tr>
<td><em>Enterobacter</em> sp.</td>
<td>1431 U/gss</td>
<td>wheat bran: flake chitin 1, moisture 80%, inoculum size 2.6 ml, incubation time 168 h.</td>
<td>30ºC</td>
<td>-</td>
<td>Dahiya et al. 2005</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td>23.6% U/gss</td>
<td>wheat bran and chitin (10:1)</td>
<td>30ºC</td>
<td>6</td>
<td>Gkargkas et al., 2004</td>
</tr>
<tr>
<td><em>Fusarium oxysporum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F3(NAGdase)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Penicillium chrysogenum</em></td>
<td>3809 &amp; 2516 U/gss</td>
<td>wheat bran &amp; 0.1% chitin</td>
<td>24ºC</td>
<td>5.0</td>
<td>Patidar et al. 2005a</td>
</tr>
<tr>
<td>PPCS1&amp;2</td>
<td></td>
<td></td>
<td></td>
<td>4.0 (PPC S1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.0 (PPC S2)</td>
<td></td>
</tr>
<tr>
<td><em>Beauveria bassiana</em></td>
<td>246.6 U/gIDS</td>
<td>Wheat bran, 1% (w/w) colloidal chitin, 75% aged sea water (5:5 w/v)</td>
<td>27ºC</td>
<td>9.2</td>
<td>Suresh and Chandra-sekaran, 1999</td>
</tr>
<tr>
<td>(marine isolate)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Beauveria felina</em> RD101</td>
<td>6.34 U/IDS per hr</td>
<td>Wheat bran and 100% MS-HCl</td>
<td>28</td>
<td>5</td>
<td>Patidar et al., 2005b</td>
</tr>
</tbody>
</table>

Agro-industrial residues are generally considered the best substrates for the SSF processes. A number of such substrates have been employed for the cultivation of microorganisms to produce host of enzymes e.g. sugar cane bagasse, wheat bran, rice bran, maize bran, wheat straw, rice straw, rice husk, corncobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, sugar beet pulp, sweet sorghum pulp, apple pomace, etc. The selection of a substrate for enzyme production in a SSF process depends
upon cost and availability of the substrate, and thus may involve screening of several agro-industrial residues. In chitinase production processes including different organisms wheat bran has most commonly been used (Table 1.5). Conversely, Sudhakar and Nagarajan (2010) have used rice bran and Barranco-Florido et al. (2002) used sugarcane pith bagasse for support in SSF.

It is crucial to provide optimized water content, and control the water activity \( (a_w) \) of the fermenting substrate, for the availability of water in lower or higher concentrations affects microbial activity adversely. Moreover, water has profound impact on the physico-chemical properties of the solids and this, in turn, affects the overall process productivity. Water activities below 0.9 do not support most bacterial growth, but yeast and fungi can grow at water activities of 0.7 and greater. Thus, the low moisture environment of many solid state fermentations favours yeast and fungi (Chisti et al., 1999). In some cases, the optimal water activities for growth and product formation differ (Prior et al., 1992). The optimal water activity depends also on factors such as agitation rate and cultivation temperature (Prior et al., 1992). Because the water activity depends on the concentration of dissolved solutes, sometimes salts, sugar, or other solutes are added to alter the water activity. Furthermore the fermentation process itself leads to changes in water activity as products are formed and the substrate is hydrolyzed. Oxidation of carbohydrates produces water (Chisti et al., 1999). During fermentation the water activity is controlled by aeration with humified air and sometimes with intermittent water spray. Aeration with water saturate air has commonly been found to increase the moisture content of the substrate. Relative humidity of the aeration gas is typically 60-80%.

The microbial biomass concentration in SSF is lower than seen in submerged culture (Tengerdy, 1985), but because there is little water, the heat generation per unit fermenting mass tends to be much greater in SSF. Temperature can rise rapidly because there is little water to absorb the heat (i.e. the mean specific capacity of the fermenting mass is much lower than that of water). Solid state fermentations are practiced without pH control (Lonsane, 1985) other than any adjustments made during substrate preparation. Other than that, the
buffering capacity of substrates is relied on to check large changes in pH during fermentation (Lonsane, 1985). Many substrates are effective buffers particularly true of protein-rich substrates.

Different types of fermenters (bioreactors) have been employed for various purposes in SSF systems. Laboratory studies are generally carried out in Erlenmeyer flasks, beakers, petri dishes, trays, jars and glass tubes (as column fermenter). The development of a simple and practical fermentor with automation is yet to be achieved for the SSF processes (Pandey, 1991).

In spite of all the above advantages, SSF is currently used only to a limited extent for enzyme and secondary metabolite production due to severe process engineering problems (Holker et al., 2004). The major problems are (a) the low amenability of the process to regulation (b) strongly heterogeneous fermentation conditions due to build up of gradients in temperature, pH, moisture, oxygen, substrate and inoculum (c) difficult to scale up mainly because of unavailability of suitable reactors (d) difficulty in determining biomass which is essential for kinetic studies and (e) complicated downstream processes for product purification resulting from the use of heterogeneous organic growth substrates (Holker and Lenz, 2005; Rahardjo et al., 2006; Singhana et al., 2009).

**Process optimisation**

When many variables control a process, the classical approach to optimize the outcome of the process is the ‘one-variable-at-a-time’ approach, that way any change could be linked to one different variable. It involves the study of behaviour of a system at several levels of one variable being studied, while maintaining rest variables at fixed levels. For each variable, the best value is found and then, the process is repeated for next variable, until all variables have been optimised. This approach is based on the assumption that effect of every variable is mutually independent and may be effective in some situations, but it is inefficient and takes too many experiments to optimize the process. Besides the influence of noise increases as the number of experiments are high.

So to investigate systems involving several factors in presence of variability and noise, a statistically designed set of experiments is required, in which all
pertinent factors are varied simultaneously and systematically in a single set of 10-20 experiments. These statistical experimental designs are more effective and widely used in many for process optimisation (Minocha et al., 2007; Kumar and Satyanarayana, 2007; Singh and Satyanarayana, 2008). An experimental design is a collection of predetermined settings of the process variables. Each process variable is called an experimental factor and each combination of setting for process variables represents a run. A response variable is a measure of process performance, and each value of the response variable is called an observation. Each experiment carefully explores the experimental space while studying many variables using a small number of observations. Statistical optimisation not only allows quick screening of a large experimental domain, but also reflects the role of each component. Using a mathematical model, the levels as well as the interactive effects of variables giving maximum response can be determined.

**Plackett-Burman design**

Plackett-Burman designs are experimental designs presented in 1946 by Robin L. Plackett and J.P. Burman. It is the most commonly used among screening designs that provides a simple model with information about dominating variables, and their ranges. This design is very useful for economically detecting large main effects, assuming all interactions are negligible when compared with the few important main effects.

According to the design, the number of variables to be studied is N-1, where N is the total number of experiments (multiple of four). When the number of variables is not N-1 where N is multiple of four, dummy or unassigned variables are included in the design, which help to calculate the statistical variation in the data. Each variable is studied at two concentrations, high (H) and low (L) values. The high and low values are chosen large enough to ensure that any effective concentration is included in the range. The high and low values for dummy variables are kept same, thus they must not have any effect of response/output. The effect of each variable or factor is the difference between the average of the measurements made at the high level of that factor and the average of the measurements made at the low level of that factor. If the
statistical variation were to be negligible the effect of dummy variables should be zero. Therefore the average effect of dummy variables is taken as a measure of the statistical error. An ‘F’ test is performed to compare the square of the effect of an individual variable with that of the dummy variable and found out the importance of the variable. The critical variables are selected on the basis of their F-values; higher F-values indicate greater influence of that variable on the product formation.

**Response surface methodology**

Once critical factors and the region of interest, where the factor’s level influence the response, is known, the next step is to determine the optimum combination of factors. This is achieved by response surface methodology. The method was introduced by G.E.P. Box and K.B. Wilson in 1951. The most popular response surface design is the central composite design. The design consists of 3 different sets of experimental runs:

1. Factorial points ($2^k$): The number of factors to be included in the design, each having two levels coded as +1 and -1.

2. The axial/star points (2k): The centre points are augmented with a group of ‘star points’ that allow estimation of curvature. A central composite design always contains twice as many star points as there are factors in the design. The star points represent new extreme values (low and high) for each factor in the design. These are coded as $+\alpha$ and $-\alpha$.

3. The centre points: Experimental runs whose values of each factor are the medians of the values used in the factorial portion. This point is often replicated in order to improve the precision of the experiment and coded as 0.

Here $k$ is the number of factors being studied. The response of individual runs is used to fit a model by least squares technique. Adequacy of the proposed model is then revealed using the diagnostic checking tests provided by analysis of variance (ANOVA). The response surface plots are employed to study the surfaces and locate the optimum. In several industrial processes, RSM is almost routinely used to evaluate the results and efficiency of the operations (Beg et al., 2003; Weska et al., 2007).
Many authors have reported increase in chitinase titres using Plackett-Burman screening design and response surface methodology. Vaidya et al. (2003) optimized medium components for chitinase production by *Alcaligenes xylosoxydans*. They found that chitin, yeast extract and tween 80 significantly affect chitinase production through PB screening and obtained 2.4 fold increase in chitinase production by optimizing their concentration in the medium by RSM. Nawani and Kapadnis (2004) used statistical experimental designs to optimize nutritional and process parameters for chitinase production by three strains of *Streptomyces*. The most significant factors identified by 2-level fractional factorial design were chitin, yeast extract, ammonium sulphate, trace elements, pH and temperature. Optimizing the values of these variables by RSM led to 9.3-29% increase in chitinase titres by the strains.

Effect of 19 different medium components on chitinase production by marine isolate *Pantoea dispersa* was studied by Plackett-Burman design and the variables chitin, peptone, yeast extract, urea, NH₄NO₃, NaCl, CaCl₂, KBr, MgSO₄.7H₂O, KNO₃ and KH₂PO₄ were found significant with confidence level at or above 95%. It was also observed that the 22nd medium gave highest chitinase production among the 24 experimental runs. Comparison of chitinase activity in this and the basal medium showed 3.95-fold and 2.31-fold higher endochitinase and chitobiase production respectively (Gohel et al., 2006).

**PURIFICATION**

A necessary prerequisite to initiate purification is to have protein in sufficiently high concentration. Different methods such as, precipitation using salts such as ammonium sulphate (Teotia et al., 2004; Guo et al., 2004), or organic solvents such as acetone (Kragh et al., 1991; Muskazli et al., 2006), lyophilisation (Watanabe et al., 1990), ultrafiltration etc. have been used for concentrating crude chitinase preparations. The most commonly used methods for purifying chitinase involves ion exchange chromatography and gel filtration (Kragh et al., 1991). A list of some chitinases purified from different microbes is presented in *table 1.6*.
## Table 1.6. Characterization of chitinases from different micro-organisms

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces erythraeus</em></td>
<td>-</td>
<td>5</td>
<td>3.7</td>
<td>30</td>
<td>-</td>
<td>-</td>
<td>Hara et al., 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mn2+, Mg2+, Zn2+, glutathione, dithiothreitol, and 2-mercapto-ethanol.</td>
<td></td>
</tr>
<tr>
<td><em>Aeromonas schbertii</em></td>
<td>-</td>
<td>4.8</td>
<td>-</td>
<td>75</td>
<td>-</td>
<td>-</td>
<td>Guo et al., 2004</td>
</tr>
<tr>
<td><em>Aeromonas hydrophila subsp. anaerogens A52</em></td>
<td>-</td>
<td>7</td>
<td>4.6</td>
<td>110</td>
<td>45</td>
<td>-</td>
<td>Yabuki et al., 1986</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Monoidoacetate, N-ethylmaleimide, Hg2+ and As3+</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cu2+, Fe2+, Hg2+ Zn2+, &amp; Fe2+</td>
<td></td>
</tr>
<tr>
<td><em>Clostridium E-16</em></td>
<td>-</td>
<td>5-7</td>
<td>77 &amp; 98</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Konagaya et al., 2006</td>
</tr>
<tr>
<td><em>Burkholderia cepacia</em> strain KH2</td>
<td>Endo</td>
<td>4.5</td>
<td>5.9</td>
<td>34</td>
<td>50ºC</td>
<td>-</td>
<td>Ogawa et al., 2002</td>
</tr>
<tr>
<td><em>Alcaligenes xylooxydans</em></td>
<td>-</td>
<td>5</td>
<td>-</td>
<td>45</td>
<td>50ºC</td>
<td>Cu2, Na2</td>
<td>Vadiya et al., 2003</td>
</tr>
<tr>
<td><em>Streptomyces helstedii AJ-7</em></td>
<td>-</td>
<td>7</td>
<td>-</td>
<td>55</td>
<td>50ºC</td>
<td>Co2+</td>
<td>Joo, 2005</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Hg2+, Ni2, Pb2+</td>
<td></td>
</tr>
<tr>
<td><em>YHS A2</em></td>
<td>Exo</td>
<td>-</td>
<td>67</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Lee et al., 2000</td>
</tr>
<tr>
<td>Organism</td>
<td>Type</td>
<td>pH</td>
<td>T</td>
<td>Temperature</td>
<td>Inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------</td>
<td>-----</td>
<td>---</td>
<td>-------------</td>
<td>---------------------------------------------------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces thermo-viaceus</em> OPC-520</td>
<td>Endo</td>
<td>8-10</td>
<td>3.8</td>
<td>40</td>
<td>70-80°C, Cu2+, Hg2+, Cd2+, Sn2+, N-Bromo-succinimide, 2-hydroxy-5-nitrobenzyl bromide, woodwards reagent K</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces griseus</em></td>
<td>-</td>
<td>5-7</td>
<td>-</td>
<td>31</td>
<td>45-50°C, -</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces sp. NK1057</em></td>
<td>Endo</td>
<td>4</td>
<td>-</td>
<td>48</td>
<td>60, Hg2+, NAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Streptomyces sp. NK1057</em></td>
<td>Exo</td>
<td>6</td>
<td>-</td>
<td>35</td>
<td>40, NAG, Hg2+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus sp. DAU101</em></td>
<td>Bifunctional Endo, exo</td>
<td>-</td>
<td>7.5</td>
<td>66</td>
<td>60°C, Mg2+, Ni2+, Co2+, Zn2+, Cu2+ and Hg2+</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus sp. 13.26</em></td>
<td>-</td>
<td>7-8</td>
<td>-</td>
<td>60</td>
<td>60°C, Co2+, -</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Rhodothermus marinus</em></td>
<td>Endo, exo</td>
<td>4.5-5</td>
<td>-</td>
<td>42</td>
<td>70°C, -</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Cellulomonas uda</em></td>
<td>Endo</td>
<td>-</td>
<td>8.5</td>
<td>70</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Vibrio alginolyticus</em> 283</td>
<td>-</td>
<td>6.5</td>
<td>-</td>
<td>65</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus sp. BG 11</em></td>
<td>-</td>
<td>7.5-9</td>
<td>-</td>
<td>-</td>
<td>45-55°C, Ca2+, Ni2+ tritonX100, Ag2+, Hg2+, dithiothreitol, b-mercaptoethanol, glutathione, iodoacetic acid and iodoacet-amide</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus pumilus</em> SG2 (Halotolerant)</td>
<td>Exo</td>
<td>4.5</td>
<td>-</td>
<td>63</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus amylo-liquifeciens</em> V656</td>
<td>7</td>
<td>5.8</td>
<td>14</td>
<td>40°C</td>
<td>Zn2+, Cu2+, Hg2+, acetone</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> W-118</td>
<td>Endo</td>
<td>6</td>
<td>6</td>
<td>20.6</td>
<td>37°C, Hg2+ and acetone</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Introduction

**Serratia marcescens NK1**
- 6.2
- 47°C
- 2001
  Nawani and Kapadnis,

**Microbiosa sp. V2**
- 3
35
60°C
Hg2+
  Nawani et al., 2002

**Gliocladium virens**
Endo
4-6
7.8
41
-  
-  
  Pietro et al., 1993

**Piromyces communis**

**OTS1 (anaerobic)**
Endo
6 (at 39°C)
4.4
44.8
-  
Hg2+, allosamidin
  Sakurada et al. 1998

### Thermophilic bacteria

**Bacillus sp. HSA, 3-1a**
- 7
22-79
60
Mg2+, Ca2+, Co2+, Fe2+, Zn2+
Natsir et al., 2010

**Bacillus sp. Hu1**
- 6.5
60
Mg2+, Ca2+, Cu2+, DTT, EDTA
Dai et al., 2011

**Oerskovia xanthineolytica**
- C1
7.5
50
Mn2+, Cu2+, Hg2+
Waghmare and Ghosh, 2010

NCIM 2839
C2
8
55

**Bacillus MH-1**
endo
6.5
5.3
5.3
71
75
Mn2+, Ca2+
Ag, Hg, (NAG)2
Sakai et al., 1998

**Ralstonia sp. A471**
ChiA Endo
5
4.7
70
70°C
Cu2+, Mn2+, Ca2+, Mg2+
Sutrisno et al., 2004

ChiB
5
45
60
-
-
Udea et al., 2005

ChiC
-
-
55
Co
Hg
Udea et al., 2009

### Hyperthermophilic bacteria and archea

**Pyrococcus furiosus**
Endo (ChiA)
6
40
90-95
-  
-  
  Gao et al., 2003
Exo (ChiB)
6
55
90-95
-  
-  

**Thermococcus chitino-phagus**
Eno (Chi70)
7
70
70
-  
-  
  Andronopoulou and
Exo (Chi50)
6
50
80
-  
-  
  Vorgias, 2004

**T. kodakaraensis**
Exo
5
134
85
-  
-  
  Tanaka et al., 1999
**Fungi**

*Monascus purpureus*

| CCRC31499 (yeast) | 7 | 5.4 | 81 | 40ºC | Fe2+ | Hg2+ | Wang et al., 2002 |

*Verticillium chlamydosporium*

| endo | 5.2-5.7 | 7.9 | 43 | - | - | Tikhonov et al., 2002 |

*V. suchlasporium*

| endo | 5.2-5.7 | 7.6 | 43 | - | - | Tikhonov et al., 2002 |

*Talaromyces flavus*

| 4/5 | - | 41/32 | 40 | - | - | Duo-Chuan et al., 2005 |

*Trichotheicum roseum*

| 5 | - | 39 | 40 | - | - | Duo-Chuan et al., 2004 |

*Rhizopus oligosporus*

| both | 4/3.5 | - | 50/52 | - | - | Yanai et al., 1992 |

*Metarhizium anisopliae*

| both | 5 | - | 60 | - | - | Kang et al., 1999 |

*Neorospora crassa*

| both | 6.7 | - | 20.6 | - | - | Zarain-herzberg et al., 1983 |

*Plectospharella sp.*

| - | 3-4 | - | 67 | 37 | - | Velmurugan et al., 2011 |

*MF-1 (low temp active)*

| Kluveromyces lactis | - | 4.5 | - | 85 | - | - | Colussi et al., 2005 |

*Aphanocladium album*

| (hyperparasitic) | - | 4 | 6.8 | 39 | 50 | - | Kunz et al., 1992 |

*Monascus purpureus*

| - | 7 | 5.4 | 81 | 40ºC | Fe2+ | Hg2+ | Wang et al., 2002 |

*Orpinomyces (anaerobic)*

| endo | 6.5 | - | - | 50 | Mg2+, Fe2+ | Hg2+, Mn2+, SDS | Novotna et al., 2008 |

*Anaeromyces (anaerobic)*

| endo | 6.5 | - | - | 40 | Fe2+ | Hg2+, Mn2+, SDS | Novotna et al., 2008 |

**Thermophilic fungi**

*Talaromyces emersonii*

| - | 5-5.5 | - | - | 65 | - | - | McCormack et al., 1991 |

*Thermomyces lanuginosus*

| - | 4.5 | - | 48 | 55 | Ca2+, Ba2+ | Fe2+, Ag2+, Hg2+ | Guo et al., 2005 |

| Na+ and K+ | Cu2+ and EDTA | Guo et al., 2008 |

*Thermoascus aurantiacus* |

| both | 8 | - | 48.4 | 50 | - | - | Li et al., 2010 |

*Chaetomium thermophilum* |

| both | 5.5 | - | 47.3 | 60 | - | - | Li et al., 2010 |

*Paecilomyces thermophila* |

| endo | 4.5 | - | - | 50 | - | Hg2+, Mn2+, SDS | Kopparapu et al., 2011 |

* recombinant proteins
Introduction

Frequently affinity chromatography using various chitinous substrates has also been employed. Macroporous beaded chitin cross linked with 2,3-epoxypropyl chloride was used as an affinity matrix for column chromatography for chitinase purification from *Streptomyces kurssanovii* (Tikonov et al., 1998). Macroporous cross-linked chitin column was used to purify chitinase from the nematophagous fungi *Verticillium chlamydosporium* and *V. suchlasporium*. The column could separate different chitinases on the basis of their affinity to chitin (Tikonov et al., 2002). Teotia et al. (2004) purified chitinases to homogeneity from *Neurospora crassa*, cabbage and puffballs by affinity precipitation with chitosan and obtained a fold purification of 27, 15 and 30 respectively. Acid swollen colloidal chitin was used for affinity purification of chitinase from *Vibrio carhariae* (Sunginta et al., 2000). An affinity matrix consisting of N,N',N"-triacetylchitotriose immobilized on agarose beads has been used for chitinase purification from *Medicago sativa* (Minic et al., 1998).

In addition, a rapid process for purification of chitinase by an aqueous two-phase extraction (ATPS) and salt fractionation from the latex of *Carica papaya* has been reported (Nitsawang and Khanasawud, 2006). The whole latex, including the insoluble material, was extracted by 5% PEG-12% ammonium sulphate. Chitinase was purified from the salt-rich bottom phase by precipitation with 11% ammonium sulfate and 20% sodium chloride, respectively. 2.4 mg enzyme/g of wet latex and 4.2 folds of purification were obtained. In a modified ATPS procedure, incorporation of macroaffinity ligand like chitosan resulted in drastic increase in partitioning of chitinase (from three different sources, *N. crassa*, cabbage and puffball) in PEG phase. A 34-fold purification and 86% recovery could be obtained in case of *N. crassa*. Similarly, cabbage enzyme was purified 20-fold with 80% activity recovery and puffball enzyme could be purified 38-folds with 88% recovery of the chitinase activity (Teotia et al., 2004).

MECHANISM OF ACTION

There are two general mechanistic pathways for acid catalyzed glycosyl hydrolysis that results in the following:
1. Retention of the stereochemistry of the anomeric oxygen at C-1 relative to the initial configuration

The catalytic mechanism of glycosyl hydrolases is discussed with reference to the retaining mechanism of hen egg white lysozyme. It involves two catalytic residues and proceeds through a geometrically distorted oxocarbonium intermediate (Fig. 1.3). The β-1,4 glycosidic bond between sugars is broken involving the protonation of the leaving group alcohol leading to an oxocarbenium ion intermediate, which is then stabilized by a second carboxylate (either through covalent or electrostatic interactions). A nucleophilic attack by water which replaces the leaving sugar group yields the hydrolysis products, which necessarily retains the initial anomeric configuration. This is commonly referred to as the double displacement mechanism of hydrolysis. It has been suggested, based on site directed mutagenesis, that Glu 204 and Asp 200 may be the catalytic residues for the chitinase from Bacillus circulans (Watanabe et al., 1993).

Family 18 chitinases are reported to yield hydrolysis products that retain the anomeric configuration at C-1. However, chitinases, chitobiases, and related enzymes from glycosyl hydrolase families 18 and 20, which act also with retention of configuration, have only a single glutamic acid in their active sites but no aspartate (Tews et al., 1997). Their x-ray crystal structure reveals the absence of a second acidic residue in the active site, which is capable of stabilizing the oxocarbenium ion. Stabilization of the oxocarbonium ion is a fundamental prerequisite of the mechanism since oxocarbonium ions have extremely short lifetime in solution. It has been reported that the retaining mechanism of these chitinases involve substrate assistance (Tews et al. 1997). That is, the N-acetyl group at position 2 of the scissile sugar may itself facilitate the reaction via formation of a transient oxazolium intermediate (Terwisscha et al., 1995, Drouillard et al., 1997).

2. Inversion of the stereochemistry

Although the x-ray crystal structure of family 19 chitinase is similar to that of hen egg white lysozyme, especially in the substrate binding and catalytic core suggesting a similar double-displacement mechanism, the hydrolysis products
for two family 19 chitinases show inversion of anomeric configuration. This
inverting mechanism was confirmed in barley chitinase using nuclear magnetic
resonance (NMR) to follow the anomeric hand of the product sugars which were
α. The difference in catalytic mechanism between hen egg white lysozyme and
barley chitinase is ascribed to the distance between the two catalytic residues.
The longer separation between the catalytic residues seems to be a structural
feature characteristic of family 19 chitinase (Fukamizo, 2000). The reaction of
inverting glycosyl hydrolases which have two largely separated catalytic
residues is often explained by a single displacement mechanism in which a
bound water molecule acts as the nucleophile (Fig. 1.3). The location of the
water molecule would lead to a nucleophilic attack to C1 from the α-side.
The crystal structure suggested that the second catalytic carboxylate may be
sufficiently close to allow coordination of a water molecule consistent with a
single-displacement mechanism (Brameld and Goddard 1998a,b). The
hydrolytic profile for hexasaccharides by this chitinase suggests preferred
binding of substrates at sites B-G (Hollis et al., 1997). Hydrolysis would occur
between sugars in sites D and E, i.e., hexasaccharides are cleaved into two
trisaccharides. Two carboxylates were hypothesized to be responsible for the
catalysis, Glu 67 as the catalytic acid and Glu 89 as a base (Blake et al., 1967;
Kelly et al., 1979). The importance of these two residues in catalysis has since
been confirmed by site-directed mutagenesis (Andersen et al., 1997).

![Double-Displacement Retaining Mechanism](image)

![Single-Displacement Inverting Mechanism](image)

**Fig. 1.3.** The hydrolysis mechanism of chitinases (a) double-displacement mechanism
proposed for family 18 chitinases and (b) single-displacement mechanism
proposed for family 19 chitinases.
MULTIPLE FORMS OF CHITINASES

Most chitinolytic microorganisms have been found to produce more than one kind of chitinase. *Trichoderma harzianum* produce seven individual chitinases: two N-acetyl-glucosaminidases (102 and 73 kDa), four endochitinases (52, 42, 33 and 31 kDa) and one chitobiosidase (40 kDa) [Haran et al., 1995; Harman et al., 1993; De La Cruz et al., 1992]. *Talaromyces flavus* produces at least two kinds of chitinases (Li et al., 2005). The mycoparasite *Stachybotrys elegans* produces two exochitinases and one endochitinase (Taylor et al., 2002). The entomopathogenic fungus *Metarhizium anisopliae* produces at least six different chitinases (Pinto et al., 1997; St. Leger et al., 1996; Kang et al., 1999; St. Leger et al., 1991). Chitinolytic system of *Bacillus circulans* WL-12 is one of the most extensively studied systems so far in prokaryotes. Six chitinases have been detected in the culture supernatant of this bacterium grown in the presence of chitin (Watanabe et al., 1990; Watanabe et al., 1992).

Research finds that these multiple chitinases have a mutually synergistic and complementary effect between them. For example, chitinases CHIT33, CHIT37, and CHIT42 of *T. harzianum* show the synergistic action on cell wall degradation (De La Cruz et al., 1992). Mixing the activities of the he endochitinase and chitobiosidase from *T. harzianum* results in the synergistic increase in antifungal activity (Lorito et al., 1993). A similar phenomenon has been reported in plant chitinases (Jach et al., 1995) and bacterial chitinases (Suzuki et al., 2002).

The chitinolytic machinery of *Serratia marcescens* comprises three chitinases ChiA, ChiB and ChiC which preferentially produce dimers, a chitin binding protein CBP21 (Kolstad et al., 2005) and a hexosaminidase which further degrades the major end product of chitinases i.e. NAG dimer. ChiA and ChiB are processive exochitinases which degrade chitin chains in opposite directions, while ChiC is a nonprocessive endochitinase. Chitinases often contain one or more non-catalytic domains called as carbohydrate binding modules (CBMs) that help in binding and degrading the insoluble substrate. CBP21 is a CBM that binds to β-chitin.
**SUBFAMILIES OF MICROBIAL CHITINASES**

Based on chitinase structure and amino acid sequence, chitinases are separated into different subfamilies, classes, or groups. Bacterial chitinases are clearly separated into three major subfamilies, A, B, and C (Watanabe et al., 1993). Plant chitinases are divided in five different classes. Class I and Class II chitinases are similar in their catalytic domains. Class I chitinases have a chitin-binding domain. This domain is separated from the catalytic domain by a hinge region. Class II chitinases lack both the chitin-binding domain and the hinge region. Class III chitinases have higher homology to fungal chitinases than to other plant chitinase classes. Class IV chitinases are similar to Class I chitinases but they are smaller in size due to certain deletions. Class V chitinases show some homology with bacterial exochitinases (Neuhaus et al., 1996).

Compared with bacterial chitinases and plant chitinases, fungal chitinases are not well classified. Within family 18 two distinct classes of fungal chitinase may be identified based on the similarity of enzymes to family 18 chitinases from plants or bacteria (Pishko et al., 1995; Takaya et al., 1998). Therefore, fungal chitinases are divided into fungal/bacterial (corresponding to Class V) chitinases, similar to chitinases found in bacteria, and fungal/plant (corresponding to Class III) chitinases, which are similar to chitinases from plants (Adams, 2004; Takaya et al., 1998; Jaques et al., 2003). Recently, according to phylogenetic analysis, it has been suggested that fungal chitinases of family 18 can be divided into three groups: groups A and group B (corresponding to class V and III of plant chitinases, respectively), whereas a novel group C comprises high molecular weight chitinases that have a domain structure similar to *Kluyveromyces lactis* killer toxins (Seidl et al., 2005).

![Fig. 1.4](image_url) Comparison of domains of the ChiB1 fungal/bacterial chitinase (433 aa) and ChiA1 fungal/plant chitinase (825 aa) of *A. fumigatus*. 1, catalytic domain; 2, Serine/threonine rich domain.
STRUCTURE OF FUNGAL CHITINASES

Most fungal chitinases belong to family 18 of the glycosylhydrolase superfamily (Henrissat, 1999). A characteristic of the family 18 chitinases is their multi-domain structure. Typically, the basic structures of fungal chitinase of family 18 are composed of five domains or regions: N-terminal signal peptide region, catalytic domain, serine/theronine-rich region, chitin-binding domain, and C-terminal extension region. Two chitinases *Rhizopus oligosporus* (chitinase I and chitinase II) have all the five distinct domains listed above (Yanai et al., 1992). However, most fungal chitinases lack the last three domains, which do not seem to be necessary for chitinase activity because naturally occurring chitinases that lack these regions are still enzymatically active. The derived amino-acid sequence of a *Saccharomyces cerevisiae* endochitinase, CTS1, shows four distinct domains: a signal sequence, a catalytic domain, a serine/theronine rich region and chitin-binding domain (Kuranda and Robbins, 1991). *T. harzianum* 33 kDa chitinase includes a putative signal peptide region and a catalytic domain, but lacks the other three domains (Limon et al., 1995) [Figure 1.5].

![Fig. 1.5](image)

*Fig. 1.5.* The structure of fungal chitinase of family 18. (a) *S. cerevisiae* endochitinase (CTS1); (b) *R. oligosporus* chitinase (CHI1); (c) *T. harzianum* chitinase (CHIT33); (1) signal peptide; (2) catalytic domain; (3) serine/theronine-rich region; (4) chitin-binding domain; (5) C-terminal extension region (Duo-Chuan, 2006).

The biochemical functions of these domains are as suggested by their names. A signal peptide is predicted to precede the N-terminal region of many mature chitinases. The signal peptide presumably mediates secretion of the enzyme and it is cleaved off by signal peptidases after the protein has been transported across the membrane. The chitinases lacking secretory signal sequence are shown to be intracellular chitinase, and they may function during morphogenesis (Takaya et al., 1998; Seidl et al., 2005). The catalytic domain of fungal chitinases, responsible for the hydrolysis of the substrate, comprises the N-terminal half of the enzyme. Sequence alignments reveal two highly
conserved regions within the catalytic domain. The two consensus regions or motifs, SxGG and DxxDxDxE, correspond to a substrate-binding site and a catalytic domain, respectively, in family 18 chitinases (Henrissat, 1991).

It is proposed that two conserved carboxylic acids are important in the retaining, substrate assisted catalytic mechanism of family 18 hydrolases (Perrakis et al., 2004). One is supposed to act as a general acid to protonate the leaving group and glutamate serves this role in a wide range of glycohydrolases (Monzingo et al., 1996). A second carboxylic acid, generally aspartate in chitinases either assists the catalytic glutamate or acts as base to stabilize the transition state (Papanikolau et al., 2001; Bortone et al., 2002).

Apart from N-terminal signal peptide region and catalytic domain, the structure of some fungal chitinases reported has serine/theronine-rich region and chitin-binding domain (ChBD), such as *Saccharomyces cerevisiae* endochitinase, CTS1 (Kuranda and Robbins, 1991), *Kluyveromyces lactis* endochitinase, KICts1p (Colussi et al., 2005), *Trichoderma reesei* Chi18-13 and Chi18-16 and *Rhizopus ologosporus* chitinase I and Chitinase II (Yanai et al., 1992). The serine/theronine-rich region of fungal chitinases is usually glycosylated with sugar chains post-translationally to yield the mature protein. The glycosylation sites may be necessary for the secretion of the protein and maintenance of its stability.

Fungal chitinases are thought to be anchored to the cell wall or their substrate through the chitin-binding domain (Kuranda and Robbins, 1991). A conserved six-cysteine motif, which likely mediates the tertiary structure or protein–protein interaction through the formation of disulfide bridges, is found in the chitin-binding domain of CTS1 and KICts1p (Kuranda and Robbins, 1991; Colussi et al., 2005). The chitin-binding domain of *Trichoderma reesei* Chi18-13 and Chi18-16 has high similarity with a fungal cellulose-binding domain (CBD), consisting of four strictly conserved aromatic amino acid residues that are implicated in the interaction with cellulose (Seidl et al., 2005; Kraulis et al., 1989). The chitin-binding domain differs from the substrate-binding site in the catalytic domain (Henrissat, 1999). The chitin-binding domain of *Kluyveromyces lactis* KICts1p functions independent of the catalytic domain (Colussi et al.,
It has been demonstrated that the presence of the chitin-binding domain in *Saccharomyces cerevisiae* CTS1 does not increase the rate of chitin hydrolysis (Kuranda and Robbins, 1991), but addition of ChBD from *Nicotiana tabacum* ChiA chitinase to *Trichoderma harzianum* chitinase Chit42, which lacks a ChBD, increases chitin-binding capacity of the native Chit42 (Limon et al., 2001).

It is not clear how the C-terminal region of fungal chitinase functions. Interestingly, it has been found using the DGPI facility at the SIB website that at the C-terminus of a *Candida albicans* chitinase, CHT2, and two *Aspergillus fumigates* chitinases, ChiA1 and ChiA, there are putative glycosyl-phosphatidylinositol (GPI)-anchor and cleavage sites. By a GPI anchor, CHT2 and ChiA1 are likely to be anchored to the cell wall or cell membrane, and to perform roles during growth and morphogenesis (Takaya et al., 1998; Jaques et al., 2003; Iranzo et al., 2002).

Studies on fungal β-N-acetyl-glucosaminidases, belonging to family 20 of the glycohydrolase superfamily, have been done only recently. Genes encoding β-N-acetyl-glucosaminidases have been cloned from several fungi, such as *T. harzianum*, *C. albicans*, and *A. nidulans* (Cannon et al., 1994; Peterbauer et al., 1996; Kim et al., 2002). Molecular cloning and sequencing of fungal β-N-acetyl-glucosaminidases reveals that fungal β-N-acetyl-glucosaminidase structure is composed of signal peptide, propeptide, zincin-like domain, catalytic domain, and C-terminal segment (Plihal et al., 2004).

There are only a few reports on chitinases from thermophilic fungi (Li et al., 2010; Guo and Li, 2006). The catalytic domains of chitinases from *Thermoascus aurantiacus* var. *levisporus* and *Chaetomium thermophilum* are similar to those of other fungal chitinases in family 18 of glycosyl hydrolases.
Alignment of genes encoding these two proteins and the most similar other fungal chitinases revealed that these chitinases share the LSIGGWT and DXXDXDXE motifs that are considered to be substrate-binding site and a chitin-catalyzing domain, respectively (Watanabe et al., 1993; Hollis et al., 2000; Li, 2006; Li et al., 2010). There was no evidence for the presence of a signal peptide in the putative amino acid sequences of TaCHIT1 and CtCHIT1. The chitinase gene from another thermophilic fungus, Paecilomyces thermophila (PtChiA) does not have a potential signal peptide but consists of a potential N-glycosylation site, N-X-S/T (Kopparapu et al., 2011).

Comparison with related fungal chitinase sequences from GenBank database identified by Blastp searches showed that TaCHIT1, CtCHIT1 and PtChiA were composed of a single catalytic domain, no other domains (chitinbinding domain and Ser/Thr rich domain) were found, similar to 33 kDa chitinase from T. harzianum and 48 kDa chitinase from thermophilic fungus T. lanuginosus (Limon et al., 1995; Guo and Li, 2006).

CRYSTAL STRUCTURE

The three-dimensional models of a Coccidioides immitis chitinase, CiX1, and an Aspergillus fumigatus chitinase, ChiB1, were among the first ones to be reported (Duo-Chuan, 2006). The active site of CiX1 is composed of conserved amino acid residues (W47,131,315,378, Y239,293, R52,295). The amino acid residue E171, by analogy to other class 18 glycohydrolases, is thought to be the catalytic amino acid, as site-directed mutagenesis of E171 eliminates any detectable enzyme activity (Hollis et al., 2000). The structure model reveals that the chitinase is an eight-stranded beta/alpha-barrel (Fig. 1.7). The eight parallel beta strands form the barrel’s core, which is surrounded by eight alpha-helices connected to the barrel. It is suggested that the barrel structure of chitinases forms a groove on the enzyme’s surface. This groove is considered as the active center, which binds sugar units of chitin, possibly (Glc-NAc)_6 moieties, which are subsequently cleaved (Drouillard et al., 1997).

Recently, the structure of chitinase CrChi1 from a nematophagous fungus, Clonostachys rosea was elucidated (Fig. 1.7). It consists of an additional α/β domain, composed of five antiparallel β-strands and two α-helices, inserted in
the loop between strand β9 and helix α8, and this domain might give the active site a groove character. Like all other Family 18 chitinases, *Clonostachys rosea* chitinase has the DXDXE motif at the end of strand β5, with Glu174 as the catalytic residue in the middle of the open end of the (β/α)8 barrel (Yang et al., 2010). While the chitinases from different organisms shared low sequence similarity, their main differences were restricted to the N- and C-terminal domains. The structures were conserved especially for the amino acid residues corresponding to the substrate-binding domain and the catalytic domain suggesting that these chitinases probably share a common catalytic mechanism (Yang et al., 2010).

![Crystal structures of chitinases of (a) Aspergillus fumigatus and (b) Clonostachys rosea.](image)

**Fig. 1.7.** Crystal structures of chitinases of (a) *Aspergillus fumigatus* and (b) *Clonostachys rosea*.

**PROTEIN ENGINEERING OF CHITINASES**

The sizes and three-dimensional conformations of proteins can be engineered to obtain better catalytic performance and operational stabilities. Such manipulations are frequently used to discover structure-function relationships, as well as to alter the activity, stability, localization, and structure of proteins. Many subtle variations in a particular protein can be generated by making amino acid replacements at specific positions in the polypeptide sequence to generate a mutant protein that may have different characteristics by virtue of the single replaced amino acid. For example, the kinetic stability of chitinase B from *Serratia marcescens* was increased by introduction of rigidifying mutation of the Gly-Ala and Xxx-Pro type. A double mutant containing two stabilizing mutations (G188A, A234P) displayed a 10 fold increase in half life at 57°C and a 4.2°C increase in apparent Tm (Gaseidnes et al., 2003).
Catalytic potential of proteins can be also altered through substitution of amino acids. Martinez et al. (2012) have reported that the mutagenesis of two GH-18 glycoside hydrolase, *B. circulans* WL-12 chitinase A1 (Bc ChiA1) and *Trichoderma harzanium* chitinase 42 (Th Chit42), and usage of *E. coli* produced building blocks to generate long chitooligosaccharides. The conserved catalytic residues of Bc ChiA1 and Th Chit42 were mutated individually, and each mutant was screened for its transglycosylation behaviour. The mutants D200A and D202A of Bc ChiA1, together with D170N and to a lesser extent D170A of Th Chit42 proved to be active for chitobiose oxazoline polymerization. These mutants have additionally retained the ability to catalyze transglycosylation reaction on natural COs, whereas their hydrolytic activity is abolished. Thus the generation of complex mixtures of various reaction products obtained due to hydrolytic activity of the enzymes and uncontrollable random transglycosylation reactions by wild type chitinases could be avoided (Martinez et al., 2012).

In addition to the substitution of amino acids at specific positions, amino acids can be deleted from the sequence, either individually or in groups. Such deletion mutants have been a useful way to create smaller proteins that contain only one or a few of these domains so that the individual properties can be studied. A chitinase from *Pyrococcus kodakarensis*, ChiA has two catalytic domains and deletion mutants having either of the two showed that these two domains are functional independently as thermostable chitinases. It was also observed that the C-terminal half of ChiA was much more thermostable than the N-terminal half. The removal of the N-terminal region might induce a more thermostable conformation (Tanaka et al., 1999).

Deletion of 304 C-terminal amino acid residues from *Aeromonas caviae* chitinase ChiA, yielded a mutant with lower optimum temperature and higher relative activity on chitin, colloidal chitin and 4-methylumbelliferyl-N-N'-N"-triacetylchitotriose (Lin et al., 2001). Removal of C-terminal regions in chitinases from *Bacillus licheniformis* (145 amino acid residues containing putative chitin binding and fibronectin domain) and *Vibrio parahaemolyticus* (30 aa residues) led to higher thermostabilities and decreased catalytic activity towards insoluble chitin substrates in both mutated enzymes. The activities towards insoluble
substrates was unaffected by truncation in both cases (Chuang and Lin, 2007; Chuang et al., 2008). The knowledge of the function of different domains and their contribution to the overall activity and stability of whole proteins can help increasing the suitability of the existing repertoire of proteins for specific applications.

Another aspect of protein engineering is joining or fusion of different sequences resulting in a hybrid, fusion, or chimeric protein, which generally has characteristics that combine those of each of the joined partners. The most common application for fusion proteins is to facilitate purification by affinity chromatography. A six histidine tag can be used to purify a protein by affinity to nickel (Lin et al., 2001; Chuang and Lin, 2007).

Another application for fusion protein can be augmenting the role of a protein for a particular application. \textit{B. bassiana} transformants expressing a hybrid enzyme (containing a chitinase and a protease of \textit{B. bassiana}) showed greater virulence when compared to the wild type (WT) or to transformants overexpressing either protease or chitinase. The expression of this hybrid enzyme reduced LC50 by 67.4\%, compared to the wild type. This is greater than the additive effects of transformants overexpressing protease and chitinase (Fang et al., 2009).

**APPLICATIONS**

The potential of chitinases for increasing the production of several useful products in the most economic way is immense. The major applications of chitinases are discussed in the following subsections.

**Biocontrol**

Biological control tactics have become an important approach to facilitate sustainable agriculture. The use of microorganisms or their secretions to prevent plant pathogens and insect pests offers an attractive alternative or supplement for the control of plant diseases. (Wang et al. 2002; Neeraja et al., 2010). Several microbial chitinases have shown antagonizing activities against plant pathogenic fungi. Mahadevan and Crawford (1997) reported the antagonistic action of \textit{Streptomyces lydicus} WXEC108 against \textit{Pythium ultimum} and \textit{Rhizoctonia solani}, which cause disease in cotton and pea. A \textit{Fusarium}
chlamydosporum strain, a mycoparasite of groundnut rust (*Puccinia arachidis*), produces endochitinase that inhibits germination of uredospores of rust fungus (Mathivanan et al. 1998). Govindsamy et al. (1998) reported the use of purified preparation of *M. verrucaria* chitinase to control groundnut rust, *P. arachidis*. Partially purified chitinase from *T. harzianum* destroys the cell wall of *Crinipellis perniciosa*, the casual agent of witches’ broom disease of cocoa (DeMarco et al. 2000). Chitinase from *B. cereus* YQ 308 inhibited the growth of plant pathogenic fungi such as *Fusarium oxysporum*, *Fusarium solani*, and *P. ultimum* (Chang et al. 2003). Chitinases from *Trichoderma aureoviride* DY-59 and *Rhizopus microsporus* VS-9 inhibited microconidial germination in *F. solani* (Nguyen et al., 2008). Two chitinases from *Bacillus pumilus* SG2 inhibited growth of *Rhizoctonia solani*, *Verticillium* sp., *Nigrospora* sp., *Stemphylium botryosum* and *Bipolaris* sp. (Ghasemi et al., 2010).

Chitinases can be added as a supplement to the commonly used fungicides and insecticides to make them more potent and to minimize the application of harmful chemical components of the fungicides and insecticides. Bhushan and Hoondal (1998) studied the compatibility of a thermostable chitinase from *Bacillus* sp. BG-11 with the commonly used fungicides and insecticides. The possibility for improving plant resistance using genetic manipulation techniques is promising. The chit42 gene of *T. harzianum*, encoding a powerful endochitinase with a much stronger antifungal activity to a wide range of phytopathogenic fungi, is expressed constitutively in tobacco, apple and potato, and these transgenic plants show a high level resistance against phytopathogenic fungi tested (Lorito et al., 1998; Bolar et al., 2000). Transgenic tobacco plants expressing the *S. cerevisiae* Cts1 chitinase can inhibit spore germination and hyphal growth of *Botrytis cinerea* (Woo et al., 1998).

Fungal chitinases are also used for insect control (Halder et al., 2012). *M. verrucaria*, a saprophytic fungus, produces a total complex of an insect cuticle-degrading enzyme (Shaikh and Desphande 1993). It has been seen that both first and fourth instar larvae of mosquito *A. aegypti*, a vector of yellow fever and dengue, can be killed within 48 h with the help of the crude preparation from *M. verrucaria* (Mendonsa et al. 1996). Overexpression of the Bbchit1 gene
from the entomopathogenic fungus *Beauveria bassiana* in transgenic *B. bassiana* can significantly enhance the virulence of *B. bassiana* for aphids (Treger et al., 1998).

**Production of single-cell protein/Bioconversion of chitin waste**

The solid waste from shellfish processing, mainly composed of chitin, can be hydrolyzed by chitinases and the resulting sugars can be utilized by other microorganisms for production of SCP and biofuels. Revah-Moiseev and Carrod (1981) used the *S. 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* (Dahiya et al., 2006). Vyas and Deshpande (1991) utilized the chitinolytic enzymes of *M. verrucaria* and *S. cerevisiae* for the production of SCP from chitinous waste reporting the total protein content to be 61%, with very low contents of nucleic acids (3.1%). Ferrer et al. (1996) used chitin acid/enzyme hydrolysate as a carbon and energy source for the growth of *Saccharomyces cerevisiae* K1V-1116 and reported that the acid and enzymatic hydrolysis route are comparable when effective production of SCP is concerned.

Cody et al. (1990) reported the applicability of yeast *P. tannophilus* and *Z. mobilis* in production of ethanol on amino sugars, glucosamine and N-acetylglucosamine. Oleaginous microorganisms like *Rhodotorula glutinis*, *Rhodococcus opacus* and *Cryptococcus curvatus* possess the ability to convert NAG into triacylglycerides and store these materials intracellularly (Zang et al., 2011).

**Isolation 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. Mizuno et al. (1997) isolated protoplast from Schizophyllum commune using the culture filtrate of *B. circulans* KA-304. Dahiya et al. (2005) reported the effectiveness of
Enterobacter sp. NRG4 chitinase in the generation of protoplasts from Trichoderma reesei, Pleurotus florida, Agaricus bisporus, and A. niger. An enzyme complex from B. circulans WL-12 with high chitinase activity was effective in generating protoplasts from Phaffia rhodozyme (Johnson et al. 1979). A chitinase and a laminarinase from the thermophilic fungus Paecilomyces variotii were used to isolate protoplasts of a thermophilic fungus, Malbranchea sulfurea (Gautam et al., 1996). In biological research, chitinases from T. harzianum are used for the generation of fungal protoplasts (Duo-Chuan, 2006).

Medical 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 and Davis 1979; Orunsi and Trinci 1985). They can also be used as potential additives in antifungal creams and lotions due to their topical applications. Chitinases of insect and fungal pathogens can also be useful as vaccine antigens. Recombinant chitinase of Haemaphysalis longicornis (hard tick) expressed in E. coli stimulated a specific protective anti-tick immune response in mice, suggesting its potential as a tick vaccine (You and Fujisaki, 2009).

In addition, chitinases are also useful in generating chitoooligosaccharides, glucosamine, and GlcNAc that have immense pharmaceutical potential owing to their strong physiological activities (Dhaiya et al., 2006). For example, Chitoooligomers show enhancing protective effects against infection with some pathogens like Listeria monocytogenes in mice. The beneficial lowering effect of oligosaccharides on plasma cholesterol may play an important role in the prevention and treatment of cardiovascular diseases (Tharanathan and Kittur, 2003). Chitin oligomers also show the antitumorigenic properties and inhibit growth of tumor cells via an immuno-enhancing effect.

The potential of glucosamine and N-acetylglucosamine in the treatment of patients with osteoarthritis has been recognized for several years (Horstman et al., 1999; Rubin et al., 2001; Matheson and Perry, 2003).
Glucosamine and NAG are not only the primary building blocks of the structural matrix of connective tissue in joints, but it also stimulates their synthesis and inhibits their degradation. In addition, they are capable of scavenging reactive oxygen species, whose levels are elevated in the synovium of arthritis patients (Chapman et al., 1986; Sato et al., 1988; Gracy et al., 1999). Glucosamine/NAG has been shown to accelerate wound healing, improve skin hydration, and decrease wrinkles. In addition, as an inhibitor of tyrosinase activation, it inhibits melanin production and is useful in treatment of disorders of hyperpigmentation. GlcNAc also shows promise as an inexpensive and nontoxic treatment in chronic inflammatory bowel disease. The oral administration of GlcNAc to children with severe ulcerative colitis and Crohn’s disease led to histological improvement, and a significant increase in epithelial and lamina propria glycosaminoglycans and intracellular GlcNAc.

**THERMOPHILIC FUNGI/MOULDS**

A key physical factor determining the sustenance of living beings on earth is temperature. The origin of life on earth occurred in extreme temperatures and microbes recovered from such extremes are until now only prokaryotes. Eukaryotes can grow only up to 60°C or so, herein too the extremes are shown by a small number of fungal species (Sharma and Johri, 1992). These thermophilic moulds when grown at high temperatures exhibit greater saturation of lipids and a different mode of lipid storage (Satyanarayana and Johri, 1999). Several enzymes of these moulds are more thermostable than the mesophilic fungi. Their proteins have been found to be stable upto 95°C or higher (Satyanarayana et al., 1992).

Many definitions of this phenomenon of thermophily in fungi occur in literature. Crisan (1959) defined thermophilic fungi as those whose optimum temperature for normal growth lies at or above 40°C. Cooney and Emerson (1964) defined ‘A thermophilic fungus is one that has a maximum temperature of growth at or above 50°C and a minimum temperature for growth at or above 20°C’. Thermophilic fungi were discovered accidently due to chance contamination of organic materials incubated at elevated temperatures. The earliest discovered species were *Mucor pusillus* from bread by Lindt (Lindt, 1886) and *Humicola*
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Lanuginosa from potato disk by Tsiklinsky (Tsiklinsky, 1899). The first extensive report on thermophily in fungi was produced by Miehe in 1907, who made a thorough study of self heating hay and described *Thermoascus aurantiacus* and *Malbranchea pulchella* var. *sulfurea* (Miehe, 1907). Thermophilic fungi grow in simple media containing carbon and nitrogen sources and mineral salts, suggesting that they do not have any special requirement for growth and they are autotrophic for all vitamins.

Thermophilic fungi are worldwide in distribution and have been reported from various types of soils and in habitats where decomposition of plant material results in high temperature. These include: composts, piles of hays, stored grains, wood chip piles, nesting material of birds and animals, snuff, and municipal refuse, and other accumulations of organic matter wherein the warm, humid, and aerobic environment provides the basic physiological conditions for their development. Man made habitats such as cooling towers, effluents from nuclear power reactors and ducts employed for thermal insulation (Johri and Satyanarayana, 1986). In these habitats thermophiles may occur either as resting propagules or as active mycelia depending on the availability of nutrients and favourable environmental conditions. Their widespread occurrence could well be due to the dissemination of propagules from self-heating masses of organic material (Maheswari et al., 1987).

Thermophilic fungi have high potential for industrial applications as a source of thermostable enzymes. Biomass-degrading enzymes from thermophilic fungi consistently demonstrate higher hydrolytic capacity (Wojtczak et al., 1987). Thermophilic fungal strains are also capable of rapid growth with minimized viscosity at relatively elevated growth temperatures thereby enhancing productivity in fermentors (Jensen and Boominathan, 1997).

Though chitinolytic enzymes are produced from a wide range of organisms, most of them cannot be applied wildly due to the high cost of isolation of enzymes from natural resources, their instability and activity within narrow temperature and pH ranges (Dai et al., 2011). The use of thermostable enzymes, which are intrinsically stable and active at high temperatures minimizes stability problems and thus cost. The thermostable biocatalysts are
also more resistant to denaturing agents, more stable to change in pH and tolerant to higher solute (reactants) concentrations. Other expected advantages of thermostable enzymes are a longer shelf life, less contamination problems and a different substrate spectrum than comparable mesophilic enzymes (Sonnleitner and Fiechter 1983; Vieille and Zeikus, 2001). Higher processing temperatures gives the benefit of achieving higher reaction rates and allows higher substrate concentrations, lower viscosity and minimization of diffusion limitations of the reactants and the products (Sonnleitner and Fiechter, 1983; Unsworth et al., 2007). Once expressed in mesophilic hosts, thermophilic enzymes are also easier to purify by heat treatment (Vieille and Zeikus, 2001).

Among various microbes, filamentous fungi are the most frequently used organisms in industrial processes. In general, filamentous fungi have 18-20 different GH family 18 proteins (Seidl, 2008). Some mycoparasitic and entomopathogenic fungi have even 30 or more GH family 18 proteins (Gao et al., 2011; Kubicek et al., 2011). The variability of fungal chitinases makes them ideal candidates for the development of enzymes acting on chitinous carbohydrates used in biotechnology (Hartl et al., 2012). As sources of thermostable enzymes, thermophilic fungi have a high potential for industrial applications. Another advantage of thermophilic fungi over their mesophilic counterparts is better growth characteristics in a fermenter. Filamentous growth in fermenters is normally accompanied by a high culture viscosity, low mixing rates, and, consequently, suboptimal transfer of, e.g., oxygen and nutrients, which may severely hamper protein productivity in large-scale fermentations. Thermophilic fungal strains are capable of rapid growth with minimized viscosity at relatively elevated growth temperatures, thereby enhancing productivity in fermenters (Jensen and Boominathan, 1997).

As compared to mesophilic fungi, data on thermophilic fungal chitinases is limited. As listed in table 1.6, these chitinases are less diverse and all show an optimum temperature in the range of 50-65°C, molecular weight of 47-48 kD and pH optimum of 4.5-5.5 (McComark et al., 1991; Guo et al., 2005, 2008; Li et al., 2010; Kopparapu et al., 2011). The genes encoding these proteins belong to family 18 of glycosyl hydrolases and consist of a single catalytic domain with conserved substrate-binding and catalyzing domain (Li et al., 2010;
Kopparapu et al., 2011). Active chitinases from *Thermoascus aurantiacus* var. *levisporus* and *Chaetomium thermophilum* have been expressed in *Pichia pastoris* (Li et al., 2010). A chitinase from *Paecilomyces thermophila* has also been expressed in *Escherichia coli* as an intracellular soluble protein (Kopparapu et al., 2011).

*Myceliophthora thermophilia* is a thermophilic fungus that grows optimally at 45°C and is able to produce some industrially important hydrolytic enzymes such as phytase, lipase, cellulose, xylanase and others (Satyanarayana et al. 1985; Singh and Satyanarayana et al. 2006). Recently the 38,744,216 bp genome of *M. thermophilia* containing 9110 protein coding genes on 7 chromosomes has been sequenced, one of the first among filamentous fungi (Berka et al., 2011). Like other fungi, it harbours a large number (>210) of glycosyl hydrolase and polysaccharide lyase and can be considered as all purpose decomposers (Berka et al., 2011). Based on the peptide sequence of extracellular proteins produced by *Myceliophthora thermophilia*, previously classified as *Chrysosporium lucknowense* C1, two chitinases (a 45 kDa endochitinase and a 70 kDa exochitinase) were identified in the culture filtrates (Visser et al., 2011). Homologous production of the 45 kDa endochitinase reached the level of 7.5 g/L using an expression vector containing chi1 (chitinase gene) promoter region and cbh1 (cellobiohydrolase gene) terminator region flanking the cloning site for chi1 gene (Visser et al., 2011). However, detailed studies relating to biochemical characterization and application potential of the chitinases has not been investigated (Visser et al., 2011).

In view of the biotechnological potential of a thermostable chitinases, the present investigation was planned and executed with the following objectives:

1. Isolation, screening and selection of a potent chitinase producing thermophilic mould
2. Optimization of enzyme production in submerged and solid state fermentations
3. Purification and characterization of chitinase
4. Testing the applicability of chitinase in the production of chitooligosaccharides and in biocontrol