CHAPTER I

INTRODUCTION
CHAPTER 1

1.1 INTRODUCTION

The chitin is a linear homopolymer of β(1→4) linked N-acetyl-β-D-glucosamine (NAG, 2-acetamido-2-deoxy-D-glucose). It is widely distributed in nature and found mainly in marine invertebrates, insects and fungi (Muzzarelli, 1977). Therefore, it is at present the subject of a number of projects directed towards the commercial exploitation of various chitinous resources. Several countries including Japan, Norway, Italy, USA and India have chitin/chitosan production plants based on shellfish waste composed mainly of chitin, CaCO₃ and protein as the raw material (Joshi, 1990; Muzzarelli, 1977b; Nicolaysen, 1980). For instance, USA has an average landings for shrimp and crab to the order of 780 and 756 million kg, respectively of which 70-80% constitutes the waste (Revah-Moiseev and Carroad, 1981). Recently, Gooday (1990a) has estimated the annual recovery of 10¹⁰ - 10¹¹ tons of chitinous resources. Whereas, Nicol (1991) has suggested the worldwide annual recovery of 37,300 tons of chitin from the processing of shrimps, prawns and lobsters.

Fungal waste (Muzzarelli et al., 1980) is the second best source of chitin used for its commercial production. The industrial fungal waste is preferred over chitinous resources from animal origin mainly because of
the uniformity in the production of fungal biomass and (if necessary) economical pretreatment. The global estimation of chitinous materials as potential chitin sources are presented in Table 1.1.

Earlier, the conventional methods of shellfish solid waste handling included ocean dumping, incineration, land filling and fish meal production after drying and milling (CRESA, 1971; Kreag and Smith, 1975; Mendenhall, 1971). However, increase in the cost of transportation, decrease in the availability of the land-fill sites and growing awareness of environmental pollution have prompted the search for an alternative method of waste disposal, such as bioconversion of chitinous waste to single cell protein (Carroad and Tom, 1978).

Some of the selected applications and uses of chitin and its derivatives have been summarized in Table 1.2. Distribution of chitin in nature and its physical and chemical properties that makes it an attractive speciality material for the applied research has been discussed in subsequent sections.
Table 1.1: Worldwide estimates of chitinous materials as potential chitin sources per year

<table>
<thead>
<tr>
<th>Chitin source</th>
<th>Quantity harvested(^b)</th>
<th>Chitinous waste</th>
<th>Chitin potential</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fraction of harvest (%)</td>
<td>Solids (%)</td>
</tr>
<tr>
<td>Shellfish</td>
<td>1,700</td>
<td>50 - 60</td>
<td>30 - 35</td>
</tr>
<tr>
<td>Krill</td>
<td>18,200</td>
<td>40</td>
<td>22</td>
</tr>
<tr>
<td>Clams and Oysters</td>
<td>1,390</td>
<td>65 - 85</td>
<td>90 - 95</td>
</tr>
<tr>
<td>Squid</td>
<td>660</td>
<td>20 - 40</td>
<td>21</td>
</tr>
<tr>
<td>Fungi</td>
<td>790</td>
<td>100</td>
<td>20 - 26</td>
</tr>
<tr>
<td>Total</td>
<td>22,740</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Modified from Muzzarelli, 1985

\(^a\) Estimates are based on mean values and are given in million kg.

\(^b\) Except for fungi, the values expressed are the ones after processing the harvested organisms for chitinous product.
<table>
<thead>
<tr>
<th>Application</th>
<th>Use^a</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromatography</td>
<td>Nucleic acid (chitosan)</td>
<td>Takeda and Tomida (1972)</td>
</tr>
<tr>
<td>matrix</td>
<td>Lysozyme (chitosan and its carboxymethylated derivative)</td>
<td>Cherkasov and Kravchenko (1968)</td>
</tr>
<tr>
<td></td>
<td>Wheat germ agglutinin (chitin)</td>
<td>Bloch and Burger (1974)</td>
</tr>
<tr>
<td>Enzyme immobilization</td>
<td>α-Amylase (chitin)</td>
<td>Flor and Hayashida (1983)</td>
</tr>
<tr>
<td></td>
<td>Glucose isomerase, urease, pronase, β-galactosidase (chitin)</td>
<td>Muzzarelli (1980)</td>
</tr>
<tr>
<td>Nutritional supplement</td>
<td>Bifidus factor (NAG-glycosides)</td>
<td>Austin et al. (1981)</td>
</tr>
<tr>
<td></td>
<td>Feed formulation (chitin)</td>
<td>Kono et al. (1987)</td>
</tr>
<tr>
<td>Agriculture</td>
<td>Nematocide (chitin)</td>
<td>Spiegel et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>Fungicide (chitin)</td>
<td>Schlumbaum et al. (1986)</td>
</tr>
<tr>
<td>Application</td>
<td>Use</td>
<td>References</td>
</tr>
<tr>
<td>----------------------</td>
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</tr>
<tr>
<td>Medical</td>
<td>Surgical sutures</td>
<td>Nakajima et al.</td>
</tr>
<tr>
<td></td>
<td>(chitin)</td>
<td>(1986)</td>
</tr>
<tr>
<td></td>
<td>Anti-tumor agents</td>
<td>Masato et al.</td>
</tr>
<tr>
<td></td>
<td>(chitosan oligo-</td>
<td>(1989)</td>
</tr>
<tr>
<td></td>
<td>saccharides)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypcholesterolemic</td>
<td>Sugano et al.</td>
</tr>
<tr>
<td></td>
<td>agent (chitosan</td>
<td>(1978)</td>
</tr>
<tr>
<td></td>
<td>acetate)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemostatic agent</td>
<td>Malette et al.</td>
</tr>
<tr>
<td></td>
<td>(chitosan)</td>
<td>(1983)</td>
</tr>
<tr>
<td></td>
<td>Blood and anti-</td>
<td>Muzzarelli et al.</td>
</tr>
<tr>
<td></td>
<td>coagulants</td>
<td>(1984)</td>
</tr>
<tr>
<td></td>
<td>(sulfated</td>
<td></td>
</tr>
<tr>
<td></td>
<td>carboxymethyl</td>
<td></td>
</tr>
<tr>
<td></td>
<td>chitosan)</td>
<td></td>
</tr>
<tr>
<td>Effluent treatment</td>
<td>Removal of colloidal</td>
<td>Muzzarelli et al.</td>
</tr>
<tr>
<td></td>
<td>and metallic</td>
<td>(1989)</td>
</tr>
<tr>
<td></td>
<td>impurities</td>
<td></td>
</tr>
</tbody>
</table>

Specific matrix used is mentioned in parentheses.
1.2 DISTRIBUTION, STRUCTURE AND PROPERTIES OF CHITIN

1.2.1 DISTRIBUTION

In nature, chitin is associated with other structural polymers like proteins, carbohydrates and lipids. In insects and other invertebrates, the observed ordered chitinous structures such as cuticles, peritrophic membrane, chaetae of annelids and of the shells of the molluscs are composites of chitin and specific proteins (Blackwell and Weih, 1984; Giraud-Gaillé and Bouligand, 1986; Jeuniaux, 1963; Kramer and Koga, 1986). They often undergo varying degree of mineralisation, i.e. calcification and sclerotization, involving interactions with phenolics and lipid molecules (Peter et al., 1986). The presence of chitin in the cyst walls of Entamoeba sp. (Arroyo-Begovich et al., 1980) and Giardia lamblia (Ward et al., 1985) offers high degree of protection to adverse conditions such as desiccation. It is also found to be present in the egg shells of nematodes (Bryden et al., 1987).

Chitin is one of the major cell wall components of fungi which constitutes the innermost layer of the cell wall (Gooday and Trinci, 1980). It is found to be covalently linked with glucans either directly or via peptide bridges (Sietsma et al., 1986; Surarit et al., 1988).

The presence of chitin or cellulose (a homopolymer of β-(1→4) linked glucose) may be used as a criterion to
establish the phylogenetic relationship between the groups of fungi such as Phycomycetes (Foster and Weber, 1960). Frey (1955) has shown by X-ray analysis that chitin and cellulose apparently do not normally occur together. However, the presence of both have been convincingly demonstrated in Rhizidiomyces bivellatus (class Hyphochytridiomycetes) (Lejohn, 1968) indicating it to be an intermediate group of Phycomycetes distinct from the cellulosic Oomycetes and chitinous Chytridiomycetes (Bartnicki-Garcia, 1968).

Cell walls of the zygomycetous fungi have been found to contain chitosan, a deacetylated derivative of chitin. It occurs in the cell walls together with pyrophosphates and glucuromannan (Bartnicki-Garcia and Lindberg, 1972; Datema et al., 1977).

1.2.2 STRUCTURE

Chitin is a polymer of unbranched chains of $\beta-(1 \rightarrow 4)$ linked 2-acetamido-2-deoxy-glucose. It may thus be regarded as a derivative of cellulose, in which C-2 hydroxy groups have been substituted by acetamido group (Foster and Weber, 1960). In chitosan, the acetamido group is deacetylated (Fig. 1.1).

Existence of more than one polymorphic form of chitin from different sources has been suggested from the X-ray diffraction analysis (Rudall, 1963). Minke and Blackwell (1978) have suggested the presence of $\alpha$-chitin in
Fig. 1.1: Structure of chitin, chitosan and cellulose.
CELLULOSE
CHITIN
CHITOSAN

R = OH
R = NHCOCH₃
R = NH₂
lobster tendon. The unit cell is orthorhombic with dimensions $a = 4.74 \text{ Å}$, $b = 18.86 \text{ Å}$, and $c = 10.32 \text{ Å}$ (fiber axis). The space group of $P_{2_1}P_{2_1}P_{2_1}$ suggested the antiparallel packing of chains. The structure observed by them was similar to the one proposed by Carlstorm (1957). He arranged the chains in sheets along the $a$ axis linked by N-H $\cdots \cdots$ O = C hydrogen bonds between the acetamido groups. The stacking of the chains is further stabilized by the hydrophobic forces between the surfaces of the glucose rings. In addition, half of the $\text{CH}_2\text{OH}$ groups are bonded to carbonyls within the same stack of chains and half are bonded to $\text{CH}_2\text{OH}$ groups on an adjacent stack. The existence of this intersheet bonding confers the stability of $\alpha$-chitins. Lotmar and Picken (1950) while studying the X-ray data of chitin from chaetae of *Aphorodite aculeata* proposed the $\beta$-form of chitin. The unit cell has dimensions $a = 9.32 \text{ Å}$, $b = 10.17 \text{ Å}$, $c = 22.15 \text{ Å}$. Rudall (1963) proposed the presence of $\gamma$-chitin in the thick cuticle lining the stomach of squid *Loligo*. Table 1.3 summarizes a comparison of these 3 distinct crystallographic types of chitin. Rudall (1963) has classified them on the basis of the number of chains present per unit cell. The distribution of the three crystallographic forms of chitin does not seem to be related to animal taxonomy (Jeuniaux, 1971). Moreover, these three types may occur in different organs of the same
Table 1.3: Structural forms of chitin

<table>
<thead>
<tr>
<th>α-</th>
<th>β-</th>
<th>γ-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occurrence</td>
<td>Chaetae of <em>Aphrodite aculeata</em></td>
<td>Thick cuticle lining the stomach of the squid <em>Loligo</em></td>
</tr>
<tr>
<td>Arthropods and fungi</td>
<td><em>Aphrodite aculeata</em> and pen of squid</td>
<td></td>
</tr>
<tr>
<td>Unit cell</td>
<td>Two chains in antiparallel directions</td>
<td>One chain. Different chains running in parallel directions.</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a 4.76 Å</td>
<td>9.32 Å</td>
<td></td>
</tr>
<tr>
<td>b 10.46 Å</td>
<td>10.17 Å</td>
<td></td>
</tr>
<tr>
<td>c 19.25 Å</td>
<td>22.15 Å</td>
<td></td>
</tr>
<tr>
<td>Properties</td>
<td>Higher degree of packing of chains and more close type of crystalline structure.</td>
<td>More readily penetrated by chemical reagents and enzymes than α-chitin</td>
</tr>
<tr>
<td>Functions</td>
<td>Replaces collagen type cuticle in <em>Loligo</em> and <em>Lingula</em></td>
<td>Associated with collagen type cuticles or with collagen secreting neighbouring tissues in the same organisms.</td>
</tr>
</tbody>
</table>

Modified from Deshpande (1986)
animal as *Loligo* and *Lingula* (Rudall, 1955). According to Rudall (1955), $\beta$-chitins (and probably $\gamma$-chitin) appear to be associated with collagen type cuticles, while $\alpha$-chitin completely replaces collagen-type cuticles.

### 1.2.3 PHYSICAL AND CHEMICAL PROPERTIES

Hackman and Goldberg (1974) reported that average molecular weight of $\alpha$-chitin from crab, *Scylla serrata* was found to be $1.036 \times 10^6$ daltons using light scattering technique.

The chitin is an amorphous solid insoluble in water, dilute acids, dilute and concentrated alkalis, alcohol and other organic solvents. It is soluble in concentrated HCl, H$_2$SO$_4$, 78 - 97% H$_3$PO$_4$ and anhydrous formic acid. There are substantial variations in solubility, molecular weight, acetyl values among chitins of different origins and prepared by different methods.

When chitin is treated with concentrated alkali at higher temperatures ($>100^\circ$C), it undergoes various degrees of deacetylation to form chitosan.

Various aprotic and other special organic solvents have been used for the characterization of chitin as a polymer in fiber manufacture. Chitin is soluble in LiCl/tertiary amide (R-CO-N = R$_2$) system which would yield 5% solution. It is a non-degradative solvent system and can be used for the purification of chitin (Austin, 1988).
Chitin is also soluble in di- and tri-chloroacetic acid and methane sulfonic acid at temperatures \(<5°C\).

1.3 **ENZYMATIC SYNTHESIS OF CHITIN AND CHITOSAN**

1.3.1 **CHITIN SYNTHESIS IN FUNGI**

Chitin is a homopolymer of N-acetyl-\(\beta\)-D-glucosamine (NAG) residues joined to each other by \(\beta-(1\rightarrow 4)\) linkages. Chitin synthesis has been reported from most of the fungi from various taxonomic groups such as *Mucor rouxii* (Ruiz-Herrera *et al.*, 1977), *Neurospora crassa* (Selitrennikoff, 1979), *Agaricus bisporus* (Craig *et al.*, 1981), *Candida albicans* (Hardy and Gooday, 1983), *Coprinus cinereus* (Montgomery and Gooday, 1985) and *Saccharomyces cerevisiae* (Sburlati and Cabib, 1986). The biosynthetic pathway leading to synthesis of chitin and chitosan from glucose has been shown in Fig. 1.2. Chitin synthase is the key enzyme which catalyzes the last step in the pathway and has been studied in detail for its metabolic regulation.

1.3.1.1 **Chitin synthase**

The enzyme chitin synthase (EC 2.4.1.16) catalyzes the formation of chitin by transferring the NAG from UDP-NAG to a growing chain of NAG. The stoichiometry of the reaction is as follows:

\[
2n \text{UDP-NAG} \rightarrow 2n \text{UDP} + [\text{NAG-\(\beta-(1\rightarrow 4)\)NAG}]_n
\]

Most of the chitin synthases reported so far showed zymogenic nature. This may be due to the fact that
Fig. 1.2: Pathway for chitin and chitosan synthesis. The enzymes involved in the metabolism of N-acetylglucosamine (NAG) and the synthesis of chitin are: (a) N-acetyl-D-glucosamine kinase, (b) N-acetylglucosamine-6-phosphate deacetylase, (c) glucosamine-6-phosphate deaminase, (d) L-glutamine D-fructose-6-phosphate aminotransferase, (e) glucosamine phosphate acetyltransferase, (f) glucosamine phosphate isomerase, (g) UDP acetylglucosamine pyrophosphorylase, (h) chitin synthase, (i) chitin deacetylase (from Araki and Ito, 1975)
Glucose

Glucose 6-Phosphate

Fructose 6-Phosphate

\[ \text{NH}_3 \] (c)

\[ \text{H}_2\text{O} \]

Glucosamine 6-Phosphate

\[ \text{CH}_3\text{COOH} \] (b)

\[ \text{H}_2\text{O} \]

NAG (d)

\[ \text{Glutamine} \]

Glutamate

\[ \text{Acetyl-SCoA} \] (e)

\[ \text{CoASH} \]

NAG 6-Phosphate

NAG 1-Phosphate

NAG (g)

ATP ADP

\[ \text{UTP} \]

PP

UDP-NAG

Chitin

Chitosan

(Modified from Chiew et al., 1980)
the activity of chitin synthase in the cell needs precise regulation with respect to time and place. In *S. cerevisiae*, Cabib and Farkas (1971) observed that the initiation and localised chitin synthesis occurred as a result of partial proteolysis of zymogenic form of chitin synthase. Most of the fungi studied so far, have reported to possess the mechanism of activation of chitin synthase by limited proteolysis (Arroyo-Begovich and Ruiz-Herrera, 1979; Cabib et al., 1988; Duran and Cabib, 1978; Gooday, 1979; Ulane and Cabib, 1976). The only reported exception of the presence of active chitin synthase was from *Coprinus cinereus* (Gooday and Rousset-Hall, 1975). However, in this case also the activation of zymogenic form by endogenous proteases even in the presence of protease inhibitors cannot be ruled out. Two hypotheses have been put forward to explain the presence of zymogenic form of chitin synthase in fungi. The enzyme may be synthesized as a single inactive chain which on proteolysis becomes active, or alternatively, the actively synthesized enzyme gets inactivated by forming a covalent bond to the proteinaceous inhibitor, once its functional utility is over (Farkas, 1979). However, the presence of a zymogenic form in cells of *S. cerevisiae* containing a cloned gene for chitin synthase supports the single chain hypothesis (Cabib, 1987).

In most of the fungi studied so far, chitin
synthase was found to be membrane bound (Duran et al., 1975; Vermeulen and Wessels, 1986). However, Bartnicki-Garcia and coworkers strongly believed in the presence of cytological apparatus which plays role in the synthesis of fungal cell walls (Bartnicki-Garcia, 1981; 1987; Bartnicki-Garcia et al., 1978; Bracker et al., 1976; Ruiz-Herrera et al., 1977). Bartnicki-Garcia (1981) has proposed that chitosomes are spheroidal particles of 40 - 70 nm diameter, which play role in the transport of chitin synthase to the cell surface. Chitin synthase is present in the zymogenic form in chitosomes and gets activated by limited proteolysis on reaching its destination, which may be either plasma membrane or the periplasmic space.

While studying chitin synthesis in *M. rouxii*, Bartnicki-Garcia (1987) has reported that chitin synthase exists in the chitosome as a complex (16S subunit, molecular weight 500,000), which can be reversibly dissociated with digitonin. The chitin made by dissociated chitosome had a short, needle-like appearance. Evidently, the spatial organization of enzyme subunits in the chitosome provide conditions conducive to the assembly of long microfibrils.

The chitin synthase requires magnesium or cobalt for activity (Gooday, 1979; Sburlati and Cabib, 1986). The enzyme may be allosterically activated by UDP-NAG, NAG and chitooligosaccharides and was inhibited by UDP (Sburlati
and Cabib, 1986). Phosphatidyl serine and lysophosphatidyl serine have been found to be activators of chitin synthase in *S. cerevisiae* (Duran and Cabib, 1978).

*Streptomyces* isolates have been reported to produce certain antibiotics which were found to be competitive inhibitors of chitin synthase activity. For instance, *Streptomyces tendae* (Dahn et al., 1976) and *Streptomyces cacaoi* (Isono et al., 1965) produce nucleoside di- and tripeptide antibiotics, which have been classified as polyoxins and nikkomycins (Goady, 1990). Other inhibitors of chitin synthase of *S. cerevisiae* which were reported to be less effective than these antibiotics are unsaturated fatty acids such as oleic and linoleic (Duran and Cabib, 1978). However, their saturated counterparts were reported to be ineffective as inhibitors. The dyes, calcoflour white M2R new and congo red were found to be the inhibitors of chitin synthase in *Geotrichum lactis* (Roncero and Duran, 1985). However, Elorza et al. (1983) have reported normal chitin synthesis in regenerating protoplasts of *C. albicans* in the presence of calcofluor white.

Sburlati and Cabib (1986) have reported the presence of two forms of chitin synthases (chs 1 and chs 2) in *S. cerevisiae*. Both the enzymes were different from each other with respect to the stimulation by cobalt and magnesium, optimum pH and the relative inhibition of activity by
polyoxins and NaCl. However, both were zymogenic in nature and required limited proteolysis for maximum activity in vitro. The structural genes CHS 1 and CHS 2 for chitin synthase 1 and chitin synthase 2 respectively, have been cloned and sequenced (Bulawa et al., 1986; Cabib et al., 1990; Silverman et al., 1988). Based on gene disruption experiments, Silverman et al. (1988) suggested the role of chs 2 in vegetative growth, i.e. septation and cell division. However, recently Bulawa and Osmond (1990) while studying the double disruption mutants in S. cerevisiae reported that chs 1 and chs 2 are not required for chitin synthesis in vivo.

1.3.1.2 Assay of chitin synthase

Chitin synthase activity can be measured by radio­metric assay where the incorporation of labelled NAG from the reaction mixture containing UDP N-acetyl[14C]glucosamine, Mg²⁺/Co²⁺, unlabelled NAG, into the insoluble product, chitin is monitored (Kang et al., 1984).

In samples, where the chitinase contamination cannot be avoided, the chitin obtained after filtration can be subjected to complete hydrolysis by purified chitinase and radioactivity of the soluble hydrolysate can be determined (Molano et al., 1979).

1.3.1.3 Vectorial synthesis

Chitin synthesis takes place on the outside surface
of the plasma membrane, which is impermeable to UDP-NAG, the precursor of chitin. Cabib et al. (1983) while studying S. cerevisiae protoplast regeneration suggested the scheme of vectorial synthesis of chitin through the plasma membrane.

It appears that UDP-NAG on approaching the plasma membrane from the cytoplasm, transfers the amino sugar with the help of synthase to a growing chain of chitin that is simultaneously extruded (Fig. 1.3). The possibility of proteins other than chitin synthase spanning the membrane cannot be ruled out. Hydrolysis of the bond between UDP and NAG may provide the energy required for the translocation (Cabib et al., 1983).

Evidence for a transient non-crystalline state of chitin during synthesis has been reported in Schizophyllum commune (Vermeulen and Wessels, 1986). The nascent and crystalline chitin have been found to differ with respect to their susceptibility to chitinase and X-ray diffraction patterns.

1.3.2 CHITIN SYNTHESIS IN ANIMALS

The biochemical pathway for synthesis of chitin from glucose or trehalose (α-D-glucosido-α-D-glucoside, a disaccharide which serves as a reserve carbohydrate) is similar to the one reported in fungi (Marks, 1973, Porter and Jaworski, 1965; Weiss-Fogh, 1970). However, the purification and characterization of chitin synthases from
Fig. 1.3: Vectorial synthesis of chitin by membrane bound chitin synthase.
Plasma membrane

Cytoplasmic face

External face

Chitin synthase

UDP-$\text{UDP-C5-} = \text{NAG}$

(From Cabib et al, 1983)

$\bigcirc = \text{NAG}$
animals is not very well studied so far. NAG and diacetylchitobiose have been reported to inhibit the chitin synthase activity from the extract of the stable fly, *Stomoxys calcitrans* (Meyer et al., 1980), whereas divalent cations have no effect on enzyme activity. However, NAG and divalent cations showed stimulatory effect on the chitin synthase activity from the extracts of *Tribolium*, *Tenebrio* and *Galleria* (Cohen and Casida, 1980). While studying the chitin synthesis in the extract of brine shrimp, *Artemia salina*, the possibility of the involvement of a lipid carrier has been suggested by Horst (1981; 1983).

### 1.3.3 CHITOSAN SYNTHESIS IN FUNGI

Chitosan, a deacetylated derivative of chitin is found to be a major component in the cell walls of zygomycetous fungi (Bartnicki-Garcia, 1968; Kreager, 1954). Most of the work has been carried out in *M. rouxii* (Araki and Ito, 1975; Calavo-Mendez and Ruiz-Herrera, 1987; Davis and Bartnicki-Garcia, 1984). The synthesis of chitosan takes place by the deacetylation of newly formed chitin (Fig.1.2). Chitin deacetylase catalyzes the deacetylation of chito-oligosaccharides (Araki and Ito, 1975). Davis and Bartnicki-Garcia (1984) suggested that chitin synthase and chitin deacetylase operate in tandem to catalyze the conversion of UDP-NAG to chitin and chitin to chitosan. However, once the chitin chains crystallize into microfibrils, they cease
to be the effective substrate for the chitin deacetylase. Calavo-Mendez and Ruiz-Herrera (1987) have also shown the involvement of chitin synthase and membrane bound chitin deacetylase in the chitosan biosynthesis in M. rouxii.

1.4 ENZYMATIC DEGRADATION OF CHITIN AND CHITOSAN

1.4.1 ENZYMATIC DEGRADATION OF CHITIN

Chitin is one of the excellent sources of carbon and nitrogen for many *Streptomyces* strains and fungi. The growth on it induces the production of several chitinolytic enzymes, however, very little information is available on the mechanism of their action and behaviour in presence of each other. This could be due to the uncertain nature of their assay systems. However, several reports on cloned chitinases from various microorganisms and the information on the detailed methods of substrate preparation and enzyme purification have now made the researchers possible to comment on mechanism of action of chitin degrading enzyme complex (Gooday, 1991).

The complete enzymatic degradation of chitin to free NAG is performed by a chitinolytic system, the action of which may be synergistic and consecutive. The endo-chitinase (poly β-1→4-(2-acetamido-2-deoxy)-D-glucoside glycanohydrolase, EC 3.2.1.14) randomly hydrolyzes the polymers of NAG, including trimers and to a lesser extent tetramers (Berger and Reynold, 1958; Jeuniaux, 1966); chitobiase (β-D-N-acetylglucosaminidase, EC 3.2.1.30) hydrolyzes
dimers efficiently and to a lesser degree oligomers (Charpentier and Percheron, 1983). The 3rd group of enzymes can be categorized as exo-chitinases. Earlier, exo-chitinases were defined as enzymes which released NAG from the non-reducing ends of chitin chains (Abeles et al., 1970; Boller et al., 1983). However, Robbins et al. (1988) suggested that the formation of NAG from chitin could in many cases be the result of hydrolysis of small oligosaccharides produced by contaminating hexosaminidase. Therefore, they proposed that exo-chitinases are the enzymes which catalyze the processive release of diacetylchitobiose units from the non-reducing ends of chitin chains and the former group of enzymes may be called as β-N-acetylglucosaminidase (EC 3.2.1.52). Thus, exo-chitinases are the enzymes which hydrolyze chitin chains from the non-reducing end releasing chitobiose (Robbins et al., 1988).

Chitin can alternatively be degraded to release GA as the final end product via formation of chitosan by enzymatic deacetylation. Enzymes involved in chitosan hydrolysis consist of chitosanases and glucosaminidases (more information is covered in Section 1.4.6) (Gooday et al., 1990a; Hillman et al., 1989).

1.4.1.1 Occurrence and roles

The primary role of chitinolytic enzymes in nature
is to degrade huge quantities of chitin for recycling. The distribution of chitinolytic bacteria in habitats such as lake water sediments, lake planktons, cray fish, insects, soil, compost, frog and bat intestines has been reported by Benton as early as 1935. Some of the potent chitinase producers from bacterial sources are *Serratia marcescens* (Monreal and Reese, 1969), *Serratia liquefaciens* (Joshi et al., 1987; 1989a), *Vibrio vulnificus* (Wortman et al., 1986), *Pseudomonas stutzeri* YPL-1 (Lim and Kim, 1990), *Cytophaga johnsoni* (Sunderraj and Bhat, 1972), *Aeromonas hydrophila* subspecies *anaerogenes* A-52 (Yabuki et al., 1986), *Micrococcus roseus* (Cody et al., 1990), *Bacillus circulans* WL-12 (Watanabe et al., 1990), *Clostridium* strains (Pel et al., 1989; 1990), *Myxococcus xanthus* (Cody et al., 1990), *Streptomyces orientalis* (Tominaga and Tsuji-Saka, 1976), *Streptomyces cinereoruber* (Ueno et al., 1990) and *Streptomyces erythraeus* (Hara et al., 1989).

Fungi known to produce chitin degrading enzymes include *Phycomyces blakesleanus* (Cohen, 1974), *Oerskovia xanthanolytica* (Jeffries et al., 1977), *Trichoderma harzianum* (Thomas et al., 1979), *Saccharomyces cerevisiae* (Correa et al., 1982), *Pycnoporus cinnabarinus* (Ohtakara and Mitsutomi, 1982), *Neurospora crassa* (Zarain-Herzberg and Arroyo-Begovich, 1983), *Beauveria bassiana* (Coudron et al., 1984), *Metarrhizium anisopliae* (St. Leger et al.,
Chitinolytic enzymes are also synthesized by protozoans, coelenterates, nematodes, molluscs and arthropods (Bradbury et al., 1987; Jeuniaux, 1966). In vertebrates, chitinases are secreted by the pancreas and gastric mucosa of insectivorous birds, reptiles and mammals (Jeuniaux, 1961). They are reported to be present in the moulting fluid of hornworm (Manduca sexta) (Daizo et al., 1983; Fukamizo and Kramer, 1985), stomach of Japanese eel (Anguilla japonica) (Kono et al., 1990, 1990a) and all the developmental stages of brine shrimp (Artemia species) (Funke and Spindler, 1989; Peter and Schweikart, 1990).

Apart from the nutritional role in fungi and bacteria, chitinases seem to facilitate microbial equilibrium in soil (Danulat and Kausch, 1984; Oranusi and Trinci, 1985; Reichardt et al., 1983). The diversity of
roles of fungal chitinases ranges from morphogenesis to autolysis (Goody, 1989; 1990; Polacheck and Rosenberger, 1975; Tracey, 1955).

The presence of chitinases in plants has been attributed to the protection from infection by pests. Chitinases are one amongst the batteries of proteins produced by plants against the pathogenic invasion and therefore are called plant pathogenesis related proteins (Molano et al., 1979; Pierpoint et al., 1990; Tahiri-Alaoni, 1990).

1.4.1.2 Purification and properties

Roberts and Cabib (1982) have purified chitinase from S. marcescens by a single step purification procedure of adsorption digestion. The procedure was first developed by Jeuniaux (1966) for Streptomyces antibioticus chitinase. It involved affinity adsorption of the enzyme on the colloidal or regenerated chitin at 0 - 4°C. In the subsequent experiment, on incubation at 30°C, the chitinases hydrolyzed the chitin to which they were adsorbed and released into the solution. With this method, six fold purification of the enzyme was achieved. Yabuki et al. (1986) have also obtained 17 fold purification of chitinase from the crude concentrate of the culture filtrate of A. hydrophila subsp. anaerogenes A-52 using the same affinity-adsorption procedure. The enzyme was further
purified to homogeneity with no trace of chitobiase activity by additional CM-Sephadex C-50 chromatography. The enzyme was reported to be endo-type. The chitobiase which remained unadsorbed on chitin was purified 20 times as compared to the culture filtrate by successive ion-exchange chromatography with CM-Cellulose and DEAE-Sephadex A-50.

Chitinase of wheat germ was purified by affinity chromatography on chitin column (Molano, 1979). Enzyme was eluted by a linear gradient of pH from 3.3 - 5.3. This was followed by a chromatography on Sephadex G-50. The enzyme acted in random manner as dimers, trimers and tetramers were formed on chitin hydrolysis. Enzyme showed much greater activity on nascent rather than preformed chitin. Even glycol chitin, a substrate used for endo-chitinase assay was not attacked by the wheat germ enzyme.

Chitinase from S. erythraeus (Hara et al., 1989) culture filtrate was purified 130 fold by chromatographies using DEAE-Cellulose, Bio-GelP-60 and DEAE-Sephadex A-25. The purified enzyme could hydrolyze the high molecular weight substrates such as glycol chitin and colloidal chitin but not \( p \)-nitrophenyl-N-acetylglucosaminide, indicating its random action. While studying the kinetic analysis after the hydrolysis of colloidal chitin, glycol chitin and chitooligosaccharides, they have suggested the presence of at least four subsites laid across the
catalytic site.

Robbins et al. (1988) have used Superose 12 column (Pharmacia LKB FPLC apparatus) for adsorption and elution followed by Sephadex G-50 to purify the *Streptomyces plicatus* chitinase cloned in *Escherichia coli*. They studied the kinetics of purified enzyme using 4-methylumbelliferyl (4-MU) oligosaccharides as substrates and compared it with three other purified enzymes from *S. plicatus*. They found that two of the enzymes formed 4-methylumbelliferone much more rapidly from 4-MU-di saccharide than from trisaccharide. These enzymes convert the 4-MU-trisaccharide to diacetyl-chitobiose and 4-MU-monosaccharide, a non-fluorescent product. The later compound is not hydrolyzed by any of the purified enzymes. However, according to them further experimentation with chitin itself as a substrate is necessary to conclusively define endo- or exo-acting enzymes.

Tsukamoto et al. (1984) purified 3 chitinases from yam (*Dioscorea opposita*) to homogeneity by ammonium sulfate fractionation, chromatographies on DEAE-Cellulose and DEAE-Sephadex A-50, chromatofocussing on a Polybuffer Exchanger and Polybuffer-74 column and gel filtration on Bio-Gel P-60. All the three enzymes were endo-type as they readily hydrolyze glycol chitin but did not act on p-hydroxyphenyl-N-acetyl-D-glucosaminide. Recently, Koga et al. (1989)
carried out kinetic analysis of chitinase E-3 from yam using both series of chitooligosaccharides \( \text{NAG}_n \); \( n = 2 \) to 6 and \( \text{p-nitrophenyl-N-acetylchitooligosaccharides} \) \( \text{p-Np-NAG}_n \); \( n = 1 \) to 5) as substrates. The results monitored on HPLC using TSK Gel G2000 PW column suggested that the enzyme differed from insect chitinases with respect to its affinity towards chitooligosaccharides and the mechanism of action on \( \text{NAG}_6 \). The enzyme was found to be a random type hydrolytic enzyme with maximum activity on \( \text{NAG}_4 \) than on \( \text{NAG}_5 \) and \( \text{NAG}_6 \), the reverse was observed for insect chitinases. The insect chitinases cleave \( \text{NAG}_6 \) by only two ways i.e. \( \text{NAG}_2 \) plus \( \text{NAG}_4 \) and two molecules of \( \text{NAG}_3 \), whereas the yam chitinase does by three ways, to \( \text{NAG} \) plus \( \text{NAG}_5 \), in addition to the above two ways.

The chitinase and chitobiase have been purified from the culture filtrate of \( \text{P. cinnabarinus} \) (Ohtakara and Mitsutomi, 1982) by chromatographies on DEAE-Sephadex A-50, CM-Sephadex C-50 and Sephadex G-100. Affinity chromatography on N-N-diacetyl chitobiose-Sepharose 4B was carried out for chitinase purification. The analysis of hydrolysis products of chitooligosaccharides by the purified enzyme showed it to be a random-acting one. It could not hydrolyze chitobiose. \( \text{NAG} \) and chitobiose were observed from the hydrolysis of colloidal chitin. Chitobiase completely hydrolyzed chitooligosaccharides to \( \text{NAG} \).
Somers et al. (1987) and Sakuda et al. (1987) while screening *Streptomyces* species with potential insecticidal activities discovered the specific inhibitors of chitinases, allosamidins. Allosamidins are pseudotristosaccharides consisting of a disaccharide of N-acetylaspartallosamine, linked to a novel amino cyclitol derivative, allosamizoline. The inhibitory effect of allosamidin was reported to be variable for the chitinases from the different sources. Koga et al. (1987) have shown allosamidin to be a strong competitive inhibitor of insect chitinase (*Bombyx mori*), whereas bacterial chitinases from *Streptomyces griseus* and *S. marcescens* were poorly inhibited. However, it was ineffective against plant chitinases from yam. Gooday et al. (1988) showed it to be a potent inhibitor of chitinase from *Onchocera gibsoni*. Fungal chitinases from *A. nidulans* and *N. crassa* were also effectively inhibited by allosamidin (Gooday, 1989a).

Properties of several chitinases/chitobiases have been listed in Table 1.4.

1.4.1.3 Mechanism of chitin degradation

Monreal and Reese (1969) compared the chitinolytic and cellulolytic systems for proposing the mechanism of degradation of the respective polymers. According to them, both systems appear to involve random attacking endo-glycanases, which produce soluble intermediates and
Table 1.4: Properties of purified chitinases and chitobiases

<table>
<thead>
<tr>
<th>Source</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Km</th>
<th>Optimum pH</th>
<th>Optimum Temperature (°C)</th>
<th>pI</th>
<th>Molecular Weight of Hydrolysate</th>
<th>Products of Hydrolysate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vibrio species</strong></td>
<td>Endo-</td>
<td>Colloidal chitin</td>
<td>-</td>
<td>6.0-8.3</td>
<td>10.5</td>
<td>3.7</td>
<td>63,000</td>
<td>NAG₂</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glycol chitin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chitobiase</td>
<td>pNP-NAG</td>
<td>250 μM</td>
<td>6.0-9.0</td>
<td>-</td>
<td>-</td>
<td>95,300</td>
<td></td>
</tr>
<tr>
<td><strong>Aeromonas hydrophila</strong></td>
<td>Endo-</td>
<td>Colloidal chitin</td>
<td>2.8 mg/mL</td>
<td>7.0</td>
<td>45</td>
<td>4.6</td>
<td>110,000</td>
<td></td>
</tr>
<tr>
<td><strong>subsp. anaeorganes</strong> A-52</td>
<td>Chitobiase</td>
<td>pNP-NAG</td>
<td>1 mM</td>
<td>7.0</td>
<td>50</td>
<td>5.4</td>
<td>105,000</td>
<td></td>
</tr>
<tr>
<td><strong>S. erythraeus</strong></td>
<td>Endo-</td>
<td>Glycol chitin</td>
<td>-</td>
<td>5.0</td>
<td>-</td>
<td>3.7</td>
<td>30,000</td>
<td></td>
</tr>
<tr>
<td><strong>S. griseus</strong></td>
<td>Endo-</td>
<td>Colloidal chitin</td>
<td>-</td>
<td>4.5</td>
<td>50</td>
<td>8.6</td>
<td>19,000</td>
<td></td>
</tr>
<tr>
<td><strong>S. plicatus</strong></td>
<td>Endo-</td>
<td>Tritiated chitin</td>
<td>-</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
<td>63,000</td>
<td>4-MU</td>
</tr>
<tr>
<td>(purified from Escherichia coli recombinant)</td>
<td></td>
<td>4-MU NAG&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.5 μM</td>
<td>4.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-MU NAG&lt;sub&gt;2&lt;/sub&gt;</td>
<td>5.0 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S. species</strong></td>
<td>Endo-</td>
<td>4-MU NAG&lt;sub&gt;3&lt;/sub&gt;</td>
<td>14 μM</td>
<td>6.3-6.8</td>
<td>-</td>
<td>4.8</td>
<td>44,000</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-MU NAG&lt;sub&gt;2&lt;/sub&gt;</td>
<td>49 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colloidal chitin</td>
<td>-</td>
<td>3.4-4.2</td>
<td>-</td>
<td>8.3</td>
<td>41,000</td>
<td></td>
</tr>
<tr>
<td><strong>P. cinnabarinus</strong></td>
<td>Endo-</td>
<td>Colloidal chitin</td>
<td>-</td>
<td>4.5</td>
<td>-</td>
<td>3.6</td>
<td>38,000</td>
<td>NAG₂</td>
</tr>
<tr>
<td><strong>N. crassa</strong></td>
<td>Endo-</td>
<td>Tritiated chitin</td>
<td>-</td>
<td>6.7</td>
<td>-</td>
<td>-</td>
<td>20,600</td>
<td></td>
</tr>
<tr>
<td><strong>A. niger</strong></td>
<td>Chitobiase</td>
<td>pNP-NAG</td>
<td>450 μM</td>
<td>2.2</td>
<td>-</td>
<td>5.4</td>
<td>65,000</td>
<td></td>
</tr>
<tr>
<td><strong>Mucor rouxii</strong></td>
<td></td>
<td>Tritiated chitin</td>
<td>-</td>
<td>6.5</td>
<td>-</td>
<td>-</td>
<td>30,000</td>
<td></td>
</tr>
<tr>
<td><strong>Bean leaf (Phaseolus vulgaris)</strong></td>
<td>Endo-</td>
<td>Colloidal chitin</td>
<td>-</td>
<td>6.5</td>
<td>-</td>
<td>9.4</td>
<td>32,500</td>
<td>NAG₂, NAG₃</td>
</tr>
</tbody>
</table>
Table 1.4 Contd.

<table>
<thead>
<tr>
<th>Source</th>
<th>Enzyme</th>
<th>Substrate</th>
<th>Km</th>
<th>pH</th>
<th>Temp (°C)</th>
<th>pH</th>
<th>Molecular weight</th>
<th>Products of hydrolysis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melon plant (Cucumis melo cv. Cantaloupe charantais)</td>
<td>Endo-</td>
<td>Tritiated chitin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.4</td>
<td>29,000</td>
<td>NAG₂, NAG₃</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NAG₆</td>
<td>0.096 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NAG₃</td>
<td>1.31 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Exo-</td>
<td>NAG₆</td>
<td>1.70 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10.4</td>
<td>34,000</td>
<td>NAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NAG₃</td>
<td>0.54 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Yam (Dioscorea opposita)</td>
<td>Endo-</td>
<td>Glycol chitin</td>
<td>-</td>
<td>3.5</td>
<td>8.5</td>
<td>-</td>
<td>4.05</td>
<td>33,500</td>
<td>-</td>
</tr>
<tr>
<td>Hornworm (Manduca sexta)</td>
<td>Endo-</td>
<td>Glycol chitin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.0</td>
<td>33,500</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Endo-</td>
<td>Glycol chitin</td>
<td>-</td>
<td>3.5</td>
<td>6.5</td>
<td>-</td>
<td>3.8</td>
<td>33,500</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Endo-</td>
<td>Glycol chitin</td>
<td>0.15 mM</td>
<td>6.5</td>
<td>-</td>
<td>-</td>
<td>75,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Endo-</td>
<td>Glycol chitin</td>
<td>0.23 mg/ml</td>
<td>6.5</td>
<td>-</td>
<td>-</td>
<td>62,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Endo-</td>
<td>Glycol chitin</td>
<td>0.23 mg/ml</td>
<td>5.2</td>
<td>-</td>
<td>-</td>
<td>52,000</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Chitobiase</td>
<td>pNp-NAG</td>
<td>177 µM</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
<td>5.9</td>
<td>60,000</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Chitobiase</td>
<td>pNp-NAG</td>
<td>160 µM</td>
<td>6.0</td>
<td>-</td>
<td>-</td>
<td>5.1</td>
<td>61,000</td>
<td>-</td>
</tr>
<tr>
<td>Japanese eel (Anguilla japonica)</td>
<td></td>
<td>Colloidal chitin</td>
<td>-</td>
<td>4.4</td>
<td>-</td>
<td>-</td>
<td>6.2</td>
<td>50,000</td>
<td>NAG, NAG₂</td>
</tr>
<tr>
<td>Red sea bream (Pagrus major)</td>
<td></td>
<td>Colloidal chitin</td>
<td>-</td>
<td>5.5</td>
<td>50</td>
<td>8.3</td>
<td>46,000</td>
<td>NAG, NAG₂</td>
<td>Kono et al. (1987a)</td>
</tr>
</tbody>
</table>

Endo-, Endo-chitinase
pNp-, p-Nitrophenyl derivatives
4-MU, 4-Methylumbelliferyl derivatives
NAGₙ, Chitooligosaccharides
glycosidases which hydrolyze the intermediates, specifically dimers to monomers. They suggested the presence of prehydrolytic factor Ch₁ analogous to C₁ in cellulose degradative mechanism for the efficient hydrolysis of chitin. Based on this, the possible mechanism of chitin degradation can be represented as follows:

```
Chitin  →  Reactive Chitin  →  Chitooligosaccharides  →  Chitobiase  →  NAG
```

(Modified from Monreal and Reese, 1969; Reese et al., 1950).

With the partially purified chitinases from *Aspergillus niger*, Otakara (1964) suggested a possible pattern for the enzymatic degradation as follows:

```
\[
\begin{align*}
\text{Chitin} &\quad (\text{High molecular weight}) \\
\text{c(Chitinase?)} &\quad \text{Chito-dextrins} \\
&\quad (\text{Low molecular weight}) \\
\text{a}_1 (\text{Chitinase}) &\quad \text{b}_1 (\text{Chitinase}) \\
\quad &\quad \text{NAG}_2 \\
\text{a}_2 (\text{Chito-dextrinase}) &\quad \text{b}_2 (\text{Chitobiase}) \\
\quad &\quad \text{NAG} \\
\end{align*}
\]
Due to the action of $a_1$ and $b_1$ enzyme fractions, low molecular weight species were produced from chitin, which were subsequently hydrolyzed to monomers by $a_2$ and $b_2$ fractions. Whereas, enzyme fraction $c$ released monomers without forming any detectable amount of oligosaccharides during the degradation of chitin.

The detail work on purification and characterization of all the chitinolytic enzymes and their mode of action singly or in combination from one source is necessary for the conclusive comments.

1.4.1.4 Transglycosylation

Usui et al. (1987) have reported for the first time the transglycosylation action for the purified chitinase from Nocardia orientalis. The enzyme catalyzed the conversion of $\text{NAG}_4$ to $\text{NAG}_6$ and $\text{NAG}_2$. The rate of transglycosylation was influenced by temperature, pH and substrate concentration in the reaction mixture. Subsequently, Nanjo et al. (1989) reported increased transglycosylation by N. orientalis chitinase in the presence of ammonium sulfate. Nanjo et al. (1990) further reported that $\beta$-$\text{N}$-acetylhexosaminidase of N. orientalis first catalyzed the hydrolysis of $\text{NAG}_2$ to NAG and subsequently the formation of $\text{NAG}_3$ and $\beta$-$(1\rightarrow6)$ linked $\text{NAG}_2$ was observed.

1.4.1.5 Chitinase assay

Chitinase activity can be qualitatively assayed
by determining the clearance zone developed around the colonies growing on the colloidal chitin agar medium (Cody et al., 1990; Ueno et al., 1990).

Assay of chitinase deserves a separate attention because the same enzyme may show variation in the activity depending on the nature of substrate. Colloidal chitin and regenerated chitin obtained after acetylation of chitosan were reported to be more susceptible to the chitinase attack than the native (crystalline) chitin (Molano et al., 1977; Monreal and Reese, 1969). Some of the widely used procedures for the quantitative estimation of chitinase activity have been discussed.

Colorimetric assay

This method is based on the determination of products released from the hydrolysis of chitin. The formation of products can be detected either by determining the total reducing sugars (Chen et al., 1982; Tsukamoto et al., 1984) or by specifically determining the NAG with p-dimethylaminobenzaldehyde (DMAB) (Reissig et al., 1955).

Abeles et al. (1970) reported the estimation of exo-type chitinase activity using colloidal chitin as a substrate. Boller and Mauch (1988) have described a procedure for the estimation of endo-chitinases from various plant sources using colloidal chitin as a substrate. The principle is, endo-chitinases hydrolyze insoluble
chitin to soluble oligosaccharides and after removal of undigested substrate, the chitooligosaccharides can be completely hydrolyzed to NAG by glycosidases which can be estimated with DMAB reagent. However, in case of some crude plant extracts, the interference of chromogenic products make the colorimetric estimation of chitinases inaccurate.

Thus, the type of enzyme assayed using colorimetric method depends on the nature of substrate, i.e. colloidal chitin will assay both endo- as well as exo-chitinases, whereas soluble chitin derivatives such as glycol chitin can be used for endo-enzymes (Hara et al., 1989).

Aribisala and Gooday (1978) have used 3-4-dinitrophenyl tetra-N-acetylchitotetraoside as a substrate to measure chitinase activity. However, Stirling et al. (1979) have pointed out that the chain length in this substrate may be short for some endo-chitinases and furthermore it is hydrolyzed by \( \beta \)-N-acetylxosaminidase.

**Viscometric assay**

The viscometric assay for chitinase is sensitive to detect a slight activity. However, the procedure is troublesome as compared to colorimetric ones.

The procedure is based on measuring the drop in the viscosity of soluble chitin derivatives such as glycol
chitin (Otakara, 1961) or carboxymethyl chitin (Alfonso et al., 1991; Hultin, 1955) using viscometer.

Since endo-enzyme splits chitin chain randomly, a few breaks in the chain would decrease the specific viscosity (\( \eta_{sp} \)) of the substrate considerably in a short time. Even after viscosity losses are essentially complete (i.e. even when viscosity losses are no longer detectable) reducing power would continue to be generated at a steady rate. The exo-acting enzymes on the other hand will have the opposite effect, since these enzymes remove only NAG or chitobiose from the non-reducing end, hence the viscosity (\( \eta_{sp} \)) fall is not rapid.

Radiometric assay

In this procedure [acetyl-\(^3\)H]chitin is used as a substrate and the water soluble products formed are separated from the insoluble chitin and their radioactivity is determined (Molano et al., 1977). This is the most sensitive method because of the possibility of using substrate of very high specific activity. Both endo- and exo-type chitinases can be assayed by this method.

Fluorometric assay

O'Brien and Colwell (1987) have described a rapid test for chitobiase activity using 4-methylumbelliferyl (4-MU)-N-acetyl-\( \beta \)-D-glucosaminide as a substrate. This assay is based on the formation of fluorescent methyl-
umbelliferone as a reaction product. Robbins et al. (1988) have used 4-MU glycosides of N-acetylglucosamine oligosaccharides (4-MU disaccharide and 4-MU trisaccharide) for the estimation of chitinase activity. On the basis of reaction products they have distinguished endo- and exo-chitinases. Hood (1991) has used 4-methyllumelliferyl chitin derivatives for measuring chitinase activity in aquatic environments.

1.4.2 ENZYMATIC DEGRADATION OF CHITOSAN

Chitosan is a non-crystalline polymer of glucosamine with varying degrees of acetylation. Chitosanases (EC 3.2.1.99) are a class of enzymes that hydrolyze chitosan. Several microorganisms have been reported to produce chitosanases. For example, Bacillus species (Uchida and Ohtakara, 1988), Bacillus circulans (Ohtakara, 1988), Myxobacter AL-1 (Hedges and Wolf, 1974), Penicillium islandicum (Fenton and Eveleigh, 1981) and M. rouxii (Alfonso et al., 1991).

Chitosanases are inducible extracellular enzymes produced by microorganisms when grown on chitosan (Hedges and Wolf, 1975; Fenton and Eveleigh, 1981). Fenton and Eveleigh (1981) have purified chitosanase from the culture filtrate of P. islandicum to homogeneity by ammonium sulfate fractionation and sequential chromatography on DEAE-Bio-Gel A, Bio-Gel P-60 and hydroxyapatite. The enzyme (molecular weight 30,000; pI 4.2) was found to be endo-acting producing mainly dimers and trimers on hydro-
ysis of chitosan. The chitosan having equal proportion of acetylated and non-acetylated sugars was found to be optimal for chitosanase activity.

Purified chitosanase from Bacillus sps. (Uchida and Ohtakara, 1988) hydrolyzed colloidal chitosan, soluble chitosan and glycol chitosan, however, it could not act on powdered chitosan, powdered chitin, colloidal chitin and glycol chitin. Hydrolysis products of soluble chitosan suggested the endo-type nature of the enzyme.

The purified chitosanases have been classified into two groups, the enzymes hydrolysing only chitosan and the enzymes which show action on chitosan and carboxymethyl cellulose. The chitosanase reported from S. griseus fall into the second group (Ohtakara, 1988). In addition to these two substrates, enzyme has also shown action on glycol chitosan and carboxymethyl chitosan. However, no hydrolysis has been reported of colloidal chitin and glycol chitin. The rapid fall in viscosity of glycol chitosan and soluble chitosan with the enzyme suggested the mode of action as endo-type.

1.5 APPLICATIONS OF CHITINOLYTIC ENZYMES

Chitin degrading enzymes have a variety of roles to play in nature. It ranges from their involvement in fungal morphogenesis to the destruction of chitin containing cells. Depending on the practical utility and economic
considerations, chitinases have a great deal of potential for applied research.

1.5.1 BIOCONTROL AGENTS

As the cell wall of many plant pathogens contain chitin, the enzymatic hydrolysis of this structural polymer will eventually lead to the lysis of the cell. Elad et al. (1984) have demonstrated with the help of scanning and transmission electron microscopy, the degradation of the walls of *Sclerotium rolfsii* by *T. harzianum* leading to the release of cytoplasmic contents. They attributed this parasitism to the production of chitinase and β-glucanase. Chet (1988) has shown the swelling and bursting of the hyphal tips of *S. rolfsii* by *S. marcescens* chitinase.

In plants, chitinases serve as an endogenous defence mechanism against the invasion by pathogens. Cline et al. (1978) have shown that soluble oligomers formed as a result of fungal and bacterial cell wall hydrolysis act as elicitors in the plant tissue to induce the production of phytoalexins which are normally absent in healthy plants. Hadwiger and Beckman (1980) demonstrated that extracts of the pea endocarp containing chitinase and chitobiase on lysing *Fusarium solani* f-sp-*pisi* and f-sp-*phaseoli* cell walls, release fragments which are strong elicitors of the isoflavinoid phytoalexin, pisatin in the pods.
The direct proof for the role of chitinase against the pathogens was obtained by Shapira et al. (1989). The partially purified chitinase from the recombinant E. coli having S. marcescens chitinase genes was found to be effective against S. rolfsii in beans and Rhizoctonia solani in cotton.

Jaworski et al. (1986) have demonstrated the protection of soybean plant from the nematode invasion by inoculating the plant root samples with the recombinant Pseudomonas fluorescens carrying the chitinase genes from S. marcescens.

1.5.2 BIOCONVERSION OF CHITINOUS WASTE TO SINGLE CELL PROTEIN

In the search for finding an alternative method of waste disposal from the shellfish processing industry, a process scheme has been developed for the bioconversion of shellfish waste chitin to yeast SCP (Carroad and Tom, 1978; Cosio et al., 1982; Tom and Carroad, 1981). The product, a protein rich material can be used as an animal and aquaculture feed supplement. Likewise, chitinases also play important role in the cultivation of edible mushrooms on commercial wheat straw/horse manure compost (Fermor and Grant, 1985).

1.5.3 THE PROTOPLAST TECHNOLOGY

Chitinases in the mycolytic preparations enhance the yield of fungal protoplasts (Moore, 1975; Morinaga
et al., 1985; Thomas and Davis, 1980; Yanagi and Takabe, 1984). Sandhu et al. (1989) reported the efficient release of *Trichoderma reesei* protoplasts by supplementing the partially purified culture filtrate of *T. harzianum* with commercial mycolytic preparation.

The chitinolytic enzymes likewise have a number of applications in different areas of research, from agriculture to medicine. The importance of chitinases in biotechnology has prompted us to carry out the present investigations.
1.6 PRESENT INVESTIGATIONS

The work presented in this thesis includes the following investigations.

1. Screening of potent fungal chitinase producers and optimization of culture conditions for maximum chitinase production by *M. verrucaria* NCIM 903.

2. Potential of the *M. verrucaria* culture filtrate for fungal mycelia degradation.

3. Enzymatic hydrolysis of chitin and utilization of hydrolysate for SCP production.

4. Use of solid state CP/MAS $^{13}$C NMR spectroscopy as a tool to monitor enzymatic hydrolysis of chitin.

5. Purification of chitinase to homogeneity and study of its physico-chemical properties.