Chapter 2

Review of literature
Review of Literature

Chitin deacetylase is an enzyme that catalyzes the deacetylation of chitin to chitosan. It belongs to the family of hydrolases, those acting on carbon-nitrogen bonds other than peptide bonds, specifically in linear amides. Deacetylation of chitin is an interesting process because it affects the crystallinity and solubility of the polymer, which play an important role in application of chitin in various fields.

2.1 Chitin

Chitin is a cationic amino polysaccharide primarily consisting of \( \beta-(1\rightarrow4) \)-linked \( N \)-acetyl-D-glucosamine (GlcNAc) units (Shen et al., 2013). It is a white, hard, inelastic, nitrogenous polysaccharide and a major source of surface pollution in many coastal areas. Approximately \( 3.7 \times 10^4 \) tons are produced by marine invertebrates alone (Mukherjee and Sen, 2006) and the annual rate of steady-state of chitin synthesis is approximately \( 10^{10} \) to \( 10^{11} \) tons worldwide (Gooday, 1994). 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 underutilized 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).

2.1.1 History

Chitin was discovered by Henri Braconnot in 1811 in mushrooms. He named it ‘fungine’. Chitin - a Greek term for tunic /envelope was first used by Odier in 1823. He found it in insects and plants and named it ‘chistine’ (Deshpande, 2005). According to Lower (1984) the term was derived from Greek word ‘chiton’ meaning a coat of mail. An English scientist in 1799, A. Hachett discovered that a “material is predominantly resistant to usual chemicals”. In 1843, it was established by Lassaigne that nitrogen is present in chitin (Jeuniaux, 1996).

2.1.2 Occurrence

In nature, chitin exists in the exoskeleton of animals such as insects and crustaceans (Rudall and Kinchington, 1973). It is also found in microorganisms e.g. in the cell walls and structural membranes of fungal mycelia (mushrooms), yeast & green algae (White et al., 1979; Bartinicki-Garcia, 1989; Mathur and Narang, 1990). Chitin is the structural component of the shells of crustaceans i.e. shrimp, crab, lobster and prawn (Austin et al., 1981). It has also been reported in Protozoans, Arachnids, Nematodes, and Tunicates (Cohen, 1987). The exoskeleton of crustaceans consists of 25-30% chitin by dry weight (Austin et al., 1981).
In Fungi, chitin appears to be ubiquitous with exception of *Schizosaccharomyces* which has small but essential amounts of chitin. Several zygomycete species of fungi chitin exits in its deacetylated form referred to as chitosan. But latter is found in the cell walls of fungi, to a lesser extent than Chitin (Ruiz-Herrera, 1978; Shimahara et al., 1984; Knorr, 1991). Chitin is present in the cell wall of most of the fungi (Fig 2.1).

![Chitin in cell wall of fungi](http://byjus.com/biology/fungal-cell-wall/)

Chitin is present as a structural component in most existing species today. Its phylogenetic distribution varies in different types of organisms. In Prokaryotes, chitin has only been reported as a possible component of streptomycete spores and the stalks of some prosthecate bacteria. In Eukaryotic microorganisms, it is present in tough structural material e.g. in cyst walls of some ciliates and amoebae; in the lorica walls of some ciliates and chrysophyte algae; in the flotation spines of centric diatoms and in the walls of some chlorophyte algae and oomycete fungi (Gooday, 1990). *Pneumocystis carinii*, of uncertain affinity, has chitin in the walls of its cysts and trophozoites (Walker et al., 1990). In Animals, chitin is present in most invertebrates but absent from vertebrates. It is probably absent from plants, but polymers rich in (1, 4)-β-linked N-acetylglucosamine have been reported in plants (Benhamou and Asselin, 1989). Apart from these sources, byproducts from citric acid and antibiotic manufacturing plants may also become an important source for chitin.

### 2.1.3 Chitin Structure

Both chemical structure and biological function of chitin has similarity with cellulose (Ruiz-Herrera, 1978). The crystalline structure of chitin has been shown to be similar to cellulose in the arrangements of inter and intrachain Hydrogen bonding (Garg and Kashyap, 2014). Chitin is a linear polysaccharide consisting of β-(1-4)-linked-2-amino-2-deoxy-D-glucopyranose units, its basic repeating unit in the chitin chain is
chitobiose, whereas cellulose is a linear homopolymer of anhydroglucose units linked by β-(1-4) glycosidic bonds, its basic repeating unit is disaccharide cellobiose (Garg and Kashyap, 2014). Chitin may be regarded as a cellulose derivative (Fig 2.2) with an acetamido group at C₂ (Suzuki, 2000). Cellulose contains hydroxyl groups at the C₂ position of the monomers. Both cellulose and chitin strengthen the cell walls of plants and supporting structures of invertebrate animals respectively.

![Cellulose and Chitin Structures](image)

**Fig 2.2 Structure of cellulose and chitin**

The molecular configuration of a single polymer chain is helicoidal (6 aminosugar residues per turn) which is stabilized by intramolecular hydrogen bonds. In the crystalline state of chitin, a number of molecules (~ 20) coalesce to form microfibrils (~ 3 nm in diameter), are generated by intermolecular hydrogen bonding. Three highly ordered crystalline structures of chitin polymorphs were described largely by X-ray diffraction studies (Jang et al., 2004; Minke and Blackwell, 1978; Rudall and Kenchington, 1973).

In the chitin crystal structure, the chains form hydrogen-bonded sheets linked by C=O and -NH groups. In addition, intramolecular hydrogen bonds are present between the neighbouring sugar rings of each chain: the carbonyl group bonds to the hydroxyl group on C₆. There is also a second hydrogen bond between the OH-group on C₃ and the ring oxygen, similar to that in cellulose (Minke and Blackwell, 1978; Garg and Kashyap, 2014). This extensive hydrogen bonding enhances the stiffness of the chitin chain (Fig 2.3).
Fig 2.3 Chemical structure of chitin with its intramolecular hydrogen bonds (dotted lines)

between the neigbouring sugar rings in chitin.

2.1.4 Different forms of chitin

Chitin has a highly ordered, crystalline structure. There is a wide variety of types of chitin but only three hydrogen-bonded crystalline forms have been characterized: α-chitin, β-chitin, γ-chitin (Fig 2.4).

In α-chitin, the chains are arranged in antiparallel fashion. In β- chitin, the chains are arranged in parallel fashion and γ-chitin has a three chain repeating unit, two 'up' & one ‘down’. The three chitin variants differ in their degree of hydration, in the size of their unit cell and in the number of chitin chains per unit cell. α- chitin is the most common type. It is found in most eukaryotic microorganisms and exoskeletons of invertebrates. Most thermodynamically stable crystalline polymorph is the α-chitin in which one polymer chain is antiparallel to the other. It has been speculated that such arrangement is due to either folding back of nascent chitin polymers or by virtue of in situ antiparallel biosynthesis at the catalytic sites (Cohen, 1987).
The distribution of the polymorphic forms is not related to a particular category of organism, as different forms may occur in one organism, providing different functional properties. Different physical structures are responsible for performing different functions e.g. squid, *Loligo*, have $\alpha$-chitin in its tough beak and radula, $\beta$-chitin in its rigid pen (endoskeleton), the polymer chains are packed in a parallel fashion (Garg and Kashyap, 2014) with reducing ends pointing to the same direction and $\gamma$-chitin in its flexible stomach lining. $\alpha$-chitin is by far the most abundant form, and is usually found where extreme hardness is required (Rudall and Kenchington, 1973). $\beta$ and $\gamma$-chitin seem to provide toughness, flexibility and mobility and may have physiological functions other than support. $\beta$-chitin is detected in *Eufolliculina uhligi*, a Protozoan and spines of diatoms, *Thalassiosira fluviatilis* whereas $\gamma$-chitin is found in cocoon of beetle (Rudall and Kenchington, 1973).

The inability of $\alpha$-chitin to swell upon soaking in water is explained by the extensive intermolecular hydrogen bonding (Minke and Blackwell, 1978). Due to lack of these interchain hydrogen bonds in $\beta$-chitin, it swells readily in water (Blackwell, 1969). Due to the close packing of chains and its strong inter- and intramolecular bonds among the hydroxyl and acetamido groups, chitin shows poor solubility (Urbanczyk et al., 1997). However, it can be converted into chitosan which is soluble in mild acidic solutions. Because chitosan is soluble in dilute acids e.g. formic acid and acetic acid and its salts are soluble in water therefore it finds wider application in industry than chitin (Simpson et al., 1994).

Chitin chains folded in one of the forms, aggregate and form microfibrils in living systems. Except the chitin of diatoms (Blackwell et al., 1967), chitin is found in nature cross-linked to other structural components. The chitin microfibrils in combination with other sugars, proteins, glycoproteins and proteoglycans form fungal septa and cell walls as well as arthropod cuticles and peritrophic matrices especially in crustaceans and insects (Kozloff, 1990). In animals, chitin is associated with proteins, while in fungal cell wall it is associated with glucans, mannans or other polysaccharides. In fungal walls, it is found covalently bound to glucans, either directly or via peptide bridges (Roberts, 1992). In insects and other invertebrates, the chitin is always associated with specific proteins, with both covalent and non-covalent bonding. Chitin has also been found in varying degrees of mineralization, particularly calcification and sclerotisation involving phenolic and lipid molecules. In both fungi and invertebrates, varying degrees of deacetylation have been determined, giving an
array of structure between chitin (fully acetylated) and chitosan (fully deacetylated) (Peter et al., 1986).

2.1.5 Chitin extraction

Chitin was first isolated and extracted as the alkali-resistant fraction from fungi by French chemist and pharmacist Henri Braccanot (Braccanot, 1811). Procedures for isolation of high-molecular weight pure chitin are very important for its use as a raw material to make high quality chitosan. Since proteins, minerals, lipids, and pigments are closely associated with chitin, so all these components have to be removed to achieve the high purity necessary for biological applications.

Chitin from the diatom spines such as *Cyclotella cryptica* and *Thalassiosira fluviatilis* are the only form reported to be 100% poly N-acetylglucosamine that is not associated with proteins and is termed Chitan. The common procedure for extracting chitin from crustacean shells involves demineralisation, deproteinisation and decoloration. Demineralization is frequently carried out by treatment with dilute acid or chelating agent; while deproteinization involves treatment with dilute alkali or Proteolytic enzymes (Muzzarelli, 1977; Simahara et al., 1989). The residues are decolorised using solvents and/or oxidants (Percot et al., 2003).

In most of the procedures, chitin is further degraded during extraction. Therefore, a great deal of interest still prevails in exploration of an efficient extraction procedure so that during this process, chitin degradation can be avoided and impurities be minimized to make the process commercially viable. In most instances, deproteinization precedes the demineralization (Hackman, 1954) without any adverse effects on product (NO et al., 1989; Sanford, 1989). Deproteinization of chitin is usually performed by alkaline treatments, although other effective reagents have also been reported. Typically, raw chitin is treated with approximately 1.0 M aqueous solution of NaOH for 1h to 72 h at temperatures ranging from 65 to 100 °C (Percot et al., 2003a). An alternative method involves the enzymatic degradation of proteins, where the residual protein in the produced chitin often remains relatively high and the reaction time is longer compared to chemical deproteination. However, Manni et al. (2010) from Tunisia demonstrated that the crude protease from *Bacillus cereus SV1* could be used effectively in the deproteinization (88.8%) of shrimp wastes to produce chitin.
Demineralisation is generally performed by acids including HCl, HNO₃, H₂SO₄, CH₃COOH, and HCOOH however HCl seems to be the preferred acid and is applied at a concentration between 0.2M and 2.0M for 1h to 48 h at temperatures varying from 0°C to 100°C. Subsequently/Afterwords, the product may be decolorized with acetone and/or H₂O₂ or NaOCl (NO et al., 1989), or Peroxide bleaches (Austin, 1988). However, preparation of chitin/chitosan involves demineralization and deproteinization of shellfish waste by the use of strong acids or bases (Liang et al., 2007; Wang et al., 2006).

Chitin has been extracted from six different local sources in Egypt. The obtained chitin was converted into the more useful soluble chitosan by steeping into solutions of NaOH of various concentrations and for extended periods of time, then the alkali chitin was heated in an autoclave which dramatically reduced the time of deacetylation. Chitin from squid pens did not require steeping in sodium hydroxide solution and showed much higher reactivity towards deacetylation in the autoclave that even after 15 min of heating a degree of deacetylation of 90% was achieved (Abdou et al., 2008).

2.2 Chitosan

Removal of most of the acetyl groups of chitin either by enzymatic or chemical treatment yields chitosan (Peniston and Johnson, 1980). A discrete nomenclature with respect to the degree of N-deacetylation has not been defined between chitin and chitosan (Muzzarelli, 1977). In general, chitin with a degree of deacetylation of above 70% is considered as chitosan (Li et al., 1996). Chitosan, a derivative of chitin, was produced in 1859, since then, research has been conducted to learn about the properties of chitin and chitosan and extend its commercial applications.

Chitosan, a deacetylated form of chitin, discovered by Rouget in 1859 is an important polysaccharide of biological origin. The history of chitosan begins with its first description by Braccanot in 1811. The term chitosan refers to a family of copolymers with various fractions of acetylated units. Many fungi including Aspergillus niger, Mucor rouxii, Rhizopus oryzae and Absidia coerulea have chitosan in their mycelial wall (Suntornsuk et al., 2002; Zhao and Wang, 1999; Synowiecki and Al-Khateeb, 1997; Wu et al., 2001; Tan et al., 1996). Chitosan provides the fungus an additional resistance to lysis by chitinolytic organisms (Aruchami et al., 1986).
2.2.1 Sources of chitosan

Chitosan has been found in a wide range of natural sources such as crustaceans, fungi, insects and some algae. Chitosan is produced from chitin by thermochemical deacetylation. This involves boiling of chitin in concentrated alkali for several hours. However enzymatic procedures for chitin deacetylation by chitin deacetylase are also in use. In nature, Chitosan is also found in cell walls of fungi of the class Zygomycetes, in the green algae *Chlorella* sp., yeast and protozoa as well as in insect cuticles (Pochanavanich et al., 2002). The cultivation of fungi (*A.niger*) can provide an alternative source of chitosan. However, the acetyl groups in chitosan produced from crustacean chitin are uniformly distributed along the polymer chain, but chitosan of similar degree of deacetylation isolated from fungal cell walls possess acetyl residues that are grouped into clusters (Singla et al., 2001). Although, a wide range of sources of chitosan is available, but it is commercially prepared mainly from crustaceans waste such as crab, shrimp, krill, and crayfish. It is because a large amount of crustacean exoskeleton is available as a byproduct of sea food processing. Annually India produces more than 100,000 tonnes of shrimp processing waste (Suresh et al., 2011). Out of which only an insignificant amount is utilized for the extraction of chitin and the rest is discarded. Disposal of crustacean shell waste has been a challenge for sea food processors. The unique, abundantly renowned properties of chitin and chitosan make it a formidable biopolymer, much too precious to be dumped as waste into the sea or in landfills. Thus effective utilization of such a waste had become an important issue both from the point of clean environment and the economic value of this unique bio-waste.

2.2.2 Structure of chitosan

The chemical name of chitosan is Poly-(1-4)-2-amino-2-deoxy-β-D-Glucopyranan and its molecular formula is \((\text{C}_6\text{H}_{11}\text{O}_4\text{N})_n\) (Mao, 2004). Chitosan forms extensive intra and intermolecular H- bonding due to the presence of one primary amino and two free –OH groups (one primary hydroxyl group at C-6 and one secondary hydroxyl group at C-3) for each repeating unit. Its nitrogen content varies from 5 to 8% depending on the extent of deacetylation; it is mostly in the form of primary aliphatic amino groups.

2.2.3 Chitosan preparation by thermochemical deacetylation

Commercial chitosan is derived from the shells of shrimp and other sea crustaceans, including *Pandalus borealis* which have chitin, as the structural element in their exoskeleton. Chitosan can be prepared from fungi or it can be prepared from
chitin by two methods viz. thermochemical deacetylation and enzymatic deacetylation. In thermochemical process (Fig 2.5), chitin is treated with highly concentrated (40-50%) solutions of NaOH or KOH at high temperatures (100-150°C) and exclusive of air for about an hour (Johnson and Peniston, 1982; Aranaz et al., 2009).

**Fig 2.5 Preparation of chitosan by thermochemical deacetylation**

The thermochemical deacetylation of chitin from crustacean shells is further of two types, heterogeneous deacetylation and homogeneous deacetylation (Sannan et al., 1975, Aranaz et al., 2009). The extent of deacetylation of chitin depends on concentration of alkali used, particle size, density of chitin and previous pretreatment.

There are two methods of thermochemical deacetylation:

1. **Heterogeneous deacetylation**: This method is used mainly in industry. This type of deacetylation involves the preferential action in the amorphous regions of the solid chitin so that crystalline native regions remain intact in the parent chitin. In this method, chitin flakes are treated with aqueous solution of NaOH (30-60%) at 80-120 °C for 4-6 h. This method can give highly N-deacetylated products during long treatment time and repeated treatments usually lead to depolymerisation (Lamarque, 2007).

2. **Homogeneous deacetylation**: Homogeneous deacetylation is carried out on pre-swollen chitin under vacuum in aqueous solution (Aranaz et al., 2009).
Alkaline chitin in the form of solution of the sodium salt of chitin is treated with 1.4% NaOH, at 25 °C. The process can produce partially N-deacetylated derivatives of chitosan/chitin, which are soluble in water. However, this method is not very efficient and a random distribution of N-acetyl groups is found in the products.

Availability of chitosan produced by these methods is restricted and seasonal for commercial use (Ashford et al., 1977). Besides, large amount of concentrated alkaline solution is used in this process which causes environmental pollution. To overcome all these problems several alternative sources of chitin/chitosan have been suggested including insects, Antarctic krill and diatoms which can be utilized as raw materials in industries (Allan et al., 1978).

Secondly, chitosan-producing fungi, particularly zygomycetes (Arcidiacono and Kaplan, 1991) which have chitosan in their cell wall, can be cultured. Chitosan has been produced by fermentation of zygomycetes such as A.coerulea (Davoust and Hansson, 1992; Rane and Hoover, 1993; Muzzarelli et al., 1994; Niederhofer and Muller, 2004), M. rouxii (White et al., 1979; Knorr and Kelvin, 1986; Chatterjee et al., 2005) and R. oryzae (Suntornsuk, 2002). The advantage of using fungi is the easy handling, harvesting and controlling to produce high quality chitosan. But in the fungal mycelia, chitosan and glucan are covalently linked. The complex of chitosan and glucan causes considerable problems in the extraction of both chitosan and glucan from fungal mycelia (Nwe and Stevens, 2002).

2.2.4 Production of chitosan from fungus

Crestini et al. (1996) compared both the solid state and submerged fermentations using Lentinula edodes and found that the solid-state fermentation method yields 50 times higher chitosan than submerged fermentation. They further found that chitosan obtained from the mycelium of fungus L. edodes have high-quality chitosan in terms of yields and degree of acetylation. The degree of acetylation of chitosan obtained from submerged fermentation increased with incubation time. They have attributed it to more susceptibility of chitin for chitin deacetylase enzyme because chitin is less crystalline during initial stages of growth. However, there was no such effect on degree of acetylation of chitosan obtained from solid-state fermentation. The quality of chitosan is considered better with low degree of acetylation due to high positive charge density and Acetylation degrees of chitosan obtained from L. edodes
ranged from 5 to 13% and that from commercial crab shell chitosan ranged from 18-22% making fungus as a better contender for good quality chitosan.

Zygomycetous fungi such as *A. coerulaea, B. poitrasii, C. elegans, M. rouxii, R. oryzae, Mucor racemosus and Gongronella butleri* for the production of chitosan has gained increased attention in recent years due to potential advantages in terms of homogenous polymer length, high degree of deacetylation and solubility over the current marine source (Ghormade et al., 2017).

**2.2.5 Extraction of Chitosan from fungal cell wall**

First of all, the cell wall is isolated from the fungal biomass by its alkali treatment (with dilute NaOH solution) at elevated temperature (e.g. 90-120%). At high temperature, the alkali dissolves proteins, lipids, and alkali-soluble carbohydrates and the cell wall remains as alkali insoluble material (AIM). After this, chitosan is separated from AIM by dissolving it in an acid solution (e.g. 2–10% acetic acid at 25–95 °C for 1–24 h). During this step, the other components of cell wall remain as alkali- and acid-insoluble material (AAIM). Finally, fungal chitosan is precipitated by increasing the pH to 9-10 and chitosan is recovered by centrifugation.

In another approach, treatment of cell wall with hot dilute Sulfuric acid solution has resulted in a high yield and more pure form of chitosan. Unlike acetic, citric, lactic and hydrochloric acids, chitosan is not soluble in dilute Sulfuric acid solutions at room temperature (Shaojie et al., 2004). However, it is soluble in hot boiling solution of Sulfuric acid. The temperature dependent Solubility of chitosan in Sulfuric acid solutions is not shared with the other components of cell wall such as chitin and polyphosphates. Therefore, by treating the cell wall with hot dilute Sulfuric acid, chitosan becomes soluble in hot acid and it can be separated from other components of cell wall by filtration. In the next, step chitosan can be recovered from Sulfuric acid by cooling. Zamani et al. (2007) reported that extraction of chitosan from cell wall of Zygomycetes Fungi by Sulfuric acid results in a product with higher purity and yield compared to traditional extraction method e.g. by acetic acid.

**2.2.6 Biosynthesis of chitosan**

Chitosan is synthesized in nature by enzymatic deacetylation of chitin. Chitin deacetylase is the enzyme that catalyzes the conversion of chitin to chitosan by the hydrolysis of acetamido groups (fig 7) of N-acetyl-D-glucosamine present in chitin. Chitin deacetylase was first identified and particularly purified from extracts of fungus *M. rouxii* by Araki and Ito (1975). Since then, the presence of this enzyme has been
reported in several other microorganisms. Chitin deacetylase occurring in marine bacteria, several fungi and a few insects (Zhao et al., 2010), which catalyze the deacetylation of chitin to chitosan by recognizing a sequence of four GlcNAc units in the chitin, one of which undergoes deacetylation.

2.2.7 Properties of chitosan

Chitosan occurs as flabby powder or flake. It is odorless and its colour varies from yellow to white. The properties of chitosan depend on two factors, degree of deacetylation (DD) and molecular weight ($M_w$). The term chitosan includes a variety of polymers that differ from each other in their average molecular weight and degree of deacetylation. The DD and $M_w$ of chitosan depend on the reaction conditions during the manufacture of chitosan from chitin. Chitosan is nontoxic, biodegradable as well as antimicrobial polymer. The unique properties of chitosan, are due to its cationic nature and solubility in acidic solutions, compared to other natural polysaccharides which are usually neutral or negatively charged (Muzzarelli, 1977). Chemically chitosan is a linear polyamine having reactive amino and hydroxyl groups. It can form chelates with transition metal ions. A large no. of applications of chitosan are due to its biodegradable, biocompatible, haemostatic, fungistatic, spermicidal, antitumor, antimicrobial, central nervous system depressant and immunoadjuvant character (Dutta et al., 2004). Due to their inherent properties, coupled with the ability to form films, alone or in combination with other polymers, chitin, chitosan and oligosaccharides are desirable food packaging materials (Dutta et al., 2011). It can be formed into fibers, films, gels, sponges, beads or nanoparticles. So, chitosan has numerous applications especially in food products, pharmaceutics, photography, paper industry and cosmetics etc. (Shahidi et al., 1999; Kumar, 2000; Dodane and Vilivalam, 1998). It can be degraded in vivo by several proteases like lysozyme; papain and pepsin etc. chitosan is water soluble and bioadhesive which readily binds to negatively charged surfaces such as mucosal membranes. Chitosan enhances the transport of polar drugs across epithelial surfaces. Purified quantities of chitosan are available for biomedical applications. One of chitosan's properties is its ability to absorb fat. This may make chitosan a useful tool for weight loss. Chitosan may also help reduce blood levels of both low-density lipoprotein and total cholesterol.

2.2.7.1 Molecular weight

Chitosan is not one chemical entity, it is a term applied to deacetylated chitins in various stages of deacetylation and depolymerization and it is therefore not easily
defined in terms of its exact chemical composition. A clear nomenclature with respect to the different degrees of N-deacetylation between chitin and chitosan has not been defined (Muzzarelli, 1973; Kumar, 2000). Chitosan is a polymer of high molecular weight. Its molecular weight can be determined by methods of chromatography (Bough et al., 1978), light scattering (Muzzarelli, 1977), and viscometry (Maghami and Roberts, 1988). Its molecular weight varies with the raw material and method of preparation. Depending on the process and grades of the product, the molecular weight of commercial chitosan was in the range of 10,000–1,000,000 Daltons. High temperature causes thermal degradation of chitosan, thereby lowering its molecular weight (Rout, 2001; Genta et al., 1998).

### 2.2.7.2 Solubility

Chitosan is unique because of its polyamine character which makes it soluble at acidic pH. The pH-dependent solubility of chitosan is due to its amino groups (—NH$_2$), which become protonated upon dissolution at pH 6 or below to form cationic amine groups (—NH$_3^+$). This causes the increased intermolecular electric repulsion, resulting in a polycationic soluble polysaccharide, with a large number of charged groups. In contrast, chitosan tends to lose its charge at higher pH, and may therefore precipitate from solution due to deprotonation of the amine groups (Fernandes et al., 2006). Chitosan is soluble in dilute acidic solutions below pH 6.0. Chitosan is soluble in organic acids such as acetic, formic, and lactic acids. The most commonly used solution is 1% acetic acid at about pH 4.0. Solubility of chitosan in inorganic acids is quite limited as it is soluble in 1% hydrochloric acid and insoluble in sulfuric and phosphoric acids. Concentrated acetic acid solutions at high temperature can cause depolymerization of chitosan. However above pH 7.0, solubility of chitosan is poor because chitosan solution forms poly-ion complex with anionic hydrocolloid resulting in the precipitation or gel formation (Kurita, 1986).

### 2.2.7.3 Cationic nature

The naturally occurring polysaccharides such as agarose, pectin, cellulose, dextrin, alginic acid, agar and carragenans are acidic in nature but chitosan are highly basic polysaccharides (Peter et al. 2010). Both, chitosan and cellulose have the β-(1, 4)-anhydroglucosidic bonds but the characteristic properties of chitosan are not shared by cellulose (Hudson et al., 1998). The presence of free amino groups makes it positively charged, that is why it reacts with many negatively charged polymers.
2.2.7.4 Biodegradability

Chitosan is liable to degradation by enzymes, such as lysozymes (present in tears, saliva, blood and milk), cellulases, chitinases, chitosanases, hemicellulases, proteases (papain and pronase), lipases and β-1, 3-glucanases. Chitosanases (EC 3.2.1.132) catalyze the endohydrolysis of β- (1, 4)-glycosidic linkages between D-glucosamine (GlcN-GlcN) residues in partly acetylated chitosan (Park et al., 1996). Their biodegradation leads to the production of non-toxic oligosaccharides of variable length which can be subsequently incorporated to glycosaminoglycans and glycoproteins to metabolite pathways or be excreted (Pangburn et al., 1982).

2.2.7.5 Biocompatibility

The most important biological property of chitosan is biocompatibility i.e. it should not be affected by the host and at the same time should not elicit any undesirable local or systemic effects. Our living tissues, including the skin, nasal epithelium and ocular membranes can easily tolerate chitosan. That is why chitosan is also used in nasal drug delivery and cosmetics (Park et al., 1996). Biocompatibility of chitosan depends on the characteristics such as natural source, method of preparation, molecular weight and degree of deacetylation (Aranaz et al., 2009).

2.2.7.6 Non-toxicity

The oral LD50 (median lethal dose) of chitosan in mice was found to be in excess of 16 g/day/kg body weight, which is higher than that of sucrose. The toxicity of chitosan depends on its purity.

2.2.8 Applications of chitin and chitosan

Chitin and Chitosan has a no. of applications such as in wastewater treatment, food industry, medical industry, biotechnology, agriculture, cosmetics, membrane industry and pulp & paper industry (Ghormade et al., 2010; Dutta et al., 2004). Due to the biodegradable nature of chitin and chitosan, they are used as carbon source for single cell protein production and biodegradable packaging materials (Synowiecki and Al-Khateeb, 2003). Chitin and its derivative are also used in removal of metal like mercury and colour from wastewater & textile mill effluents respectively. The only natural cationic gum i.e. Chitosan becomes viscous on being neutralized with acid. It also has fungicidal and fungistatic properties. That is why it is used in creams, lotions and permanent waving lotions etc (Kumar, 2000). Chitosan has been found to be
having antibacterial activity against *vibrio cholera* and *E.coli* (Logesh et al., 2012). The various applications of chitin and chitosan are given in Table 2.1.

**Table 2.1 Applications of chitin and chitosan polymers**

<table>
<thead>
<tr>
<th>Application</th>
<th>Uses</th>
<th>Reference</th>
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<tr>
<td>Medical applications</td>
<td>Wound Healing/Wound Dressing, Burn Treatment, Artificial Skin, Ophthalmology, Trauma, Dermatitis, Canker, Artificial tendon, Artificial kidney ,contact lenses, drug delivery, catheter</td>
<td>Shigemasa and Minami, 1995</td>
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<tr>
<td>Pharmaceutical materials</td>
<td>As antitumour, blood anticoagulant, antigastritis, haemostatic, hypcholesterolaemic and antithrombogenic agents, in drug- and gene-delivery systems, and in dental therapy</td>
<td>Pokhrel, 2015; Dodane and Vilivalam, 1998</td>
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<td>Agriculture</td>
<td>Seed- and fruit-coating, fertilizer and fungicide</td>
<td>Illum, 1998</td>
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<td>Agriculture</td>
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<td>Hirano, 1996</td>
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<td>Agriculture</td>
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<td>Arbia et al., 2013</td>
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<tr>
<td>Food and feed additives</td>
<td>Clarification and de-acidification of fruits and beverages, colour stabilization, reduction of lipid adsorption, natural flavour extender, texture-controlling agent, food preservative and antioxidant, emulsifying, thickening and stabilizing agent, livestock and fish-feed additive, and preparation of dietary fibres</td>
<td>Hirano, 1996</td>
</tr>
<tr>
<td>Food and feed additives</td>
<td>Media Immobilization of enzymes, as a matrix in affinity and gel permeation chromatography and as enzyme substrates</td>
<td>Honda et al., 1999</td>
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<tr>
<td>Chromatographic and analytical reagents</td>
<td></td>
<td>Dodane and Vilivalam, 1998</td>
</tr>
<tr>
<td>Photography</td>
<td>As fixing agent in colored photography- Improve diffusion</td>
<td>Dutta et al., 2004</td>
</tr>
<tr>
<td>Cosmetics</td>
<td>Skin- and hair-care products</td>
<td>Hirano, 1996</td>
</tr>
<tr>
<td>Water treatment</td>
<td>Recovery of metal ions and pesticides, removal of phenols, proteins, radioisotopes, dyes, recovery of solid materials from food-processing wastes</td>
<td>Hirano, 1996; Shahidi et al., 1999</td>
</tr>
<tr>
<td>Others</td>
<td>Chitosan-coated paper, manufacturing material for fibres, cottons, films and sponges</td>
<td>Hirano, 1996</td>
</tr>
</tbody>
</table>
2.3 Chitinous waste management

As chitin and chitosan have a number of applications in various fields like Wastewater Treatment, Food, Biomedicine, Agriculture, Textile and paper, Biotechnology and Cosmetics etc. Therefore, the processing of chitinous waste to useful products becomes necessary. Chitinous waste is managed either by conventional method, chemical method or biological method. Conventional method makes use of disposing the waste either by burning or landfilling. But it leads to environment pollution as burning results in the production of carbon dioxide and carbon monoxide, which augments the global warming. Land filling is destructive owing to the slow degradation and formation of intoxicating pollutant of ground water i.e. ammonia (Muzzarelli, 1997). Alternatively, chitinous waste can be converted in to chitin by using chemical processes. These include demineralization and deproteinization by a strong acid or base but using these chemicals might result in the partial deacetylation of chitin and hydrolysis of the polymer. The production of chitosan from chitin by chemical method has many drawbacks: such as more energy consumption, waste of concentrated alkaline solution, broad and heterogeneous range of soluble and insoluble products (Jung and Park, 2014). In addition, it has adverse environmental implications as it produces more acidic and basic residues, which are then discharged into the environment, usually without treatment and without a view to re-use (Brine and Austin, 1981; Simpson et al., 1994; Wang et al., 1997).

The chief source of chitinous waste is the seafood industry. In order to preserve the carbon–nitrogen balance in the ecosystem, its recycling is enormously important. The insolubility of chitin and its inertness to most chemical agents necessitate the use of more feasible biological processes. Due to the insoluble and inert nature of chitin to a majority of chemicals, it becomes necessary to make use of workable biological processes. Keeping all the above facts in view, enzymes such as chitinase, chitosanase and chitin deacetylase which can be effectively used for the biodegradation and bioconversion of chitinous waste are discussed in this review. More emphasize is given to chitin deacetylase as it can be used in bioconversion of chitosan from chitin. The biological production of chitosan offers high specificity, simpler manipulation without pollution of the environment.

2.4 Enzymatic utilization of chitin

Enzymatic hydrolysis of chitin has been proposed as an alternative method for chitin utilization during the past few decades. Enzymes with hydrolytic activity on
Chitin include chitinase and chitin deacetylase. Chitosanase act on chitosan produced by enzymatic deacetylation of chitin by chitin deacetylase. These chitinolytic and chitosanolytic enzymes all have different modes of action and specificity for substrate size. The complete chart of hydrolysis of chitin by using different enzymes is shown in Fig 2.6. Enzymatic hydrolysis seems to be generally preferable to chemical methods because the reaction is performed under more gentle conditions and the degree of deacetylation of the product is homogeneous.

Fig 2.6 Enzymatic utilization of chitin

2.4.1 Chitinase

Chitinase enzymes are an important group of chitinolytic enzymes produced in nature which play the key role in biological hydrolysis of chitin. Chitinase was first described by Bernard (1911) in orchids bulbs in which it behaves like a thermosensitive and diffusible antifungal factor. The presence of chitinase in animals was first noticed in snails by Flach et al. (1992). Organisms like fungi, insects, plants, bacteria, viruses and animals have been reported to express the chitinase enzyme (Dahiya et al., 2006; Wang et al., 2009). The microbial source of chitinase includes Serratia marcescens, Xanthomonas maltophilia, Stenotrophomonas maltophilia, Paenibacillus illinoisensis (bacterial source) and Myrothecium verrucaria & Trichoderma species (fungal sources). Several other genus of bacteria like Streptomyces (Blaak et al., 1995; Suzuki et al., 2002), Alteromonas (Tsujibo et al., 1993), Escherchia (West et al., 1984),
Aeromonas (Sitrit et al., 1995) Pseudoalteromonas (Delpin and Goodman, 2009), Cyanobacterium (Prasanna et al., 2010), Aeromonas sp. No. 10S24 (Ueda et al., 1995), Pseudomonas aeruginosa K187 (Wang et al., 1997), Bacillus circulans WL12 (Mitsutomi et al., 1998) were also reported to produce chitinases. Chitinase were also found in insects & nematodes (involved in molting process) and human (reported in serum & gastric juices) (Sharma et al., 2011). Humans and plants do not contain any chitin, but still they express chitinases. The two well-known human chitinases are chitotriosidase (CHIT-1) and acidic mammalian chitinase (AMCase). The functions of CHIT-1 and AMCase are unknown, but they are thought to aid in the defense of chitin containing pathogens (Vega et al., 2012). In plants, chitinases provide defense against fungal and insect pathogens (Wang et al., 2012; Taira et al., 2002; Karasuda et al., 2003; Santos et al., 2004; Wang et al., 2009).

2.4.1.1 Mechanism of action of chitinase

Chitinase enzyme split the β-1, 4-N-glycosidic bond of chitin and generate Chitooligosaccharides (CHOS) (Songsiriritthigul et al., 2010, Neeraja et al., 2010b). Chitooligosaccharides are further converted into NAG and monosaccharides by the action of chitobiases (Suginta et al., 2005). The hydrolysis of the glycosidic linkage by chitinase is an acid catalyzed nucleophilic substitution reaction which takes places at the anomic carbon. The reaction can lead to either retention or inversion of the anomic configuration and occurs by one of the two general pathways: single displacement pathway or double displacement pathway (Adrangi et al., 2010). Both hydrolysis reactions require a pair of carboxylic acids at the active site of enzyme. First carboxyl group (catalytic group) that acts as a proton donor and the second carboxylic acid may act either as a base (as in the single-displacement mechanism) or a nucleophile (as in the double-displacement mechanism). In both mechanisms, the position of the proton donor is within hydrogen-bonding distance of the glycosidic oxygen (Aam et al., 2010; Udaya Prakash et al., 2010).

Double-displacement mechanism of hydrolysis

It is a two-step reaction, were the first step involves the protonation of the glycosidic oxygen (by the catalytic acid) and a congruent nucleophilic attack on the anomic carbon atom by the nucleophile (the second carboxylic acid). This attack leads to breakage of the glycosidic linkage and the formation of a covalent linkage between the anomic carbon and the catalytic nucleophile. Subsequently, this intermediate is hydrolyzed by a water molecule that approaches the anomic carbon
from a position close to that of the original glycosidic oxygen, leading to retention of the anomeric carbon configuration (Aam et al., 2010). In this mechanism, the β-(1, 4) glycosidic oxygen is first protonated which leads to the production of an oxocarbenium ion intermediate. It is then stabilized by a second carboxylate either through covalent or electrostatic interactions. This is followed by Nucleophilic attack of water which releases the hydrolysis products. Thus, the initial anomeric configuration is retained that means the stereochemistry of the anomeric oxygen at C-1 relative to the initial configuration is not undergoing inversion (Dahiya et al., 2006; Adrangi et al., 2010).

**Single-displacement mechanism of hydrolysis**

It is a “one-step” reaction, where the protonation of the glycosidic oxygen occurs simultaneously with a nucleophilic attack on the anomeric carbon by an activated water molecule. This water molecule is located between a carboxylic group and the anomeric carbon and it is activated by the carboxylic group that acts as a base. Since the water molecule approaches the anomeric carbon from the side of the catalytic base, this mechanism leads to inversion of the anomeric configuration. Chitinases belonging to family GH 19 use the inverting mechanism (Aam et al., 2010). The inversion of the anomeric configuration of the hydrolyzed GlcNAc residue is brought about by the single-displacement mechanism. The absence of a second acidic residue in the active site, which is capable of stabilizing the oxocarbenium ion leads to the Inversion of the stereochemistry by this method (Dahiya et al., 2006; Adrangi et al., 2010).

**2.4.1.2 Types of Chitinases**

Chitinases can be grouped into two major categories: Endochitinases and Exochitinases

Endochitinases (EC 3.2.1.14) are the chitinases which cleave randomly at internal sites of chitin. Thus it leads to the production of low molecular mass multimers of GlcNAc, such as chitotetraose, chitotriose, and diacetylchitobiose.

Exochitinases are the chitinases which cleave off chitobiose (GlcNAc)\(_2\) or chitotriose (GlcNAc)\(_3\) from the reducing or the nonreducing end of the chitin chain.

Exochitinases are further divided into two subcategories:

Chitobiosidases (EC 3.2.1.29) catalyze the progressive release of diacetylchitobiose starting at the non-reducing end of chitin microfibril.
β- (1, 4) N-acetyl glucosaminidases (EC 3.2.1.30) cleaves the oligomeric products of endochitinases and chitobiosidases and generates monomers of GlcNAc (Dahiya et al., 2006; Brurberg et al., 2000).

Chitinases are members of the superfamily of O-glycoside hydrolases (GH). The classification of glycoside hydrolases into families is based on amino acid sequence similarities. This classification reflects the structural features of these enzymes better than their sole substrate specificity and help to reveal the evolutionary relationships between these enzymes (Henrissat and Bairoch, 1996). Based on sequence homology the families 18, 19, 23, and 48 of glycosyl hydrolases (GH) belong to Chitinases while GH3, GH18, GH20, and GH84 comprise β-N-acetylhexosaminidases (Cantarel et al., 2009; Tsujibo et al., 2000). Family 20 includes the β-N-acetylhexosaminidases from bacteria, Streptomyces, and humans. In Family 18, the chitinase enzymes from prokaryotic and eukaryotic organisms were included. But family 19 included the chitinases only from higher plants and Gram-positive bacterium Streptomyces. Both the families contain endo-chitinases, as well as exochitinases (Brurberg et al., 2000; Dahiya et al., 2006; Karasuda et al., 2003). The chitinases of the GH18 and GH19 has different amino acid sequence. Their 3-D structures and molecular mechanisms are completely different. Therefore, they are considered to be evolved from different ancestors (Dahiya et al., 2006).

Chitinases comprises a huge and diverse group of enzymes. They vary in their molecular structure, substrate specificity, and catalytic mechanism (Kasprzewska, 2003).

Bacterial chitinases are included in GH18, GH19, and GH23 families. Bacterial chitinases have been classified into group A, B and C group. But group A chitinase genes are more abundant in nature than enzymes in groups B or C (Metcalfe et al., 2002). Most of the bacterial chitinases isolated and sequenced are from family GH 18 except chitinase (C-1) belongs to the family GH19. Chitinase (C-1) was isolated from S. griseus IIUT 6037 (Ohno et al., 1996). Unlike other chitinases, this (C-1) chitinase can only hydrolyze GlcNAc-GlcNAc and GlcNAc-glucosamine linkages. Because, it’s catalytic site is different from other microbial chitinases. The amino terminal region of chitinase C-1 serves as binding site of chitin and also share sequence homology with non-catalytic domain of other bacterial lytic enzymes (Hamid et al., 2013). Family GH19 represented by only few bacterial genera like purple bacteria and actinobacteria (Udaya-Prakash et al., 2010). Only one GH23 chitinase has been identified isolated
from *Ralstonia sp. A-471* and comprises an N-terminal chitin-binding domain linked to a C-terminal catalytic domain (Ueda et al., 2009).

Mammalian chitinases also belong to family GH18. They are divided on the basis of their enzymatic activity as non-enzymatic chitinase like protein and truly active chitinases (Bussink et al., 2007). Chitotriosidase was first mammalian chitinase to be identified which is produced by macrophages (Boot et al., 1995). It provide defense against chitin-containing pathogens (Bussink et al., 2006). Chitotriosidase may also show some protective effects against atherosclerosis (Kitamoto et al., 2013). Another chitinase named acidic mammalian chitinase (AMCase) has been isolated and shown to be expressed primarily in the gastrointestinal tract and lungs of both mouse and human (Boot et al., 2001). The GH18 family members consists of triose-phosphate isomerase fold in their N-terminal catalytic domain, characterized by the \((\beta/\alpha)\)-barrel structure, contains the \(\beta\)-strand consists of a conserved sequence motif \(\text{DXXDXDXE}\), where \(\text{D} = \text{aspartic acid, E = glutamic acid, and X = any amino acid}\) which forms the active site of the enzyme (Chou et al., 2006). In chitinase-like proteins glutamic acid is substituted to glutamine, leucine, and isoleucine which are responsible for the lack of chitinolytic activity in these enzymes. Although, the conserved residues meant for binding of chitin on the triose-phosphate isomerase barrel are not affected, so they can still bind with high affinity to chitin (Webb et al., 2001).

Plant chitinases have been categorized into I-VII classes. Class I, II, IV, VI, and VII are included in GH19 family where as class III and V are included in GH18 family. The Class I, II, IV, VI, and VII chitinases containing globular domains and class III and V chitinases domain are formed by 8 \(\alpha\)-helices and 8 \(\beta\)-strands (Fukamizo et al., 2003; Ohnuma et al., 2011, 2012). Plant chitinases protect them against the attack of phytopathogens (Hamid et al., 2013). For example, chitinase isolated from moth beans has been tested against mycopathogen *Macrophomina Phaseolina* strain 2165 (Garg and Gupta, 2010). Chitinases can also act as calcium storage proteins. The class III chitinase from *Punica granatum* seeds that binds calcium ions with high capacity has been reported by Yang et al. (2011).

All Insect chitinases are included in family GH18. They have been categorized into 8 groups namely I-VIII on the basis of amino acid sequence similarities (Arakane and Muthukrishnan, 2010; Zhang et al., 2011a). Groups I and II contain different numbers of catalytic and CBM domains while group III contain two catalytic domains and a single CBM domain. Groups IV, V, VII and VIII contain single catalytic domain
only whereas group VI chitinases have one catalytic domain, one CBM and a long C-terminal stretch with a high percentage of serine and threonine residues (Adrangi et al., 2013). The insect’s chitinase has been reported in *Manduca sexta*, tobacco hornworm and *Bombyx mori*. During ecdysis in insects the endochitinases randomly break the cuticle to chitooligosaccharides. These chitooligosaccharides are then hydrolyzed by exoenzymes to N-acetylglucosamine. Insect chitinases are inhibited by allosaminidin (Koga et al., 1997).

Chitinase enzyme from fungal cells belongs to GH18 family and divided into subgroups A, B and C like bacterial chitinases based on amino acid sequence homology (Adrangi et al., 2013). Subgroup A chitinases contains a single catalytic domain and no CBMs. Subgroup B chitinases contain catalytic domain along with either a C-terminal CBM or an unstructured serine/threonine-rich domain. Subgroup C chitinases consist of an N-terminal CBM linked to a C-terminal catalytic domain. Subgroup A and C are processive in nature whereas Subgroup B chitinases are non-processive in nature (Hartl et al., 2012). Endochitinase isolated from *Trichoderma* have been reported as the most effective biocontrol agent against soil-borne mycopathogens (Hamid et al., 2013).

### 2.4.2 Chitosanase

Chitosanases are the enzymes that specifically hydrolyze chitosan but not chitin, to generate low molecular weight chitosan and chitosan oligomers (Gupta et al., 2012; Kim & Rajapakse, 2005). Chitosanase is produced from different microorganisms including bacteria, fungi and cyanobacteria and plants. Chitosanase was first described in 1973 from different soil microorganisms since then several studies have been carried out on its occurrence, production, purification and characterization (Somashhekar and Joseph, 1996). The different sources of chitosanases are given in Table 2.2. Promising application of this enzyme in food and pharmaceutical industries has increased due to the popularity of chito-oligosaccharides and their bioactive properties possessed. This enzyme hydrolyzes the 2-amino-2-deoxy-\(D\)-glucoside linkages to degrade chitosan into *N*-chitooligosaccharides (Tanabe et al., 2003; Hutadilok et al., 1995). Chito-oligosaccharide possesses higher advantages over its polymer i.e. chitosan. They are biologically more active and water soluble. Therefore, chito-oligosaccharide is widely used for functional food ingredient and other pharmaceutical products (Chasanah et al., 2009). Chitosans oligosacharide are also produced by chitinase enzyme but the difference between chitinase and chitosanase is narrow as both can act on different
degrees of deacetylated chitosan, but chitosanase prefers highly deacetylated chitosan, whereas chitinase prefers highly acetylated chitosan (Somashekar and Joseph, 1996).

Table: 2.2 Sources of Chitosanase

<table>
<thead>
<tr>
<th>Source</th>
<th>Reference</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacteria</strong></td>
<td></td>
<td><strong>Fungi</strong></td>
<td></td>
</tr>
<tr>
<td>Serratia sp.</td>
<td>Wang et al., 2008b</td>
<td>Aspergillus sp.</td>
<td>Zhang et al., 2012</td>
</tr>
<tr>
<td>Janthinobacterium sp.</td>
<td>Johnsen et al., 2010</td>
<td>Gongronella sp.</td>
<td>Wang et al., 2008a Zhou et al., 2008</td>
</tr>
<tr>
<td>Paenibacillus sp.</td>
<td>Zitouni et al., 2012</td>
<td>Trichoderma sp.</td>
<td>da Silva et al., 2012</td>
</tr>
<tr>
<td>Acinetobacter sp.</td>
<td>Wang et al., 2011</td>
<td><strong>Cyanobacteria</strong></td>
<td></td>
</tr>
<tr>
<td>Streptomyces sp.</td>
<td>Jiang et al., 2012</td>
<td>Anabaena</td>
<td>Gupta et al., 2012</td>
</tr>
<tr>
<td></td>
<td></td>
<td>fertilissima RPAN1</td>
<td></td>
</tr>
<tr>
<td>Bacillus sp.</td>
<td>Gao et al., 2008; Wang et al., 2009; Zitouni et al., 2012</td>
<td></td>
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</table>

Enzyme Nomenclature Commission created the definition of chitosanase (EC 3.2.1.132, Chitosan N-acetyl glucosaminohydrolase) in 1990 and amended it in 2004. According to the sequence-based classification of glycoside hydrolases created by Henrissat and developed into the CAZY database, enzymes with chitosanase activities belong to families 3, 5, 7, 8, 46, 75 and 80. Among these, only families 46, 75 and 80 include enzymes specific for hydrolysis of chitosan. The GH46 family members have been characterized most extensively compared with other chitosanases, especially those from Bacillus and Streptomyces have been studied in terms of their catalytic features, enzymatic mechanisms and protein structures. The family itself was officially created in 1996 (Henrissat, 1996) and classified as EC 3.2.1.132 in the IUBMB Enzyme Nomenclature List (Wang et al., 2008a).
Chitosanase brings about the endohydrolysis of the $\beta-1, 4$-linkages between N-acetylglucosamine (GlcN) residues in a partly acetylated chitosan. It acts on the reducing end of chitosan molecule and produces chitosans oligosaccharides (COS) exclusively as the end product. They can hydrolyse various types of links in the chitosan molecule (Kim and Rajapakse, 2005). Chitosanases are classified into three distinct subclasses viz. Subclass I chitosanases, Subclass II chitosanases and Subclass III chitosanases on the basis of their specificity of the cleavage positions for the partially acetylated chitosan. Subclass I chitosanases split both GlcN–GlcN and GlcNAc–GlcN linkages. Subclass II chitosanases can cleave only GlcN–GlcN linkages. However, subclass III chitosanases split both GlcN–GlcN and GlcN–GlcNAc linkages. Endohydrolysis of GlcN–GlcN links in chitosan is common and the only constant property to all the known chitosanases. Also, GlcNAc–GlcNAc links in partly acetylated chitosan are not recognized by them. This selective behaviour might be controlled by rigid substrate recognition by the chitosanases of different subclasses at the position where cleavage of the substrates may occur. The presence of the reducing and non-reducing ends and degree of deacetylation of chitosan determine the specificity of chitosanases with respect to the cleavage of four different glycosidic linkages in partially N-deacetylated chitosan (Kim and Rajapakse, 2005). New type of enzyme exo-$\beta$-D-glucosaminidase, which attack chitosan and/or chitosans oligosaccharides (COS) from the non-reducing termini to remove successive GlcN residues, have been reported from different microorganisms (Fukamizo et al., 2006). The other names of this enzyme are exochitosanase and GlcNase. These exo-hydrolytic enzymes can degrade GlcN–GlcNAc but not GlcNAc–GlcNAc linkages.

Microbial chitosanases with different biological roles have been found in nature. Chitosan degrading microorganisms are widely distributed in nature and for their nutritional purpose secrete chitosanase extracellularly to degrade chitosan (Somashekar and Joseph, 1996). Chitosanase together with chitinase, chitin deacetylase and glucosaminidase is involved in the decomposition and recycling of enormous quantity of crustaceans shell produced in nature. Since chitosan is the major structural component in cell wall of Zygomycetes fungi, chitosanases contributed significantly in the determination of the degradation of chitosan and morphogenesis of this class of fungi. In addition to this, plants synthesise chitosanase as a defensive mechanism against phytopathogens especially against Zygomycetes (Hsu et al., 2012).
2.4.3 Chitin deacetylase

As defined in the CAZY database (http://afmb.cnrs-mrs.fr/~cazy/CAZY), Chitin deacetylase is a polysaccharide deacetylase which is a member of Carbohydrate Esterase 4 (CE-4s) superfamily. Chitin deacetylase share the NodB homology domain or polysaccharide deacetylase domain, a conserved domain in the primary structure of CE4 family (Zhao, 2010). Based on the sequence homology, CEs are classified in to 14 families. The family 4 is by far the largest of the CE families, with over 1000 open reading frames. There are five members in this family namely, rhizobial Nod B chitooligosaccharide deacetylases (EC 3.5.1.-), Chitin deacetylase (EC 3.5.1.41), peptidoglycan N-acetylglucosamine deacetylases (pgdA) (EC 3.1.1.-), acetyl-xylan esterases (EC 3.1.1.72) and xylanases (Coutinho and Henrissat, 2002; Chambon et al., 2017; Blair et al., 2006). The CDA, rhizobial Nod B chitooligosaccharide deacetylases and pgdA catalyzes the hydrolysis of N-linked acetyl groups from GlcNAc residues of their substrates namely chitin, Nod B factors and peptidoglycan respectively. Whereas acetyl xylan esterases and xylanases catalyze the hydrolysis of O-linked acetyl groups from O-acetylxylose residues of acetyl xylan. The members of the CE4 family are metal-dependent enzymes (Blair and Aalten, 2004).

Chitin deacetylase catalyzes hydrolysis of the acetamido group in the N-acetyl glucosamine units of chitin and chitosan, thus generating glucosamine units and acetic acid (Ischaidar et al., 2014). Chitin deacetylases have been divided into two subgroups. Intracellular chitin deacetylases are secreted into the periplasmic space e.g. M. rouxii and A. coerulea whereas extracellular chitin deacetylases are secreted into the culture medium e.g. Colletotrichum lindemuthianum and Aspergillus nidulans.

2.4.3.1 Sources of chitin deacetylases

Chitin deacetylases have been reported in insects, bacteria and several fungi. It is also reported in a few crustaceans and actinomycetes. Araki and Ito (1975) was the first to discover chitin deacetylase from the extracts of M. rouxii. Davis et al. (1984) found that the enzyme was associated with cell wall synthesis of M. rouxii by converting nascent chitin into chitosan.

The different sources of chitin deacetylase are summarized in Table 2.3.

2.4.3.1.1 Fungal chitin deacetylase

The chitin deacetylase have been reported in several fungi. Petlamul and Prasertsan (2012) checks the efficacy of chitin deacetylase from Metarhizium anisopliae and Beauveria bassiana against a cutworm Spodoptera litura (Lepidoptera:
Noctuidae). Wang et al. (2009) expressed the heat-Stable chitin Deacetylase gene from *A. nidulans* was subcloned in Escherichia coli BL21 by using pET28a vector. The recombinant protein was then purified by metal affinity chromatography using a His-bind column. Araki and Ito (1975) and Kafetzopoulos et al. (1993a) have been carried out the partial purification and characterization of CDA from *Mucor rouxii*. Pareek et al. (2014) evaluated chitin deacetylase from *Penicillium oxalicum* SAEM-51 for bioconversion of chitin to chitosan. Martinou et al. (2002) purified and characterized a cobalt-activated CDA (Cda2p) from *Saccharomyces cerevisiae* and observed that deglycosylation of the enzyme resulted in complete loss of enzyme activity which was restored by addition of 1 mM CoCl2. The role of a heat stable CDA in *A. nidulans* was studied by Alfonso et al. (1995). Deising et al. (1995) purified chitin deacetylase from plant pathogenic fungus *Uromyces uicipiae-fabae* and detected the five isoforms of the enzyme with apparent molecular masses of 48.1, 30.7, 25.2, 15.2 and 12.7 kDa. Gao et al. (1995) purified and characterized the CDA from the zygomycetous fungus *A. coerulea* whereas Tsigos and Bouriotis, (1995) purified and characterized CDA from the culture filtrate of the plant pathogenic fungus *C. lindemuthianum*. Zhang et al. (2014) optimized fermentation conditions for producing highly active CDA from *Rhizopus japonicus* M193 by using Plackett–Burman design based on the response surface methodology followed by the Taguchi design with orthogonal. Matsuo et al. (2005) analyzed the role of *Schizosaccharomyces pombe* cda1+ gene (SPAC19G12.03), which encodes a protein homologous to chitin deacetylases, in spore formation of *S. pombe*.

Chambon et al. (2017) efficiently produced and purified recombinant *Sinorhizobium meliloti* NodB CD (chitinoligosaccharide deacetylase) from *E. coli* as a thioredoxin fusion protein, under soluble and catalytically active form and used it for in vitro synthesis of lipo-chitinoligosaccharide precursors which are key molecules for the establishment of plant-microorganisms symbiosis. Cai et al. (2013) carried out parametric optimization of extracellular Chitin Deacetylase production by *Scopulariopsis brevicaulis*. Martinou et al. (1993) carried out 169-fold one-step purification of chitin deacetylase by immunoaffinity chromatography. Cord-Landwehr et al. (2016) identified a chitin deacetylase from an endophytic fungus *Pestalotiopsis* sp. (PesCDA) and expressed it in *E. coli* to study the role of the chitin deacetylase in the survival of *Pestalotiopsis* sp. in plants by avoiding the plant immune system. Suresh et al. (2011) produced extracellular chitin deacetylase from native soil isolates of *P.*
monoverticillium CFR 2 and Fusarium oxysporum CFR 8 under solid state fermentation using commercial wheat bran (CWB) and shrimp processing by-products (SPP) as solid substrate. Pacheco et al. (2013) produced and purified the chitin deacetylases from the phytopathogenic fungus C. gloeosporioides.

Christodoulidou et al. (1996) studied the role of CDA1 and CDA2 genes in the formation of the ascospore wall of S. cerevisiae. Chakraborty et al. (2016) isolated the yeast C. laurentii strain RY1 from Kombucha tea while attempting to isolate nitrogen fixing bacterial strains on medium that have no combined nitrogen. They studied the effect of nitrogen on the chitin deacetylase production from C. laurentii strain RY1.

Table: 2.3 Sources of chitin deacetylase

<table>
<thead>
<tr>
<th>Fungi</th>
<th>Reference</th>
<th>Crustaceans</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metarhizium anisopliae</td>
<td>Petlamul and Prasertsan, 2012</td>
<td>Penaeus monodon</td>
<td>Sarmiento et al., 2016</td>
</tr>
<tr>
<td>Beauveria bassiana</td>
<td>Petlamul and Prasertsan, 2012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspergillus nidulans</td>
<td>Wang et al., 2009</td>
<td></td>
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</tr>
<tr>
<td>Penicillium oxalicum SAEM-51</td>
<td>Pareek et al., 2014</td>
<td>Anopheles gambiae</td>
<td>Dixit et al., 2008</td>
</tr>
<tr>
<td>Aspergillus nidulans</td>
<td>Alfonso et al., 1995</td>
<td>Apis mellifera</td>
<td>Dixit et al., 2008</td>
</tr>
<tr>
<td>Uromyces uiciei-fabae</td>
<td>Deising et al., 1995</td>
<td>Helicoverpa armigera</td>
<td>Campbell et al., 2008</td>
</tr>
<tr>
<td>Absidia coerulea</td>
<td>Gao et al., 1995</td>
<td>Tribolium castaneum</td>
<td>Dixit et al.2008</td>
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<td>Colletotrichum lindemuthianum</td>
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<td>Rhizopus japonicus M193</td>
<td>Zhang et al., 2014</td>
<td>Trichoplusia ni</td>
<td>Guo et al., 2005</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>Matsuo et al., 2005</td>
<td>Drosophila melanogaster</td>
<td>Luschnig et al., 2006</td>
</tr>
<tr>
<td>Sinorhizobium meliloti</td>
<td>Chambon et al., 2017</td>
<td></td>
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</tr>
<tr>
<td>Pestalotiopsis sp.</td>
<td>Landwehr et al., 2016</td>
<td>Bacteria</td>
<td></td>
</tr>
<tr>
<td>Cryptococcus laurentii strain RY1</td>
<td>Chakraborty et al., 2016</td>
<td>Bacillus cereus</td>
<td>Raval et al., 2013</td>
</tr>
</tbody>
</table>
2.4.3.1.2 Bacterial chitin deacetylase

There are a few reports on chitin deacetylase production from bacteria. Chitin deacetylase have been isolated and characterized from acidophillic *Bacillus* sp. (Natsir, 2000; Rahayu, 2000) and *B. stearothermophilus* (Toharisman et al., 2001). Toharisman and Suhartono (2008) carried out partial purification and characterization of chitin deacetylase from *B. thermoleovorans* lw-4-11. Ravikumar and Perinbam (2016) isolated a novel marine bacteria *B. cereus* TK19 which have the ability of producing a promising amount of chitin deacetylase in a shorter time of 48 h under optimized conditions. The optimization of the medium components for chitin deacetylase production from *B. amyloliquefaciens* Z7 by using Plackett–Burman design and Box–Behnken response surface methodology (RSM) was carried out by Yuanhao et al. (2014). Under the optimal conditions, the CDA activity of *B. amyloliquefaciens* Z7 increased distinctly from 18.75 to 27.48 U/ml (46.6% increase in total yield). But Zhou et al. (2010) carried out fermentation optimization of chitin deacetylase production from *B. amyloliquefaciens* by conventional method. Kaur et al. (2012) isolated two
chitin deacetylase producing bacteria from flora of seashore. Based on the morphological and physiochemical analysis, the two isolates were identified as *Bacillus* sp. and *Serratia* sp.. Raval et al. (2013) cloned the gene for chitin deacetylase from *B. cereus* into pET22b vector and the resulting construct with six His tag was subsequently expressed in *E. coli* pLysS cells. The expressed chitin deacetylase was used for enzymatic modification of chitosan. Ischaidar et al. (2014) determine the pathogenecity of chitin deacetylase from *B. licheniformis* HSA3-1a as biotermiticide.

The chitin deacetylase gene was found to be involved in the chitin catabolic cascade of Vibrios (Jung et al., 2008). In Vibrio cholera, the chitobiose induced genes were required for the transport and catabolism of non-acetylated chitin residues whereas the chitin deacetylase was found to be active with chitin oligosaccharides (Meibom et al., 2004). Further, it was suggested that the oligomers generated by deacetylases could play a role in cellular communications (Li et al., 2007). From the gene bank sequence data, the chitin deacetylase from Vibrios and photobacteria were found to be highly conserved.

### 2.4.3.1.3 Insect chitin deacetylase

All arthropods that contain chitin in their exoskeleton appear to have genes that encode chitin deacetylases (CDAs) or closely related enzymes (Zhu et al., 2016). The genomes of the red flour beetle, *Tribolium castaneum*, the fruit fly (*Drosophila melanogaster*), the malaria mosquito (*Anopheles gambiae*) and the honey bee (*Apis mellifera*) contain 9, 6, 5 and 5 genes, respectively, that encode proteins with a chitin deacetylase motif (Dixit et al., 2008; Luschnig et al., 2006). In *D. melanogaster*, the elongation of tracheal tubes was restricted by proteins containing CDA domains, presumably by modification of terminal GlcNAc of the elongating chitin chain (Luschnig et al., 2006; Wang et al., 2006). The 5 major classes of CDA like proteins were reported in *T. castaneum* and one of them was found to be specifically expressed in the gut (Dixit et al., 2008). Most of the reported insect CDAs and/or CDA like proteins were associated with the midgut peritrophic membrane (PM) and evenly distributed throughout the entire length of PM. These enzymes were detected in larval midgut tissue during the feeding period and the presence of these enzymes may be essential for increased absorption of nutrients. In *Mamestra configurata*, the CDA activity was detected from gut proteins expressed in E. coli (Toprak et al., 2008). A novel midgut peritrophic membrane protein (Tn PM-P42) from the cabbage looper, *T. ni* was identified that possessed a putative polysaccharide deacetylase domain with
sequence similarities to the CDA domains from fungi like *Amylomyces rouxii*, *S. cerevisiae* CDA1 and CDA2 and nodulation protein B from chitooligosaccharide deacetylase of *S. meliloti* (Guo et al., 2005).

### 2.4.3.1.4 Other chitin deacetylase

Chitin deacetylase have been studied and characterized from fungi, bacteria and insects. But chitin deacetylase from actinomycetes and crustaceans has been not reported yet. Luong et al. (2013) isolated a total of 100 actinomycete strains from soil samples in Cuc Phuong National Park in Ninh Binh Province, Vietnam on ISP-4 agar incubated at 30°C for 7 days. Fifty isolates show chitinase activity on the agar plates with clear zone over 0.5 cm. Ten isolates produced both chitinase and chitin deacetylase. Based on 16S rRNA gene sequence similarities the highest chitin deacetylase and chitinase producing strain I2 has been identified as *Streptomyces xylophagus*, with sequence similarity of 99.9% (1449/1450 bp). Sarmiento et al. (2016) for the first time cloned the full length cDNA of chitin deacetylase gene from the gills of black tiger shrimp *Penaeus monodon* (crustacean) that survived White Spot Syndrome Virus (WSSV) infection.

### 2.4.3.2 Chitin deacetylase assay

For measuring enzyme activity, the use of a reliable and reproducible method is the main objective. Several procedures have been developed for the assay of chitin deacetylase. This review summarizes many of current methods used to determine the chitin deacetylase activity. The different assays for determining chitin deacetylase activity are radiometric assay, colorimetric method and detection by electrophoresis. The radioactive assay is more rapid and simpler than the colorimetric assay (Araki and Ito, 1975). This assay is very sensitive and deals with the quantitative release of radioactively labeled acetic acid (tritiated) from glycol chitin. Yuanhao et al. (2014) estimated chitin deacetylase activity by using partially O-hydroxyethylated chitin (glycol chitin) radiolabelled in N-acetyl groups as substrate. There are some drawbacks of this assay. Firstly, it is not easy to evaluate the extent and distribution of derivatization (O-hydroxyethyl groups) in commercially available glycol chitin. Secondly, the effect of derivatization on enzyme activity cannot be determined (Martinou et al., 1995). Furthermore, the radiometric method cannot be employed in case of non-radio labeled natural substrates.

The colorimetric method is probably the most common because of the high standards of accuracy and reliability that can be obtained from modern
spectrophotometers. The use of different colorimetric methods using different substrates and reagents has been reported by several workers. Several substrates like ethylene glycol chitin, glycol chitin, water soluble chitosan (WSCT-50), hexa (N-acetyl) chitohexaose and N-acetylchitopentose has been used as a model substrate for determination of chitin deacetylase activity (Kafetzopoulos et al., 1993a; Amorim et al., 2005; Luong et al., 2013). Toharisman and Suhartono (2008) estimated chitin deacetylase activity by using glycol chitin as a substrate by the method of Tokuyasu et al. (1996). The concentration of glucosamine residues produced by the deacetylation reaction was estimated by oxidation using NaNO₂, followed by a spectrophotometric method using indole/HCl (Dische and Borenfreund, 1950; Ravikumar and Perinbam, 2016; Ischaidar et al., 2014). The use of p-nitroacetanilide as the substrate for measuring chitin deacetylase activity has also been reported. It is a new method for chitin deacetylase assay. The reaction mixture consist of p-nitroacetanilide, diluted enzyme solution, and 0.2 M phosphate buffer (pH 7.0).Chitin deacetylase activity was determined by measuring amount of p-nitroaniline released from p-nitroacetanilide at OD 400nm (Sun et al., 2014; Liu et al., 2016). Aguila et al. (2012) estimated the chitin deacetylase activity by using synthetic chitooligosaccharides (di-N-acetyl chitobiose, tri-N-acetylchitotriose, and tetra-N-acetyl chitotetraose) or crystalline shrimp chitin. In brief, each synthetic substrate or crystalline shrimp chitin was incubated with the purified recombinant chitin deacetylase and bovine serum albumin (BSA) for 1 h at 50°C. The reaction was stopped by heating at 100°C for 10 min. The amount of primary amine formed was determined spectrophotometrically after derivatization with o-phthalaldehyde (OPA). One unit of enzyme activity is defined as the amount of the enzyme required to produce 1 µmol of primary amine per minute under the above conditions. The use of MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) for measuring the chitin deacetylase activity has also been reported (Wang et al., 2010; Nahar et al., 2003; Cai et al., 2013).

Chitin deacetylase activity can also be assayed by the native or denaturing polyacrylamide gel electrophoresis (PAGE) (Trudel and Asselin, 1990). Several molecular forms of enzymes involved in chitin hydrolysis have been described because of the availability of activity staining after PAGE (Trudel and Asselin, 1990, Laemmli et al., 1970). This approach is based on the capacity to distinguish between chitin (intact acetylated substrate) and chitosan (deacetylated substrate) (Araki and Ito, 1975, 1988). Several methods were tested using several dyes that would allow distinguishing
chitin and chitosan embedded in polyacrylamide gels (Deising et al., 1995). Howard et al. (2003) reported that Calcofluor white M2R was found to interact more strongly with chitosan than with chitin. Thus the zone of chitin deacetylase activity appears as bands of bright blue fluorescence against a blue fluorescent background. They noted the same result with glycol chitin when the later was embedded in polyacrylamide gels. Trudel and Asselin (1990) also reported that Calcofluor white M2R staining with chitosan was much more fluorescent under UV and this observation was the basis of the first criterion used to detect chitosan generated from chitin by enzymatic means. Luong et al. (2013) estimated chitin deacetylase activity using WSCT-50 as substrate. They used HPLC method for determining the acetic acid released in the enzymatic deacetylation.

2.4.3.3 Purification of chitin deacetylase

Kafetzopoulos et al. (1993a) carried out purification of chitin deacetylase from *M. rouxii* using Q-Sepharose and S Sepharose with an overall yield of 11.9% and a purification fold of 97.3. The specific activity of pure protein was 2.968 U/mg. Gao et al. (1995) purified chitin deacetylase from *A. coerulea* using ammonium sulfate, Butyl Toyopearl, Gigapite and DEAE-Toyopearl with a purification factor of 516 and overall yield of 4.1%. The specific activity increase from 0.022 to 11.37 U/mg. Chitin deacetylase from *C. lindemuthianum* has been purified to homogeneity using Q-Sepharose, S300 and Mono S column with a purification fold of 110, overall yield of 16% and specific activity of 0.219 U/mg (Tsigos et al., 1995). Shrestha et al. (2004) reported the specific activity of 72 U/mg of the pure protein from *C.lindemuthianumUPS9*. Tokuyasu et al. (1996) purified chitin deacetylase from *C. lindemuthianum*, reached a 994-fold purification with a recovery of 4.05% and specific activity of 18.4 U/mg. Chitin deacetylase from *B. thermoleovorans* LW-4-11 has been purified to homogeneity with a purification fold of 4.28, overall yield of 36.7% and specific activity of 43.7 mU/mg (Toharisman and Suhartono, 2008). Martinou et al. (1993) carried out purification of chitin deacetylase from *M. rouxii* by using immunoaffinity chromatography. The enzyme was purified to homogeneity with a purification fold of 1333.0, overall yield of 35% and specific activity 25U/mg. Chitin deacetylase from *A. nidulans* has been purified to homogeneity using desalting PD-10, Mono Q HR 5/5 and Superose 12 HR 10/30 with a purification fold of 17.4 and specific activity increase from 1.4 to 25.0 mU/mg (Alfonso et al., 1995).
2.4.3.4 Biochemical characteristics of chitin deacetylases

Chitin deacetylase has been isolated and purified from several fungi and bacteria. But the chitin deacetylase from fungi have been amply documented and well studied in terms of their purification and characterization. The properties of these purified chitin deacetylases, such as molecular mass, optimum pH and temperature, effect of metal ions, and substrate specificity, have also been investigated.

All chitin deacetylases are glycoproteins and exist in the N-glycosylated form (20–70%) (Deising et al., 1995). The fungal chitin deacetylases are secreted either into the periplasmic space or into the culture medium depending on their function. The intracellular chitin deacetylases were mostly found in the Zygomycetous fungi which contain chitosan as the major component of their cell wall. The chitin deacetylases from *M. rouxii*, *A. coerulea*, *A. nidulans* and *Cunninghamella bertholletiae* were found to be secreted into the periplasmic space and were generally associated with cell wall modification. The presence of extracellular CDAs was reported in plant and insect pathogenic fungi namely *C. lindemuthianum* and *M. anisopliae* respectively (Kauss et al., 1982; Nahar et al., 2004) and was reported to be associated with their protection against plant and insect chitinases elicited by the chitin oligomers. The extracellular CDA from *A. nidulans* was found to be secreted into the culture medium during the phase of autolysis (Alfonso et al., 1995). Further, the CDA activity of *C. lindemuthianum* was detected once the mycelial growth was completed after 8 d incubation in the medium. The secretion of CDA was correlated to the induction of black spore formation in *C. lindemuthianum* apart from its role in self defense (Tokuyasu et al., 1996).

2.4.3.4.1 Molecular mass

The molecular mass for most of chitin deacetylases is in the range of 25–80 kDa, although a 150 kDa molecular mass was found in *C. lindemuthianum* DSM 63144 (Kauss et al., 1982) and less than 150 kDa in *C. lindemuthianum* (Tsigos et al., 1995). The molecular mass of chitin deacetylase was reported 75 kDa in *A. coerulea* (Gao et al., 1995), 27.5 and 27.3 kDa in *A. nidulans* (Alfonso et al., 1995), 31.5-33 kDa in *C. lindemuthianum* (Tokuyasu et al., 1996), 25 kDa in *C. lindemuthianum* (Shrestha et al., 2004), 31 kDa in *Flammulina velutipes* (Yamada et al., 2008), 70 kDa in *G. butleri* (Maw et al., 2002), 75 kDa in *M. rouxii* (Araki and Ito 1975), 75-80 kDa in *M. rouxii* (Hunt et al., 2008), 75 kDa in *R. circinans* (Gauthier et al., 2008), 100 kDa in *R. nigricans* (Jeraj et al., 2006), 43 kDa in *S. cerevisiae* (Martinou et al., 2002), 55 kDa in *S.
brevicaulis (Cai et al., 2006) and 97.6 kDa (CDA1) and 221.7 kDa (CDA2) in A. butleri dr. (Nadarajah et al., 2006). The molecular weight of purified chitin deacetylase from M. rouxii was ~ 80 kDa (Kafetzopoulos et al., 1993a).

The two bands of 30 and 32 kDa were observed in purified chitin deacetylase of B. cereus (Raval et al., 2013). Chitin deacetylases also exist in multiple isoforms. For instance, five isoforms (12.7, 15.2, 25.2, 30.7 and 48.1 kDa) of chitin deacetylase were produced from the broad bean rust fungus of Uromyces viciae-fabae (Deising and Siegrist, 1995). In addition, four isoforms (64, 35, 30 and 26 kDa) in R. nigricans (Trudel and Asselin, 1990), three isoforms of chitin deacetylase from M. racemosus (64, 30 and 26 kDa), three isoforms of chitin deacetylase (70, 37 and 26 kDa) in M. anisopliae (Nahar et al., 2004) and two isoforms of chitin deacetylase (59 and 50 kDa) in Mortierella sp. DY-52 (Kim et al., 2008) have been reported. The chitin deacetylase isolated from the metagenomic library with 10 clones show molecular weight of 43kDa (Liu et al., 2016).

2.4.3.4.2 Optimum pH

Enzymes are affected by changes in pH. The most favourable pH value, the point where the enzyme is most active is known as optimum pH. The pH optimum depend on many factors such as reaction time, temperature, type of substrate, substrate concentration and the chemical properties of the medium in which the reaction is being carried out, such as ionic strength, type and source of the enzyme and its purity (Whitaker, 1994).

The optimum pH of most extracellular chitin deacetylase is neutral or in the alkaline ranges from 7–12, while most intracellular chitin deacetylase have optimal pH values in the 4.5–6 range. Wang et al. (2010) cloned a gene encoding chitin deacetylase from A. nidulans and expressed in E. coli BL21. The recombinant chitin deacetylase showed high activity at pH 6.0–9.0 and the optimal pH was 8.0. The pH optima of chitin deacetylase from C. lindemuthianum appeared quite similar, with optimal pH 8.0 of S. cerevisiae (Shrestha et al., 2004; Martinou et al., 2002). Optimal enzyme activity of chitin deacetylase at pH 8.5 has been reported in C.lindemuthianum (Kauss et al., 1982; Tsigos et al., 1995) and M. anisopliae (Nahar et al., 2004). The optimum pH 7.0 was reported in A. nidulans (Alfonso et al., 1995), F. velutipes (Yamada et al., 2008), M. racemosus and R. nigricans (Trudel and Asselin, 1990) where as optimum pH 7.5 was reported in S. brevicaulis (Cai et al., 2006). The optimum pH 5.0 was reported in A. coerulea (Gao et al., 1995) where as optimum pH 5.5 was reported in M. rouxii
(Araki and Ito, 1975) and Mortierella sp. DY-52 (Kim et al., 2008). Uromyces viciae-fabae and R. circinans exhibit pH optima at 5.5-6.0 (Deising and Siegrist, 1995 and Gauthier et al., 2008) and C. lindemuthianum exhibit pH optima at 11.5-12.0 (Tokuyasu et al., 1996). The chitin deacetylase isolated from the metagenomic library with 10 clones show an optimum pH 7.4 (Liu et al., 2016). The chitin deacetylase from B. thermoleovorans LW-4-11 exhibit an optimum pH 6.0 (Toharisman and Suhartono, 2008) whereas CDA from B. cereus showed an optimum of pH 7.0 (Raval et al., 2013). C. berthollitae and M. rouxii exhibit their optimal activity at pH 4.5 (Amorim et al., 2005; Kafetzopoulos et al., 1993a; Hunt et al., 2008).

2.4.3.4.3 Optimum temperature

In general, temperature plays a very important role in all reactions; it affects the stability of the enzymes and their corresponding substrates, the availability of substrates and cofactors, the affinity of the enzyme towards the substrate, the activators and inhibitors as well as the formation of by-products (Whitaker, 1994).

The optimal temperature is 50–60°C for nearly all extracellular as well as intracellular chitin deacetylase. The recombinant chitin deacetylase from A. nidulans show optimum temperature 50°C and retains more than 60% of the enzyme activity within a range of 30–70°C (Wang et al., 2010). The temperature optima of 60°C were reported in C.lindemuthianum (Tokuyasu et al., 1996; Shrestha et al., 2004), F. velutipes (Yamada et al., 2008) and Mortierella sp. DY-52 (Kim et al., 2008). The temperature optima of 50°C was reported in C. berthollitae (Amorim et al., 2005), S. cerevisiae (Martinou et al., 2002), M. rouxii (Hunt et al., 2008; Kafetzopoulos et al 1993a), A. nidulans (Alfonso et al., 1995), C. lindemuthianum (Tsigos et al., 1995) and A.coerulea (Gao et al., 1995). The optimum temperature of 55°C was reported in S. brevicaulis (Cai et al., 2006). M. anisopliae and R. circinans exhibit their optimal activity at 37°C (Nahar et al., 2004 and Gauthier et al., 2008). The partially purified chitin deacetylase from B. thermoleovorans LW-4-11 exhibit a temperature optima at 80°C (Toharisman and Suhartono, 2008). It might be the highest optimum temperature for chitin deacetylase as all temperature for this enzyme reported so far was 50°C.

2.4.3.4.4 Effect of metal ions

The effect of different metal ions on the activity of chitin deacetylase from different sources has been reported by many workers. Chitin deacetylase activity was influenced by different metal ions like Li⁺, K⁺, Na⁺, Ag⁺, Mg²⁺, Cu²⁺, Ca²⁺, Zn²⁺, Mn²⁺,
Hg$^{2+}$, Co$^{2+}$, Ni$^{2+}$, Fe$^{2+}$ and Fe$^{3+}$. These metal ions could be either an activator or an inhibitor to different chitin deacetylases.

Gao et al., 1995 observed that purified chitin deacetylase from *A. coerulea* was not inhibited by 1 or 10mM EDTA or by Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$, Zn$^{2+}$, Co$^{2+}$ but it was strongly inhibited by Fe$^{3+}$ at a concentration of 1mM. Gauthier et al. (2008) reported that the activity of purified chitin deacetylases from *R. ciracinans* slightly enhanced by Mn$^{2+}$ and Mg$^{2+}$ but strongly inhibited by Cu$^{2+}$. The recombinant chitin deacetylase from *B. cereus* was inactive in the absence of Co$^{2+}$ In the presence of Mg$^{2+}$, Mn$^{2+}$, Ca$^{2+}$ and Zn$^{2+}$ not much restoration of activity observed while Cu$^{2+}$ imparted a negative effect on activity (Raval et al., 2013). Activation of chitin deacetylase in the presence of Co$^{2+}$ was reported in *B. cereus* (Raval et al., 2013), *B. subtilis* (Kobayashi et al., 2012), *P. oxalicum* (Pareek et al., 2012) and *S. cerevisiae* (Martinou et al., 2002). Chitin deacetylase from *B. thermoevorans* LW-4-11 was activated by EDTA. It was inhibited by Li$^+$, Mn$^{2+}$, Zn$^{2+}$, Co$^{2+}$ and Ni$^{2+}$ at 1mM concentration (Toharisman and Suhartono, 2008). The purified chitin deacetylase from *C. lindemuthianum* was inhibited by Mn$^{2+}$, Zn$^{2+}$ and Cu$^{2+}$ even at low concentration (1mM) whereas it was not inhibited by Li$^+$, K$^+$, Mg$^{2+}$, Ca$^{2+}$ and Na$^+$ up to 50mM concentration (Tsigos et al., 1995). The chitin deacetylase from *C. lindemuthianum* was inhibited by Mn$^{2+}$, Ni$^{2+}$, Cu$^{2+}$ and Fe$^{2+}$. The enzyme activity was not affected by Ca$^{2+}$ and Mg$^{2+}$. The enzyme activity was enhanced by Zn$^{2+}$, Co$^{2+}$ and EDTA (Tokuyasu et al., 1996). The chitin deacetylase from *A. nidulans* was inhibited by Cd$^{2+}$, Ca$^{2+}$, Zn$^{2+}$, Co$^{2+}$, Mg$^{2+}$, Mn$^{2+}$, Sn$^{2+}$, Pb$^{2+}$, NH$_4^+$, CN$^-$, citrate, tartrate, EDTA and it was not affected by Ag$^+$ (Alfonso et al., 1995).

Chitin deacetylase activity was inhibited by carboxylic acids, particularly acetic acid (Kafetzopoulos et al., 1993a). The chitin deacetylase of periplasmic origin was observed to be inhibited by acetate and this inhibition was supposed to be due to the localization of the periplasmic enzymes. The intracellular chitin deacetylase of dimorphic fungus *M. rouxii* was shown to be inhibited by sodium acetate (Kafetzopoulos et al., 1993a). On the contrary, the extracellular chitin deacetylases did not show any change in activity in presence of acetate viz. the extracellular chitin deacetylase from *C. lindemuthianum* and *M. anisopliae* were not inhibited in the presence of sodium acetate. The chitin deacetylase from *M. anisopliae* exhibited activity in presence of sodium acetate (1-5 mM) (Nahar et al., 2004) whereas 96% and 70% chitin deacetylase of *C. lindemuthianum* was retained in presence of 100 mM and 800 mM sodium acetate, respectively (Tokuyasu et al., 1996). Further, the extracellular
chitin deacetylase from *A. nidulans* showed increase in chitin deacetylase activity in the presence of lower concentrations of acetate (0.4-4mM) but not at higher concentrations (40mM) (Alfonso et al, 1995).

2.4.3.4.5 Substrate specificity

The substrate specificity of chitin deacetylase has been reported by many workers. The specificity of chitin deacetylase depends upon the degree of deacetylation and length of the substrates. It is well known that the high crystallinity of chitin microfibrils, by the hydrogen bond-stabilized packaging of chitin polymer, greatly impedes the access of the enzyme to the deacetylation reaction site in the chitin molecule. Enzymatic deacetylation is profoundly affected by the physical properties of the substrate such as crystallinity, degree of deacetylation, particle size and origin (Jung and Park, 2014). The soluble or colloidal forms of these polymers were found to be faster deacetylated than crystalline forms which were less accessible to the enzyme. In case of *M. anisopliae*, maximum chitin deacetylase activity was detected on a soluble form of chitin, ethylene glycol chitin (Nahar et al., 2004). Chitin deacetylase from *A. corymbifera* DY-9 was active towards water-soluble chitin (WSCT-50), glycol chitin, chitosan (DD 71%–88%), and chitin oligosaccharides with DP 2-7. chitin deacetylase displayed little activity on chitin flakes, chitin powder, swollen chitin, or β-chitin powder. Chitin oligosaccharides were a comparatively good substrate because their solubility increased availability and accessibility for the chitin deacetylase. Deacetylation rate on (GlcNAc)$_{2-6}$ was size-dependent; greater lengths produced a higher rate of activity. These results suggest that solubilization of chitin is a limiting factor for enzymatic bioconversion of chitin to chitosan by chitin deacetylase. Extracellular CDA from *Mortierella* sp. DY-52 was active on WSCT (DD 50%), glycol chitin and crab chitosan (DD 71%–88%) and also on *N*-acetylglucosamine oligomers (GlcNAc)$_{2-6}$ (Jung and Park, 2014).

It was observed that the length of the polymer i.e. degree of polymerization was important for enzyme action. Chitin oligomer of sufficient length is required for the enzyme to act on the substrate. The chitin oligomers with degree of polymerization higher than two are required for the enzyme to act on the substrate. For instance, *M. rouxii* and *C. lindemuthianum* had maximum affinity towards the substrates with at least four GlcNAc residues (Kafetzopoulos et al., 1993a; Tokuyasu et al., 1996). It was suggested that minimum 3-4 consecutive GlcNAc residues were needed for the chitin deacetylase activity (Alfonso et al., 1995; Kafetzopoulos et al., 1993a; Tokuyasu et al.,
The formation of the enzyme-substrate complex in case of *M. rouxii* induced the deacetylation that took place at the non-reducing end residue of the oligomer (Tsigos et al., 1999). Following the first deacetylation, the enzyme catalysed sequentially the hydrolysis of the following acetamido groups before it was dissociated and formed a new active complex with another chitin oligomer. In case of tetra N-acetylchitotetraose (GlcNAc)$_4$ and penta-N-acetyl chitopentaose (GlcNAc)$_5$, the geometry of the enzyme – substrate complex favoured the complete deacetylation of the substrate, while for shorter or longer oligomers the reducing – end residue remained intact (Tsigos et al., 2000). The enzyme could not effectively deacetylate chitin oligomers with a degree of polymerization lower than three. Tetra-N-acetylchitotetraose (GlcNAc)$_4$ and penta-N-acetyl chitopentaose (GlcNAc)$_5$ were fully deacetylated by the enzyme, however, in tri-N-acetylchitotriose (GlcNAc)$_3$, hexa-N-acetylchitohexaose (GlcNAc)$_6$ and hepta-N-acetylchitohptaose (GlcNAc)$_7$, the reducing end residue remained intact. It was suggested that CDA needed minimum (GlcNAc)$_3$ - (GlcNAc)$_4$ consecutive residues for the activity (Tsigos et al., 1999). Similarly, the chitin deacetylase from *S. brevicaulis* possessed the deacetylating activity for the N-acetylchitooligosaccharides.

The substrates such as acetyl xylan, peptidoglycan and soluble chitin were tested for activity of chitin deacetylase from *M. rouxii* and both a native and a truncated form of acetyl xylan esterase from *Streptomyces lividans*. All enzymes tested were determined to be active on acetyl xylan and soluble chitin while inactive on peptidoglycan, which means that not only chitin but also acetyl xylan could be handled by chitin deacetylase (Caufrier et al., 2003). This happened as both chitin deacetylase and acetyl xylan esterase have a similar catalytic domain which is different from that of peptidoglycan deacetylase. Sequence alignment together with recently reported structural analysis suggests that one disulfide bond, tethering the N-terminal and C-terminal ends, is conserved in chitin deacetylase of *M. rouxii, C. lindemuthianum* and acetyl xylan esterase of *S. lividans*. This conserved region was absent from the peptidoglycan deacetylase of bacterial *Streptococcus pneumoniae* and *B. subtilis* (Blair et al., 2004, 2006; Taylor et al., 2006).

Deacetylation by chitin deacetylase is apparently substrate size-specific. GlcNAc was converted into GlcN by chitin deacetylase from *Thermococcus kodakaraensis* KOD1, but (GlcNAc)$_2$ was converted into GlcN-GlcNAc, neither GlcNAc-GlcN or GlcN-GlcN. Only the non-reducing residue of (GlcNAc)$_2$ has been
deacetylated. Chitin deacetylase from *C. lindemuthianum* ATCC 56676 converted (GlcNAc)2 not into (GlcN)2 but into hetero-disaccharide GlcN-GlcNAc, and transformed (GlcNAc)3 and (GlcNAc)4 into the deacetylated products (GlcN)3 and (GlcN)4, respectively (Jung and Park 2014). Alfonso et al. (1995) found that chitosan oligosaccharides, (GlcN)2-6, were produced from chitin by the joint action of endochitinase and chitin deacetylase from *A. nidulans*, suggesting that deacetylation mainly occurs after chitin oligosaccharide production by the endochitinase.

### 2.4.3.5 Mechanism of action

Different patterns of enzymatic action of chitin deacetylase from different sources on chitin substrates have been reported. The mechanism of action of chitin deacetylase from *M. rouxii* on chitin oligosaccharides having degree of polymerization (DP) 2-7 has been studied (Kafetzopoulos et al., 1993a). To study the mechanism, the sequence of chitin oligomers after enzymatic deacetylation was identified by using two specific exoglycosidases in conjunction with high-pressure liquid chromatography (HPLC). The results were further verified using 1H-NMR spectroscopy. Using 1H-NMR and 13C-NMR spectroscopy, it was found that the enzyme hydrolysed the acetyl groups of the substrate according to a multiple-attack mechanism. It means that the enzyme forms an enzyme–polymer complex and further catalyses the hydrolysis of several acetyl groups before it dissociates and forms a new active complex with another polymer chain.

It was found that the exo-type chitin deacetylase from *M. rouxii* hydrolyzed the acetyl groups of the substrates of either chitosan polymers or chitin oligomers according to a multiple attack mechanism. The exo-type enzyme could only effectively deacetylate chitin oligomers with a DP higher than two and the first deacetylation takes place at the non-reducing end residue of the oligomer. Among the chitin oligomers with degree of polymerization (1–7), (GlcNAc)4 and (GlcNAc)5 could be fully deacetylated, whereas the reducing-end residue of (GlcNAc)3, (GlcNAc)6 and (GlcNAc)7 always remains intact (Martinou et al., 1998; Tsigos et al., 1999). The maximum number of acetyl groups that can be hydrolysed by the enzyme during the multiple-attack process is three. This is the maximum number of successive deacetylations that could be achieved by the enzyme because the maximum number of the consecutive N-acetyl-D-glucosamine residues that were found in this substrate polymer was three (Martinou et al., 1998).
In contrast to exo-type chitin deacetylase, the endo-type chitin deacetylase from *C. lindemuthianum* ATCC 56676 catalyzed the hydrolysis of acetamido groups according to a multiple chain mechanism. It means that the enzyme forms an active enzyme–polymer complex and catalyses the hydrolysis of only one acetyl group before it dissociates and forms a new active complex. The endo-type enzyme could fully deacetylate (GlcNAc)$_2$ and (GlcNAc)$_4$ whereas the reducing-end residue of (GlcNAc)$_2$ could not be deacetylated (Tokuyasu et al., 1997). In a further study, Tokuyasu and his colleagues carried out a structural analysis of the partially deacetylated products of (GlcNAc)$_2$–6 formed by the recombinant non-glycosylated chitin deacetylase from *C. lindemuthianum*. (GlcNAc)$_4$ could be exclusively deacylated to the product of GlcNAc-GlcNAc-GlcN-GlcNAc by this recombinant chitin deacetylase in an initial deacetylation process (Tokuyasu et al., 1999).

The enzyme was suggested to possess four enzyme subsites (-2, -1, 0 and +1) in the catalytic domain that recognize and interact with GlcNAc residues of the substrate. The enzyme strongly recognizes a sequence of four GlcNAc residues of the substrate and the N-acetyl group in the GlcNAc residue positioned at subsite 0 is exclusively deacetylated. Among the four subsites, only subsite -2 strongly recognized the N-acetyl group of the GlcNAc residue of the substrate, while the deacetylation rate was not affected when either subsite -1 or +1 was occupied with a GlcN residue instead of GlcNAc residue (Tokuyasu et al., 1999; Hekmat et al., 2003). Similarly, the structure of chitin deacetylase from *C. lindemuthianum* was determined by mass spectroscopy and it was suggested that CLCDA possessed a highly conserved substrate binding groove, with subtle alterations that influenced the substrate specificity and substrate affinity. For the enzyme to be active, at least 0 and +1 subsites should be occupied by (GlcNAc)$_3$ (Blair et al., 2006).

### 2.4.3.6 Molecular studies

The chitin deacetylase genes have been cloned and characterized in several fungi such as *M. rouxii* (Kafetzopoulos et al., 1993b), *C. lindemuthianum* (Shrestha et al., 2004; Tokuyasu et al., 1999), *S. cerevisiae* (Mishra et al., 1997), *S. pombe* (Matsuo et al., 2005), *R. circinans* (Gauthier et al., 2008) and *F. velutipes* (Yamada et al., 2008). For the first time, the CDA gene was isolated, characterized and sequenced from *M. rouxii* by Kafetzopoulos et al. (1993b). The CDA gene from *C. lindemuthianum* was isolated and was over expressed in *E. coli* using a signal sequence for chitinase gene from *S. lividans* (Tokuyasu et al., 1999). Further, the CDA gene from the dimorphic
human pathogenic fungus *C. neoformans* was isolated by screening the genomic database library with a primer constructed towards the N-terminal region of the purified protein (Biondo et al., 2002). The CDA genes have also been cloned from insects such as *D. melanogaster* (Luschnig et al., 2006), *M. configurata* (Toprak et al., 2008), *T. castaneum* (Dixit et al., 2008) and *T. ni* (Guo et al., 2005).

Multiple forms of chitin deacetylase genes have been reported in several fungi. Two genes CDA1 and CDA2 were identified in *S. cerevisiae* which showed protein sequence homology with *M. rouxii* chitin deacetylase gene (Christodoulidou et al., 1996; Mishra et al., 1997). Similarly, three putative chitin deacetylase genes (RC, D2 and I3/2) were isolated and sequenced from the cDNA library of *R. circinans* by Gauthier et al. (2008).

Multiple forms of chitin deacetylase genes have also been reported in insects. The presence of nine transcripts of CDA was reported in *T. castaneum* (Arakane et al., 2009) whereas two transcripts of CDA (CfCDA2a CfCDA2b) were estimated in *Choristoneura fumiferana* (Quan et al., 2013).

### 2.4.3.7 Application of chitin deacetylase

The following major applications of chitin deacetylase make this enzyme advantageous for industrially important.

#### 2.4.3.7.1 Chitin deacetylase in transgenics

Chitin deacetylase could also be employed in transgenics. Recently, the paper and textile industries shows a keen interest in developing the chitin: chitosan and chitosan: cellulose blends. It was proposed that the genetic manipulation of plants can be carried out by expression of chitin synthase and chitin deacetylase genes. Such transgenic plants with altered cell wall can be used as a single source of cellulose, chitin and chitosan for industrial uses and improved disease resistance (Dhugga et al., 2000). It will be one of the best applications of chitin deacetylase.

#### 2.4.3.7.2 Biocontrol of insect pests

Chitin deacetylase in combination with other hydrolytic enzymes could be used in integrated pest management for the control of pests and pathogens. In *M. anisopliae*, chitin deacetylase was reported to play a dual role in modifying the insect cuticular chitin as well as in altering its own cell wall for defense from insect chitinases. Similarly, chitin deacetylase could also be a versatile tool in the biological control of insect pests. For instance, chitin deacetylase proved to be important in initiating pathogenesis of *M. anisopliae* (a kind of insect-pathogenic fungus) by softening the
insect cuticle to aid mycelial penetration. Chitin deacetylase herein may have a dual role in modifying the insect cuticular chitin for easy penetration of fungal pathogen as well as in altering its own cell walls for defense from insect chitinase (Nahar et al., 2004). In the biological control of pest insects, chitin deacetylase proved to be a potential target for an insecticide. Chitin deacetylase is a major protein secreted in the peritrophic matrix of the arthropod gut during feeding; it can modify the chitin component in such a way as to protect the gut from parasite invasion, and intercept toxins like lectins. Thus, the inhibition of this enzyme represents a potential way to control the pest insects.

Thus, chitin deacetylase could be a versatile tool in the biological control of insect pests. In C. lindemuthianum, the Cu$^{2+}$ was found to be inhibitory to chitin deacetylase (Tokuyasu et al., 1996). Therefore, the fungicides containing copper could be effectively used for the pathogen control.

### 2.4.3.7.3 Biological control of fungal human pathogen

The fungal cell wall acts as an excellent target for antifungal agents in the biological control of fungal human pathogen. It provides structure and integrity to cell and also needed for the localization or attachment of known virulence factors, including the polysaccharide capsule, melanin, and phospholipase. Thus, fungal cell wall is critical for host-pathogen interactions. The chitosan produced by the enzymatic removal of acetyl groups from nascent chitin is an important component of the cell walls of certain fungi and helps maintain cell integrity. Thus, chitin deacetylases and the chitosan made by them may prove to be excellent antifungal targets (Baker et al., 2007; Brosson et al., 2005; Das et al., 2006). The inhibition of chitin deacetylase could support the fungal cell wall hydrolysis by plant chitinases, thus the control of the plant pathogenic fungi becomes feasible (Tokuyasu et al., 1996).

### 2.4.3.7.4 Bioconversion of chitin to chitosan

Chitin deacetylase play a significant role in utilization of chitin. As chitin is not readily soluble, it has limited industrial applications (Kurita, 1986). On the contrary, the deacetylated form of chitin i.e. chitosan is relatively soluble (Patil et al., 2000). Bioconversion of chitin to chitosan by chitin deacetylase is an economical and environmentally friendly process. Chitin was confirmed to be a very poor substrate for the enzyme, but modifications of chitin either by physical or chemical methods increases the efficiency of the enzymatic deacetylation for the production of chitosans. Beaney et al. (2007) modified the chitin either by physical or chemical methods and
then treated it with extracellular deacetylase from *C. lindemuthianum* for 24 hours (reportedly not inhibited by liberated acetate). Modifications of the chitins affected the degree of deacetylation to various extents: it was found that the dissolution and drying method used in modifying the chitins had significant impact on the final efficiency of the enzymatic deacetylation reaction. The most successful preparation made by freeze-drying a colloidal chitin suspension increased the degree of enzymatic deacetylation by 20 fold. Thus it can be concluded that degree of crystallinity of the chitins must be reduced to enable enzymes to access the internal polysaccharide structure. Chitosans with low DD can be further deacetylated with *M. rouxii* deacetylase (Martinou et al., 1995, 1997). Aye et al. (2006) carried out the enzymatic deacetylation of various chitins using the chitin deacetylase isolated from *R. oryzae*. Yamada et al. (2008) found that the recombinant chitin deacetylase of *F. velutipes* catalyses deacetylation of *N*-acetyl-chitoooligomers, from dimer to pentamer, glycol chitin and colloidal chitin.

**Conclusions**

The interest in chitin deacetylases is increasing day after day as the application of molecular biological techniques provides the tools to tailor and manipulate chitin deacetylases. It helps in production of chitin deacetylases with novel properties that can be used more efficiently in biocontrol of insect pests and for the preparation of chitosan polymers and oligomers. Future developments in both basic research and biotechnological applications are awaited with great interest.