2. REVIEW OF LITERATURE

The Report of the Commission on Enzymes of the International Union of Biochemistry (1961) termed "cellulase" as the trivial name for $\beta$-(1,4)-glucan-4-glucanohydrolases, i.e., for enzymes which hydrolyse $\beta$-1,4-glucans at linkages which are not restricted to terminal linkages. Cellulase was earlier reviewed by Emert et al., (1974) and Whitaker (1971). Hydrolysis and utilization of cellulose to provide energy to the organism was thought to be carried out exclusively by microorganism. But now it appears that some animal species, including termites and cray fish produce their own indigenous microflora (Watanabe & Tokuda, 2001). Li et al., (Report, 1961) mention that their endoglucanase can be coded according to the Commission on Enzymes of the International Union of Biochemistry as a $\beta$-(1,4)-D-glucanohydrolase (E.C.3.2.1.4). It indicates the substrate, as a $\beta$-(1,4) polymer of D-glucose, which is split by hydrolysis of $\beta$-(1,4)-glucosidic linkages at random attack, not confined to the terminal ends.

2.1 Cellulose

Plants have high cellulose content, approximately 35 to 50% of plant dry weight (Lynd et al., 1999). Cellulose is present in pure state in cotton balls. In most cases, cellulose fibres are embedded in a matrix of other structural biopolymers, primarily hemicelluloses and lignin, which comprise 20 to 35 and 5 to 30 % of plant dry weight (Lynd et al., 1999; Marchessault & Sundararajan, 1993; Van Soest, 1994). Although these matrix interactions vary with plant cell type and with maturity (Wilson, 1993), they are a dominant structural feature limiting the
rate and extent of utilization of whole, untreated biomass materials. Cellulose is synthesized in nature as individual molecules (linear chains of glucosyl residues), which undergo self-assembly at the site of biosynthesis (Brown & Saxena, 2000). Approximately 30 individual cellulose molecules are assembled into large units known as elementary fibrils (protofibrils), which are packed into larger units called microfibrils, and these are in turn assembled into the familiar cellulose fibres. Regardless of orientation of adjacent chains, they are stiffened by both intra-chain and inter-chain hydrogen bonds. Adjacent sheets overlie one another and are held together (in Cellulose I, the most abundant form of cellulose in nature) by weak intersheet van der waals forces. Despite the weakness of these interactions, their total effect over the many residues in the elementary fibril is considerable (Pizzi & Eaton, 1985). The crystalline nature of cellulose implies a structural order in which all of the atoms are fixed in discrete positions with respect to one another.

Although cellulose forms a distinct crystalline structure, cellulose fibres in nature are not purely crystalline. The degree of crystallinity is variable. Distribution of crystallinity is "lateral" which portrays a population of cellulose fibres in statistical terms as a continuum from purely crystalline to purely amorphous, with all degrees of order in between (Marchessault & Howsmon, 1957). Apart from crystalline and amorphous regions, cellulase fibres contain various types of irregularities, such as twists of the microfibrils, or voids such as surface micropores, large pits and capillaries (Blouin et al., 1970; Cowling, 1975; Fan et al., 1980a; Marchessault & Sundararajan, 1993). Schematic representation of a
cellulose fibre is shown in Figure 2.1. The fibres are hydrated by water when immersed in aqueous media and some micropores and capillaries are sufficiently spacious to permit penetration by cellulytic enzymes (Stone et al., 1968). Purified celluloscs used for studies of hydrolysis and microbial utilization vary considerably in fine structure. The choice of substrate thereby affects the results obtained. Microcrystalline celluloscs (e.g. Avicel and Sigma cell) are nearly pure cellulose, and the dilute acid treatment used in their preparation removes both hemicelluloscs and the more extensive amorphous regions of the cellulose fibres. Like plant cellulose, bacterial cellulose is highly crystalline, but the two celluloscs differ in the arrangement of glucosyl units within the unit cells of the crystallites (Atalla & Vanderhart, 1984) and genetic evidence suggests that the two celluloscs are synthesized by enzymatic machinery that differs considerably at the molecular level (Brown & Saxena, 2000). The two celluloscs also differ substantially in rate of hydrolysis by fungal cellulases (Hayashi et al., 1997) and in rate of utilization by mixed ruminal bacteria (Schofield et al., 1994; Weimer et al., 2000). The variable structural complexity of pure cellulose and the difficulty of working with insoluble substrates have led to the wide use of the highly soluble cellulose ether, carboxymethylcellulose (CMC), as a substrate for studies of endoglucanase production (Lynd et al., 2002).

2.2 Glycoside hydrolase families

Enzymes are designated according to their substrate specificity, based on the guidelines of the International Union of Biochemistry and Molecular Biology. The cellulases are grouped with many of the hemicelluloscs and other
Figure 2.1: Schematic representation of a cellulose fibre (Robinson, 1987)

approx 36
β-glucan
chains

open amorphous region

β-glucan chains
polysaccharides as O-glycoside hydrolases (EC.3.2.1.x). With several thousand glycoside hydrolases being identified, alternative classifications into families were suggested based on amino acid similarity (Henrissat, 1991). This classification has been updated several times (Henrissat & Bairoch, 1993, 1996), but with the exponential growth in the number of glycoside hydrolases identified, Coutinho & Henrissat (1999) have begun to maintain and update the classification of glycoside hydrolases families at the Expasy server (http://afmb.cnrs-mrs.fr/~pedro/CAZy/db.html). Families were defined based on amino acid sequence similarities. There is usually a direct relationship between the amino acid sequence and the folding of an enzyme, and as the tertiary structures of many proteins were added, it became clear the families contain basic enzyme folds (Henrissat et al., 1995). Classification into families defines the modules of such enzymes and resolves the contradiction about substrate specificity for multifunctional enzyme. The family classification also sheds light on the evolution of glycoside hydrolases. Some families contain enzymes with different substrate specificity e.g. family 5 contains cellulases, xylanases and mannanases. This suggests divergent evolution of a basic fold at the active site to accommodate different substrates. At the same time cellulases are found in several different families [families, 5, 6, 7, 8, 9, 10, 12, 44, 45, 48, 61 and 74] suggesting convergent evolution of different folds resulting in the same substrate specificity. Family 9 contains cellulases of bacteria, fungi and plants (Del Campillo, 1999) and animals (protozoa & termites) (Watanabe & Tokuda, 2001). In contrast family 7 contains only fungal hydrolases and family 8 contains only
bacterial hydrolases. The classification of cellulases into families allows easy access to information on structure, mechanism and evolutionary origin. Henrissat et al., (1998) recently proposed a new nomenclature for hydrolases in which the first three letters designate the preferred substrate, the next digits designate the glycoside hydrolase family, and the following capital letters indicate the order in which the enzymes were first reported. For e.g., the three enzymes cellobiohydrolase I (CBH I), CBH II and endoglucanase I of Trichoderma reesei are designated Cel 7A (CBH I), Cel 6A (CBH II) and Cel 16B (EG I). However, this new nomenclature has not been completely accepted due to lack of substrate specificity, for instance the distinction between endoglucanase and CBH activity.

2.3 Mechanism of cellulase degradation by enzymes

Several models have been described to study the enzymatic mechanism by which cellulose is being hydrolyzed. Reese et al., (1950) had stated that many microorganisms are able to hydrolyze modified cellulosics, but only some of them are able to attack native cellulose such as mature, dried cotton. They suggested that a C$_1$-C$_x$ system should make the latter true cellulolytic organism capable of completely solubilizing native cellulose. Mandels and Reese (1964) have described their C$_1$-C$_x$ concept in the following schematic way:

\[
\text{Cellulose} \xrightarrow{\text{C}_1} \text{reactive cellulose} \xrightarrow{\text{C}_x} \text{cellobiose} \xrightarrow{\beta\text{-glucosidase}} \text{glucose}
\]

(\text{hydrocellulase}) \hspace{1cm} (\beta\text{-endogucanase}) \hspace{1cm} \text{hydrolytic}
The hydrolytic C₉ is a β-1,4-glucanase, able to attack all celluloses in the range from soluble cellulose derivatives to celluloses “swollen” by alkali or acids or by means of mechanical treatments such as grinding. When the substrate does not offer hindrance to the approach of the enzyme molecule, and the end effects of very short chains are absent, the enzyme is of a random-splitting type. Norkrans (1967) has reported that the endoglucanase is not specific to the bond being broken but to the reducing end unit being split off. Two species of *Trichoderma*, namely *Trichoderma viridae* and *Trichoderma koningii*, were found to give cell-free preparations capable of solubilizing cotton fiber (Mandels & Reese, 1964; Halliwell, 1965; Li et al., 1965). Halliwell obtained complete solubilization of cotton fibers with quantitative conversion to glucose within 19 days by a culture filtrate from *Trichoderma koningii*. In the first phase (7 days) short fiber fragments were produced and only minor quantities of soluble products. When soluble and insoluble products each constitute 40 to 50% of the weight of the initial substrate; the quantity of sugars increases at the expense of the insoluble fragments. Enzyme preparations from *Myrothecium verrucaria* could not solubilize the short fragments formed. Cellulose powder was solubilized by *Trichoderma* filtrate at the same rate as cotton fibers (19 years), hydrocellulose required almost four times that period.

According to the model of native cellulose as a fringe micelle, fragmentation of fibres has been interpreted as a chain shortening in the same way as enzymic attack by cellulase from *Penicillium variabile* (Norkrans, 1967). Amberlite XE-64 fractionated an enzyme preparation from *Penicillium variable* into a fraction with
activity towards carboxymethylcellulose and a fraction with activity towards carboxymethylcellulose and α-cellulose; the latter did not give cellobiose as a hydrolysis product. A fraction with activity towards carboxymethylcellulose but not towards swollen cellulose or powdered cellulose has also been reported to be obtained from *Aspergillus saitoi* (Jurášek, 1967). Mandels and Reese worked with *Trichoderma viridae*. After treating dewaxed cotton slivers for 45 days with culture filtrates from this organism, a weight loss of 60% was noted. This is 30 times or more the loss caused by *Myrothecium verrucaria* or *Chaetomium globosum*. Cotton was found to be the most resistant of the pure celluloses. Woody materials were also resistant unless they were thoroughly ground by ball milling. Filter paper or newspaper showed considerable breakdown in 1 day, thus 50g of newspaper gave rise to 12.8g of sugars, mainly glucose, but also cellobiose and xylose, when treated with *Trichoderma* filtrate. After repeated chromatograms on DEAE- dextrans, they succeeded in separating components into C₁, Cₓ and β-glucosidase activity. They assumed C₁ “to act in a way to permit an increased moisture uptake, hydrating the cellulose and pushing apart the closely packed chains”, to make the linkages accessible for the action of Cₓ (β-1, 4 - endoglucanase). Li et al., (1965) maintain that the key property of the C₁ component is its capacity to attack highly crystalline cellulose. Consequently, they used crystalline aggregates of hydrocellulose, or the commercially available Avicel, which constitutes an easily handled “eucellulosic” substrate, for testing a cellulase system. The starting enzyme was obtained from wheat bran-sawdust cultures of *Trichoderma viridae*. They have been able to separate this crude
preparation into different components with distinct enzymic properties. Although none of the components alone could account for the enzymic overall process of the crude preparation, appropriate combinations did. The components are hydrocellulase, endoglucanase and exoglucanase (Norkrans, 1967).

Hydrocellulase correspond to the C₁ component, being the only one having the capacity to attack crystalline cellulose at any appreciable rate. The primary product of attack is cellobiose. At the first step of purification, on Avicel column, irrigated by sodium citrate buffer of pH 4.8, the hydrocellulase was completely retained, contrary to all the other components (Norkrans, 1967). This component showed an approximate molecular weight of 60,000. Endoglucanase has a minimum molecular weight of 26,000 (based on amino acid composition, one methionine per molecule) and a most probable molecular weight of 52,000 according to sedimentation data. It was relatively thermo-stable and it behaved just as a typical Cₓ enzyme. The optimum length of its substrate chain was at least 6 glucosyl unit according to Kₓ values derived from hydrolysis of glucomers varying from cellobiose through cellohexose. By using terminally substituted sugars (cellotetrosyl- and cellopentosyl sorbitol), higher rates of hydrolysis for interior linkages could be shown (Whitaker, 1954). Exoglucanase has a molecular weight of 76,000, approximated form sedimentation data. All of the thirteen β-glucosides tested were hydrolysed.

The difficulties in studying enzymatic hydrolysis of cellulosic materials are attributable to the complex property of cellulose and its constituents as well as to
the multiplicity and complexity of the cellulase system (Lee et al., 1982; Ryu & Mandels, 1980). Various kinetic models have been developed to elucidate the mechanism of hydrolysis of cellulase. One model is based on the structural features of the substrate (Fan et al., 1980b; Grethlein, 1985) while other based on properties of the cellulase enzyme and the mass transfer in reaction system. Kinetic model of the full time course of hydrolysis including enzyme adsorption has been developed by (Lee & Fan, 1983; Converse et al., 1988, Converse, 1993). A kinetic study of the hydrolysis of celluloses by Endoglucanase I and Exoglucanase II purified from Trichoderma viridae cellulose was performed (Kim et al., 1995). It was found that a more reactive endoglucanase acts randomly, mainly on amorphous or modified cellulose such as carboxymethylcellulose to produce glucose. The exoglucanase removes a cellobiose unit from the non-reducing end of the cellulose chain. This compound attacks neither the amorphous or modified cellulose nor the crystalline cellulose to any significant extent (Kim et al., 1994).

Earlier studies reported on the production of cellulases by microorganisms includes: bacteria: Sporocytophaga myxococccoides, Ruminococcus albus, and a thermophilic bacterium; a streptomycete: Streptomyces antibioticus (Enger & Sleeper, 1965); molds: Rhizopus spp. various species of Penicillium, Trichoderma, and Aspergillus terreus and Penicillium variable (Pal and Ghosh, 1965), a thermophilic strain of Aspergillus fumigatus, Pyrenochaeta terrestris (Horton & Keen, 1966); Mycorrhizal fungi and higher fungi: Fomes annosus. Various cellulase-producing microorganisms are listed in Table 2.1. The cellulase
Table 2.1 List of various cellulase producing microorganisms

**Fungi**
- Acremonium cellulolyticus
- Aspergillus aculeatus
- Aspergillus fumigatus
- Aspergillus niger
- Aspergillus oryzae
- Chrysosporium lignorum
- Chrysosporium lucknowense
- Fusarium solani
- Humicola insolens
- Irpex lacteus
- Melanocarpus albomyces
- Merulius lacrymans
- Mucor circinnelloides
- Myrothecium verrucaria
- Neisseria sicca
- Orpinomyces joyonii
- Penicillium citrinum
- Penicillium funiculosum
- Penicillium notatum
- Penicillium variable
- Pervotella spp.
- Phanerochaete
- Piromyces equi
- Rhizopus delmar
- Rhizopus oryzae
- Schizophyllum commune
- Sclerotinia sclerotiorum
- Sclerotium rolfsii
- Sporotrichum cellulophilum
- Sporotrichum dimorphosporum
- Talaromycyes emersonii
- Thermoascus aurantiacus
- Thermoascus longibrachiatum
- Thielavia terrestris
- Trichoderma koningii
- Trichoderma reeseei
- Trichoderma viridae
- Volvariella volvaceae

**Bacteria**
- Alicyclobacillus acidocaldarius
- Bacillus spp.
- Bacteroides succinogens
- Cellulomonas
- Cellvibrio fulvus
- Clostridium acetobutylicum
- Clostridium thermocellum
- Pervotella spp.
- Rhodo thermus
- Ruminococcus albus
- Streptomyces sp.
- Thermobifida fusca

**Actinomycetes**
- Rhodothermus
- Streptomyces sp.
- Thermoactinomyces sp.
- Thermomonospora curvata
in the digestive juice of the edible snail, *Helix pomatia*, is prominent in the early literature on cellulases but its origin has been a matter of some controversy. Later it was suggested to be produced by the snail itself and not by its microflora (Jurášek, 1967).

For the purpose of ease and lucidity the findings on cellulases are classified based on the source from which it is obtained.

**2.4 Fungi as a source of Endoglucanase**

Fungi endoglucanase forms nearly one third of the research work carried out with endoglucanase/cellulases. Consequently the work done on fungi endoglucanase forms the basis and model for studies of other endoglucanase. Detailed reviews on fungi Endoglucanases have been published (Lynd et al., 2002; Norkrans, 1967). Amino acid composition indicates a molecular weight of 31,000. The ability of fungi to digest cellulose has been reported (Bucht & Eriksson, 1969; Keilich et al., 1969). Fungi are the well-known agents of decomposition of organic matter in general and of cellulosic substrates in particular (Carlile & Watkinson, 1997; Montegut et al., 1991). A number of species of the anaerobic *Chytridomycetes*, the primitive group of fungi, are well known for their ability to degrade cellulose in the gastrointestinal tracts of ruminant animals. Aerobic fungi are also capable of cellulolysis. Within the approximately 700 species of zygomycetes only certain members of the genus *Mucor* have been shown to possess significant cellulolytic activity. They are better known for their ability to utilize soluble substrates. The much more diverse subdivisions *Ascomycetes*,...
Basidiomycetes and Deuteromycetes (Carlile & Watkinson, 1997) contain large numbers of cellulolytic species. *Bulgaria, Chaetomium* and *Helotium* (Ascomycetes); *Coriolus, Phanerochaete, Poria, Schizophyllum* and *Serpula* (Basidiomycetes) and *Aspergillus, Cladosporium, Fusarium, Geotrichum, Myrothecium, Paecilomyces, Penicillium* and *Trichoderma* (Deuteromycetes) are some of the genera that have received considerable study with respect to their cellulolytic enzymes and/or wood-degrading capability.

Carboxymethylcellulase has been identified in rotting fungi (Bucht & Ericksson, 1969; Keilich et al., 1969). Cowling (1975), studying wood decay, makes the following statement about the white-rotting *Polyporus versicolor*, "the organisms degraded the crystalline and amorphous cellulose simultaneously".

Several isoenzymes of cellulase have been studied. Table 2.2 lists the works carried out and the number of isoenzymes of cellulases identified in each of these organisms.

Selby et al., (1963) suggested that the cellulase system of *Myrothecium verrucaria* contained at least two enzyme types that differed in the rate and extent of their attack on fibrous cellulose such as cotton yarn. It was suggested that the A enzyme necessary for extensive degradation of cotton was present in small amounts only in the culture filtrate as normally prepared, and that it was "exhausted" by exposure to cotton yarn. Another cellulase, the B enzyme, was not so deactivated, but was able to weaken cotton to a limited extent only. The B activity was measured as carboxymethylcellulase activity, the A activity as loss of
Table 2.2 List of microorganism that produce cellulase - isoenzymes.

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>No. of Isoenzymes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Myrothecium verrucaria</em></td>
<td>2</td>
<td>Selby et al., 1963</td>
</tr>
<tr>
<td><em>Myrothecium verrucaria</em></td>
<td>3</td>
<td>Selby &amp; Maitland, 1965</td>
</tr>
<tr>
<td><em>Chrysosporium lignorum</em></td>
<td>5</td>
<td>Erriksson &amp; Pettersson, 1975</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Almin et al., 1975</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Erriksson &amp; Rzedowski, 1989 a, b</td>
</tr>
<tr>
<td><em>Rhizopus oryzae</em></td>
<td>2</td>
<td>Murashima et al., 2002</td>
</tr>
<tr>
<td><em>Thermoascus aurantiacus</em></td>
<td>3</td>
<td>Tong et al., 1980</td>
</tr>
<tr>
<td><em>Polyporus vesicolor</em></td>
<td>2</td>
<td>Pettersson &amp; Porath, 1963</td>
</tr>
<tr>
<td><em>Trichoderma reesei</em></td>
<td>2</td>
<td>Bhikhabhai et al., 1984</td>
</tr>
<tr>
<td><em>Humicola insolens</em></td>
<td>7</td>
<td>Schülein, 1997</td>
</tr>
<tr>
<td><em>Penicillium citrinum</em></td>
<td>11</td>
<td>Olutiola, 1976</td>
</tr>
<tr>
<td><em>Trichoderma viridae</em></td>
<td>2</td>
<td>Okada, 1975</td>
</tr>
<tr>
<td><em>Trichoderma koningii</em></td>
<td>2</td>
<td>Iwasaki et al., 1964</td>
</tr>
<tr>
<td><em>Vertinia sclerotiorum</em></td>
<td>2</td>
<td>Waksman, 1991</td>
</tr>
<tr>
<td><em>Ilyvis</em></td>
<td>5</td>
<td>Enger &amp; Sleeper, 1965</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Chaudhary et al., 1997</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>Irwin et al., 1993</td>
</tr>
</tbody>
</table>
tensile strength in cotton yarn. When *Myrothecium verrucaria* filtrates have been fractioned by gel filtration on Sephadex G-75 (Selby & Maitland, 1965), three major cellulolytic components were obtained. The middle component (II) with a molecular weight of about 30,000 represented 90% of the total carboxymethylcellulase activity and thus seemed to contain the B enzyme. Reruns after exposure to cotton showed that it only slightly affected this activity. The other two components (I and III), having molecular weights of about 55,000 and 5300, respectively, were maintained responsible for the activity of the filtrate toward cotton, and were removed or deactivated by exposure to it, behaving as an A enzyme in these respects. These observations accord with the previously reported behavior of the whole culture filtrate.

In the previous publication (Selby et al., 1963), it was suggested that the loss of A could result in genesis of B, possibly for the combination of A with the products of cellulolysis. No evidence of such inter-conversions was found. Thus if A activity is lost by the formation of a soluble enzyme-carbohydrate complex, the change in molecular weight was too small to be detected by gel-filtration on Sephadex G-75. C₁ component is essential for the extensive degradation of cotton, but is without significant action of its own, either on cotton, carboxymethylcellulose, or cellobiose. It acts synergistically with Cₚ. The small solubilizing power of C₁ acting alone is enhanced when the incubation with cotton takes place on a dialysis membrane (Norkrans, 1967).
Five endoglucanases of *Chrysosporium lignorum* have been characterized and reported (Almin et al., 1975; Eriksson and Pettersson, 1975 and Erriksson and Rzedowski, 1960 a,b). A cellulolytic enzyme has been purified from *Penicillium notatum* by tannin precipitation, extraction with pyridine acetate buffer, IEC, 80% ammonium sulphite precipitation and Sephadex G75 chromatography (Pettersson & Porath, 1966). The purification procedure of this cellulase is shown in Table 2.3. The enzyme was active towards carboxy methylcellulose, hydroxyethylcellulose and ethylhydroxyethylcellulose, cellulodextrins. An endoglucanase was isolated from culture of fungus *Mucor circinelloides* (NRRL 26519) and properties studied (Saha, 2003). Two extracellular endoglucanases produced by *Rhizopus oryzae*, isolated from the soil, had been purified and characterized under family 45 endoglucanases (Murashima et al., 2002).

Cellulases from aerobic fungi have been extensively studied than those of any other physiological group and fungal cellulases currently dominate the industrial applications of cellulases (Gusakov et al., 1992; Nieves et al., 1998; Sheehan & Himmel, 1999). In particular, the cellulase system of *Trichoderma reesei* (teleomorph: *Hypocrea jecorina*, intitially called *Trichoderma viridae*) has been the focus of research for 50 years (Mandels & Reese, 1957; Reese, 1956; Reese & Mandels, 1971, Reese et al., 1950). It is complete in that it can convert native cellulose as well as derived celluloses to glucose (King & Vessal, 1969). Cellulase I and III isolated from *Thermoascus aurantiacus* were active towards CMC (Tong et al., 1980). CMC has been used as effective substrate for
### Table 2.3 Purification procedure of cellulase from Penicillium notatum (Pettersson & Porath, 1966)

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml)</th>
<th>$A_{280}$</th>
<th>Total $A_{280}$</th>
<th>Cellulase activity per ml (U)</th>
<th>Total cellulase activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Recovery of main material</th>
<th>Total recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>692</td>
<td>217</td>
<td>150000</td>
<td>33.0</td>
<td>22800</td>
<td>0.152</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Chromatography on DEAE Sephadex A-25</td>
<td>1720</td>
<td>13.2</td>
<td>22700</td>
<td>9.11</td>
<td>15700</td>
<td>0.690</td>
<td>69</td>
<td>88</td>
</tr>
<tr>
<td>(NH4)2SO4 (80%)</td>
<td>74</td>
<td>56</td>
<td>4140</td>
<td>197</td>
<td>14600</td>
<td>3.51</td>
<td>64</td>
<td>100</td>
</tr>
<tr>
<td>Gel filtration on Sephadex G-75 &amp; concentration</td>
<td>8</td>
<td>146</td>
<td>1168</td>
<td>1280</td>
<td>19200</td>
<td>8.77</td>
<td>45</td>
<td>86</td>
</tr>
<tr>
<td>Electrophoresis &amp; concentration</td>
<td>5.5</td>
<td>164</td>
<td>902</td>
<td>1760</td>
<td>9680</td>
<td>10.7</td>
<td>42</td>
<td>-</td>
</tr>
</tbody>
</table>
A 23 KDa endo-1, 4-β-glucanase (Cel 12 A), a wall hydrolytic enzyme from *Trichoderma reesei* with potent ability to induce extension of heat – inactivated type I cell walls have been identified and studied. This endoglucanase (Cel 12A) belong to glycoside hydrolase family 12. Extension of heat inactivated walls from Cucumber (*Cucumis sativus*) hypocotyls was induced by Cel 12A after a distinct lag time and was accompanied by a large increase in wall plasticity and elasticity (Yuan et al., 2001). A thermophilic fungus *Melanocarpus albomyces* was reported to produce a 20 KDa endoglucanase found particularly useful in textile industry. Its crystal structure has been determined (Hirvonen & Papageorgiou, 2003). Its crystal structure proves it to be classified under glycoside hydrolase family 45.

The reports on *Trichoderma viridae* cellulase are numerous and varied (Li et al., 1965; Kim et al., 1994; Shoemaker & Brown, 1978). Li et al., (1965) have reported fractionation of a commercial enzyme preparation into (a) a “hydrocellulase” fraction with high activity toward Avicel, (b) an “endoglucanase” fraction with high activity toward carboxymethylcellulose (and the soluble oligoglucosides, including cellobiose) and (c) an “exoglucanase” fraction which, unlike the endoglucanase, degraded the soluble oligoglucosides by cleaving glucose units from the nonreducing end. The separations depended on (1) adsorption of the “hydrocellulase” on Avicel in the presence of citrate buffer
(and desorption by water) and (2) adsorption of the "endoglucanase" on alkali-swollen cellulose in the presence of phosphate buffer (and desorption by water).

The molecular weight of the "hydrocellulase" was estimated from its sedimentation rate to be about 60,000, that of the "endoglucanase" to be about 50,000 and that of the "exoglucanase" to be about 76,000.

Mandels and Reese (1964) reported a partial separation of activity toward cotton linters and activity toward carboxymethylcellulose by a fractionation on DEAE-Sephadex (A-50). Chromatography on powdered cellulose partially separated activity towards cotton fibres, filter paper and carboxymethylcellulose. Chromatography on a gauze column separated the fraction with high activity toward cotton into two fractions with different activities towards vegetable cell walls. Three to four fractions with varying activities towards filter paper and carboxymethylcellulose was obtained by electrophoresis in a block of starch grains. Chromatography of the crude enzyme on Amberlite XE-64 gave three fractions with activity toward cotton, filter paper, and carboxymethylcellulose; the major fraction on refractionation on DEAE-Sephadex (A - 50) gave five fractions with activity towards the above substrates and towards soluble oligoglucosides including cellobiose (Jurášek, 1967).

Extensive purification of *Myrothecium verrucaria* cellulase enabled Whitaker et al., (1954) to state that the cellulase was an ellipsoid-shaped protein, approximately 50 x 200 Å in size with a molecular weight of 63,000; by later improved procedures this was amended to 49,000 (Datta et al., 1963). Molecular
weights as low as about 10,000 have been found. Selby and Maitland (1965) even mentioned 5300 for component III, an estimation that however, might be quite preliminary as it is only deduced from a Sephadex column, calibrated with proteins and substances of known molecular weights in a range from 670,000 to 255. Even a molecular weight of about 5000, presumably corresponding to protein with a molecular diameter not smaller than 15 Å in diameter, seems to be too large at least for action within crystalline cellulose. Pores of about 100Å in diameter, communicating with pores of 10Å, have been demonstrated in native cellulose (Frey-Wyssling, 1959). Two cellulases were reported to be purified from the culture filtrates of *Polyporus versicolor*. The enzyme was concentrated by dry Sephadex G-25 and fractionated on Sephadex G-75; one fraction (D) when refractionated on Sephades G-75, gave an apparently homogeneous enzyme (B1) (Jurášek, 1967).

Cellulase was separated from culture filtrates of *Myrothecium verrucaria* by two methods. In the first method enzyme was precipitated by ammonium sulfate and freed of noncellulase impurities by elution from Sephadex G-75, precipitation with polymethacrylic acid, and displacement from Amberlite CG-50 with a gradient of urea in citrate buffer. In the second method enzyme was precipitated with ammonium sulfate and freed of noncellulase impurities by precipitation with polymethacrylic acid, elution from DEAE cellulose with 7M urea-phosphate buffer and elution from Sephadex G-75. The two methods gave products with identical specific activities towards CMC. The use of buffered 7 M urea for the elution from DEAE cellulose required comment. It was used to prevent enzymic attack
on the resin; if this was not prevented, the enzyme became complexed with DEAE substituted oligoglucosides and there upon became subject to proteolysis; the consequent fall in molecular weight (e.g., from 50,000 to 7,000) was accompanied by loss of activity towards CMC (Whitaker et al., 1963; Datta et al., 1963). Chromatography on calcium phosphate separated two components with activity toward carboxymethylcellulose from a commercial Aspergillus niger enzyme preparation; treatment with laminarin removed the trace β(1 → 3) glucan activity (Jurášek, 1967).

An endoglucanase from Thermomonospora curvata was purified to electrophoretic homogeneity by ammonium sulphate precipitation, ion exchange chromatography, size exclusion HPLC (Lin & Stutzenberger, 1995). Two endoglucanases from Trichoderma reesei strain QM 9414 was purified by Bhikhabhai et al., 1984). Study was carried out by adding peptide tag (Trp-Pro-Trp-Pro) to endoglucanase I of Trichoderma reesei to change the partitioning in aqueous two-phase system comprising of thermo-separating ethylene oxide (EO) – propylene oxide (PO) random copolymer EO-PO (50:50) (EO50PO50) and dextran. The fusion position at the end of cellulose binding domain, with the spacer Pro-Gly was shown to be optimal (Collen, 2001). Endoglucanase of T. reesei have been reported as industrially important. A fusion protein Egl (Cel 7B) (EG I - hydrophobin I) from T. reesei culture filtrate has been extracted and purified in a one step PEG- sodium/ potassium phosphate two phase aqueous system (Collen et al., 2002). The cellulase system of the thermophilic fungus Humicola insolens has been found to possess a group of enzymes that allows
the efficient utilization of cellulose (Table 2.2). The *H. insolens* cellulose system
is homologous to the *T. reesei* system and also contains at least seven
enzymes; two cellobiohydrolases [CBH I & CBH II] and five endoglucanases
[Endoglucanase I, Endoglucanase II, Endoglucanase III, Endoglucanase V,
Endoglucanase VI] (Schülein 1997). Tong et al., 1980 have purified three
cellulases of molecular weight 79000 (Cel III), 49000 (Cel II) and 34000 (Cel III)
from Thermophilic fungus *Thermoascus aurantiacus*. The effect of acetic acid
and furfural on the cellulose production of a filamentous fungus *Trichoderma
reesei* RUT C30 was studied by Szengyel & Zacchi (2000). A low mol wt
endoglucanase (13,000) has been purified from the cellulose complex of
*Trichoderma koningii* and its properties studied (Churilova et al., 1980). The
localization of cellulase in the hyphae of fungus *Achlya ambisexualis* was studied
by (Nolan & Bal, 1974). Endoglucanase I from culture fluid of *Volvariella
volvacea* was purified by ion exchange and gel filtration chromatography (Ding et
al., 2002). Garcia et al., (2002) has resolved the catalytic domain from *T. reesei*
using anion exchange chromatography. The pure fractions were analysed using
lectins and electrospray MS. Isolated N-glycans were analysed by fluorophore
assisted carbohydrate electrophoresis and amine adsorption HPLC. Shi et al.,
(2002) purified cellulose of *Aspergillus niger* using affinity membrane followed by
anion exchange on POROS 20HQ.

Eriksson & Pettersson studied an electrophoretically homogeneous β-endoglu-
canase from *Penicillium notatum*, having no activity against native cellulose. The
molecular weight is 78,000 and the amino acid composition is known, giving
evidence for the presence of two half cysteines. The enzyme is composed of a single polypeptide chain, internally crosslinked by two cysteines as well as by chloride ions. The activity could be restored by cysteine residues in a disulfide bridge. The enzyme was strongly inhibited by mercuric ions. The activity could be restored by cysteine as well as by chloride ions. By electrolytic reduction the disulfide bridge, present in the enzyme, was shown to be essential for the activity. The β-endoglucanase was not attacked by exopeptidases under non-denaturing conditions, indicating a solid structure; on the whole, cellulases are so strikingly stable to, e.g., pH and temperature, that it surprises veteran protein chemists, although the susceptibility of the enzyme increases, naturally enough, with purification. Treatment with endopeptidases caused a pronounced decrease in activity. Active fragments could not be obtained with endopeptidases. An electrophoretically and ultracentrifugally homogenous cellulase from Myrothecium verrucaria, active with respect to ground or swollen cellulose, carboxymethylcellulose, and a series of oligoglucosides (Whitaker et al., 1963) were found to have 14 cysteine residues.

From anaerobic fungus Piromyces spp. Strain E2 three dominant proteins was purified by cellulose affinity method consisting of steps of EDTA washing followed by elution with water (Steenbakkers et al., 2002). Studies have shown that isoenzymes of endoglucanase from Trichoderma longibrachiatum differed in their ability to be adsorbed on CMC, amorphous and crystalline celluloses. Moriyoshi et al., (2003) have studied the role of endoglucanase from Neisseria sicca (SB) in synergistic degradation of cellulose acetate. Its purification
procedure is shown in Table 2.4. They have isolated Endoglucanase I of 50 KDa mol wt, optimum pH 5-6 and temperature 45°C. It’s $K_m$ and $V_{max}$ on CMC was determined to be 0.448% and 13.6 μmol/min/mg (Table 2.5). Olutiola (1976) has isolated a cellulose complex of 11 components, four high mol wt and 7 low mol wt from culture filtrates of *Penicillium citrinum*. In an enzymatic study on cellulose system of *Trichoderma viridae* two cellulases, cellulose II A and II B have been purified (Okada, 1975). To study the ability of *Thermobifida fusca* cellulases (Cel 6B, Cel 9A and Cel 5A) to bind BMCC than on Avicel, various compositions of mixtures of purified cellulases (one Endoglucanase and two CBH) were tried on the saccharification of microcrystalline cellulose (Baker et al., 1998). Two distinct cellulases (Cellulase I & II) were isolated from the water extract of a wheat bran culture of *Trichoderma koningii* (Iwasaki et al., 1964). The enzyme was precipitated by ammonium sulfate, freed of noncellulase impurities by DEAE-Sephadex and Amberlite CG-50, and fractionated on hydroxylapatite into cellulase I (eluted with 0.001M phosphate) and cellulase II (eluted with 0.1M phosphate buffer). They differ in substrate specificity (Table 2.6). *Trichoderma koningii* produces a powerful cellulase which hydrolyses native cellulose completely to glucose (Halliwell, 1965). Waksman (1991) has purified from *Sclerotinia sclerotiorum* two endoglucanases with mol wt 48KDa and 34KDa active towards CMC.

Anaerobic chytrid fungi are only found in the rumens of herbivorous animals (Orpin, 1977) and produce highly active cellulases (Borneman et al., 1989; Chen et al., 1997; Wilson & Wood, 1992; Wood et al., 1986). Ye et al., (2001) has
Table 2.4 Purification of the Endo-1, 4-β glucanase from *Neisseria sicca SB*

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Purification (Fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>40300</td>
<td>4040</td>
<td>0.100</td>
<td>1.00</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate fractionation</td>
<td>11200</td>
<td>3890</td>
<td>0.347</td>
<td>3.47</td>
<td>96.3</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>3190</td>
<td>2880</td>
<td>0.903</td>
<td>9.03</td>
<td>71.3</td>
</tr>
<tr>
<td>Q-Sepharose</td>
<td>512</td>
<td>293</td>
<td>0.572</td>
<td>5.72</td>
<td>7.25</td>
</tr>
<tr>
<td>Phenyl Toyopearl</td>
<td>31.4</td>
<td>34.3</td>
<td>1.09</td>
<td>10.9</td>
<td>0.85</td>
</tr>
<tr>
<td>Mono Q</td>
<td>9.38</td>
<td>17.2</td>
<td>1.83</td>
<td>18.3</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Source: Moriyoshi et al., 2003
<table>
<thead>
<tr>
<th>Source of Endoglucanase</th>
<th>Mol wt KDa</th>
<th>Substrate</th>
<th>Specific activity (U/mg)</th>
<th>( K_m ) (%)</th>
<th>( V_{max} ) (μmol/min/mg)</th>
<th>Temp. (°C)</th>
<th>pH</th>
<th>Isoelectric pH</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Neisseria sicca SB</em> (Moriyoshi et al., 2003)</td>
<td>41</td>
<td>Cellulose acetate</td>
<td>1.82</td>
<td>0.242</td>
<td>2.24</td>
<td>60</td>
<td>6-7</td>
<td>4.8</td>
</tr>
<tr>
<td>Blue mussel <em>Mytilus viridis</em> (Xu et al., 2000)</td>
<td>20</td>
<td>CMC</td>
<td>10.4</td>
<td>-</td>
<td>-</td>
<td>30-50</td>
<td>4-6</td>
<td>7.6</td>
</tr>
<tr>
<td><em>Rhodotorula glutinis</em> KUJ 2731 (Oikawa, 1975)</td>
<td>40</td>
<td>CMC</td>
<td>-</td>
<td>1.1</td>
<td>556</td>
<td>50</td>
<td>-</td>
<td>8.57</td>
</tr>
<tr>
<td><em>Volvariella volvacea</em> (Ding et al., 2002)</td>
<td>42</td>
<td>CMC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>55</td>
<td>7.5</td>
<td>7.7</td>
</tr>
</tbody>
</table>
Table 2.6: Properties of *Trichoderma koningii* cellulases

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Mol.wt</th>
<th>Inactivation by heat</th>
<th>Activity on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Glycol cellulose</td>
</tr>
<tr>
<td>Cellulase I</td>
<td>26,000</td>
<td>60°C, 1hr</td>
<td>Yes</td>
</tr>
<tr>
<td>Cellulase II</td>
<td>50,000</td>
<td>70°C, 1hr</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Source: Iwasaki et al., 1964
purified cellulase from ruminal fungus *Orpinomyces joyonii*, cloned it in *Escherichia coli* and purified to 88 fold by chromatography on High Q and hydroxyapatite. The polysaccharide hydrolases produced by anaerobic fungi are amongst the most active that have been described to date; they are capable of degrading a wide range of polysaccharides and can completely solubilize both amorphous and highly crystalline cellulose (Li & Heath, 1993; Selinger et al., 1996; Wubah et al., 1993). The anaerobic fungus *Piromyces equi* was isolated from the caecum of a pony (Munn, 1994; Orpin, 1981). The cellulose-hemicellulose degrading system of *P. equi* consists of a large multienzyme complex, which accounts for upto 90% of the cellulase, mannanase and xylanase activities produced by the fungus. This complex consists of atleast 10 polypeptides ranging from 50 to 190 KDa, including a 97 KDa putative scaffolding protein (Ali et al., 1995; Fanutti et al., 1995; Hazlewood & Gilbert, 1998). A new thermostable endoglucanase, inactivated only by 20% at 64°C for 6 hours in the absence of substrate was reported to be formed when the cultivation temperature of a thermophilic culture of *Allesheria terrestris* was increased from 40 to 48-49°C (Kvesitadze et al., 1996). Its molecular weight and isoelectric point were 69KDa and 6.4 respectively. The cellulase of *Aspergillus terreus* showed no heterogeneity on Sephadex G-100 or an ion-exchange chromatography; the purified enzyme hydrolyzed carboxymethylcellulose and alpha-cellulose (Pal & Ghosh, 1965). Highly cellulytic anaerobic hyperthermophiles are found in the genera *Thermotoga* (Liebl, 2001) and *Caldicellulosiruptor* (Rainey et al., 1994).
and cellulases isolated from these organisms are often highly thermostable (Bok et al., 1998).

It has been reported that yeast, Rhodotorula glutinis KUJ 2731, isolated from soil effectively produce an extracellular endoglucanase. The molecular mass was found to be 40 KDa, isoelectric point 8.57, and activation energy 20.9KJ/mol. The enzyme was found to be active at temperatures 0-70°C with highest initial velocity at 50°C. The enzyme catalyzed hydrolysis of CMC with an apparent $K_m$ of 1.1% and $V_{max}$ of 556 μmol/ min/ mg (Table 2.5). The enzyme was capable of catalyzing the transglycosylation of p-nitrophenyl-β-cellotrioside to cellotetraose (Oikawa, 1998). Biotinylation of intact Saccharomyces cerevisiae cells with a non permeant reagent (Sulfo-NHS-LC-Biotin) allowed identification of seven cell wall protein that were released from intact cells by dithiothreitol (DTT). Three of these proteins were identified to be β-exoglucanase I, β- endoglucanase and chitinase (Cappellaro et al., 1998). Endoglucanases/ cellulases have been used in yeast cell wall lysis. In a study to determine the cell wall composition of S. cerevisiae it was found that a combination of chitinase and recombinant β-1,3-glucanase released all the chitin and 60-65% of β-1,3-glucan, recombinant endo-β-1,6-glucanase of T. harzianum release all the β-1,6-glucan and finally laminariase and β-glucosidase released the remaining β-1,3-glucans present in the cell wall of Saccharomyces cerevisiae (Magnelli et al., 2002).

An endo-β-1,4-endoglucanase from edible mushroom, Volvariella volvaceae, has been purified, characterized and expressed in Pichia pastoris (Ding et al., 2002).
Almin and Eriksson (1968) have developed the theoretical and semiempirical basis for a viscometric method of determining the activity of enzymes acting, preferably at random, in the degradation of polymers. This method measures the activity in absolute terms, i.e., the number of bonds broken per unit time. By this method, the molecular activity of a purified sample of *Penicillium notatum* endoglucanase, of molecular weight 78000, acting on sodium carboxymethylcellulose (DS 0.83) at 25°C has been determined to be 29 bonds per second.

Some metals inactivate endoglucanase whereas few others activate. Endoglucanase from hyphomycete *Chalara paradoxa* was inhibited by detergents, EDTA, Hg$^{2+}$, Ag$^{2+}$, some extent by 10mM Zn$^{2+}$, Fe$^{2+}$ and Mg$^{2+}$, but stimulated by Mn$^{2+}$ (Lucas et al., 2001). Cellulase of *Trichoderma viridae* was found to be completely inhibited by 1mM Hg$^{2+}$, partially by 1mM Ag$^+$ and Cu$^{2+}$. Mg$^{2+}$, Fe$^{2+}$ and several other metal ions showed no inhibition at this concentration (Okada, 1975). Many unspecific protein-denaturing inhibitors are active also against cellulases. Inhibitors, which act on free SH-groups, also inhibit cellulases. Mandels and Reese (1964) have dealt extensively with the very interesting natural inhibitors of cellulase, chiefly belonging to phenolics, tannins, and types of polymeric leucoanthocyanins. They are probably widespread in plant tissues. About one-fifth of the plants screened contained reasonable amounts more than 12 times the amount found in wood of *Eucalyptus rostrata*, the first localized source of natural cellulase inhibitors. Cellulases from different sources vary markedly in their resistance to these inhibitors. Mandels
and Reese discuss the significance of these natural inhibitors as a factor involved in the resistance of plants. Inhibitors of cellulases have possible applications as agents for protecting cellulosic materials from microbial attack (Jurášek et al., 1967).

Cellulolytic enzymes are generally considered to be formed only in the presence of cellulose. According to Mandels and Reese (1960), however not cellulose itself but the soluble cellobiose, an hydrolysis product, is the true inducer of cellulase in cellulose cultures. Other compounds having β-glycosidic linkages, such as lactose and salicin, can serve as inducers. However, not all soluble compounds with the β-glycosidic linkages are inducers. Glucose and cellobiose offered in amounts as large as those used in standard growth experiments generally depress the yield of cellulolytic enzymes (Norkrans, 1967). Horton and Keen (1966) studied the sugar repression on cellulase synthesis in the case of Pyrenoacheta terrestris, a fungus involved in the formation of onion pink root. P. terrestris has been observed to produce carboxymethylcellulase in infected onion roots. The enzyme is also produced in quantities in cultures of the fungus containing cellulose. Cellulase production would be depressed as long as glucose or other soluble carbohydrates attacked by ‘constitutive’ enzymes are present. The cell wall, however, constitutes a cellulose substrate, which will induce an increase in cellulase production when the plant, for some reason, no longer forms a surplus of soluble carbohydrates (Norkrans, 1967).

Cellulase has been immobilized using polyurethane foam (Chakrabarti & Storey, 1988). The enzyme exhibiting cellulolytic (endoglucanase) activity at alkaline pH
isolated form the acremonium species has been patented both for its application in improving pulp drainage and for deinking recycled paper (Anderson, 2000). The degradation of cellulose is an increasingly important problem in waste treatment. Purified endoglucanases from *Trichoderma reesei*, *Penicillium verruculosum* and *Chrysosporium lucknowense* ability to bind indigo has demonstrated the best washing performance in the process of enzymatic denim treatment (Gusakov et al., 2001).

Cellulases, β-glucanases and β-glucosidases of fungi are used in the production of syrups and coffee whiteners. The United Kingdom Ministry of Agricultural Fisheries and Food (MAFF) in 1982 have recommended cellulase of *Trichoderma viridae* in the permitted list, to be used in food (Robinson, 1987). Endoglucanase finds application in textile industry (Hirvonen & Papageorgiou, 2003). Disintegration of plant tissue has been experimentally provoked with cellulase preparations. Gross (1990) succeeded in preparing protoplasts by removal of cell walls from carragenophyte *Gigartina corymbifera* using cell wall digesting enzymes. Protoplasts obtained showed vigorous cytoplasmic streaming, and formed a good experimental material for attacking a number of plant physiological problems such as wall formation, ion transport, and water balance. They also serve as a useful tool in crop improvement. Cellulases are used as cell wall disintergrators in order to increase the digestibility of proteins, fruit juices, essential oils, agar-agar from seaweeds, etc. For a long time, cellulases have found application in the pharmaceutical industry as a digestive aid (Norkrans, 1967).
The baking quality of rye flour and the shelf life of rye bread can be improved by partial hydrolysis of the rye pentosans. Technical pentosanase preparations are mixtures of β-glycosidases (1,3- & 1,4-D-xylanases). Solubilization of plant constituents by soaking in an enzyme preparation (maceration) is a mild and sparing process. Such preparations usually contain exo- and endo- cellulases, alpha and beta mannosidases and pectolytic enzymes. Examples of the utilizations are: production of fruit and vegetable purées (mashed products), disintegration of tea leaves, or production of dehydrated mashed potatoes. Some of these enzymes are used to prevent mechanical damage to cell walls during mashing and thus, to prevent excessive leaching of gelatinized starch from the cells, which would make the puree too sticky. Glycosidases (cellulases and amylases from A. niger) in combination with proteinases are recommended for removal of shells from shrimp. The shells are loosened and then washed off in a stream of water (Belitz & Grosch, 1999). Glycanases secreted by Aspergillus terreus CCMI 498 and Trichoderma viridae CCMI 8 were effective in deinking mixed office waste paper (Marques et al., 2003). Aspergillus niger, Aspergillus oryzae, Rhizopus delemar, Rhizopus oryzae and Penicillium emersonii are the fungi capable of producing endoglucanase finding wide application in the food processing (Belitz & Grosch, 1999).

2.5 Endoglucanase from bacteria

Cellulolytic bacteria can be observed to comprise of several diverse physiological groups. (1) Fermentative anaerobes, typically gram positive (Clostridium,
Ruminococcus and Caldicellulosiruptor) but containing a few Gram negative species, most of which are not (Fibrobacter) (2) Aerobic Gram postitive bacteria (Cellomonas and Thermobifida) and (3) Aerobic gliding bacteria (Cytophaga and Sporocytophaga). Generally only a few species within each of the above named genera are actively cellulolytic. The cellulolytic capability among the organisms that differ in oxygen relationship, temperature and salt tolerance indicates wide availability of cellulose across natural habitats. In aquatic habitats, hydrolysis of cellulose by secreted cellulases may be feasible e.g. by the predominance of Cytophaga, which is known to actively secrete cellulases in culture (Kauri & Kushner, 1985; Li & Gao, 1997; Malek et al., 1988). Two alkaline carboxymethylcellulases were partially purified and exhibited pH optima of 10.0 and molecular mass of 54 and 46KDa. The enzymes were stable upto 60 and 80°C, respectively (Garnier-Sillam et al., 1985). Horikoshi’s group has characterized another cellulase from alkalophilic Bacillus strain 1139. It exhibited a pH optimum of 9.0 and still showed much activity at pH 10.5 (Bracke et al., 1978). This extracellular enzyme had a particularly high content of aspartic acid. Cloned into Escherichia coli the enzyme had the same properties as the cellulase from Bacillus strain 1139, but its molecular mass was 94 rather than 92 KDa, perhaps indicating processing at a different sites from that in the Bacillus strain of origin (Bracke & Markovetz, 1978). The evidence that non-cellulolytic bacteria Clostridium acetobutylicum contains a complete cellulosomal gene cluster system that is not expressed, due to disabled promoter sequences (Schwarz, 2001), complicate the taxonomic picture. Among the bacteria, there is a distinct
difference in cellulolytic strategy between the aerobic and anaerobic groups. With relatively few exceptions (Rainey et al., 1994; Svetlichnyi et al., 1990) anaerobes degrade cellulose primarily via complexed polycellulosome organelles of the thermophilic bacterium Clostridium thermocellum (Schwarz, 2001). A strictly anaerobic Gram negative bacterium Pervotella spp. is found to play a significant and essential role in the utilization of plant cell wall materials. A broad specificity endoglucanase/xylanase were partially purified along with a 66KDa endoxylanase by a two step HPLC procedure. The non-concentrated periplasmic fraction from Pervotella bryantii cultivation medium was separated into six fractions in a gradient HPLC system employing anion exchange principle. In the second step, fraction four was further separated into four fractions (A, B, C, D) by HPLC and these were identified as enzymes of molecular weights 88, 66, 45 and 29 KDa by SDS PAGE. One fraction (4B) was identified as endoxylanase (66KDa) and the next (with two bands) as a broad specificity endoglucanase/xylanase (Logar et al., 2000).

Wharton's et al., (1965) investigated cellulolytic activity in cockroach, Periplaneta americana and demonstrated cellulases of both insect origin and bacterial origin in the alimentary tract. The former originated in the salivary glands and the latter was concentrated in the midgut. CMC was the substrate. No attempts were made to isolate cellulolytic bacteria or to estimate the number present. Isolates were identified as Klebsiella oxytoca, Clostridium freundii, and species of Eubacterium, Clostridium, and Serratia. None of the isolates, nor a cellulolytic reference strain of Ruminococcus albus used as a control, digested Whatman
No. 1 filter paper in these experiments. When enzyme assays were performed with ball-milled cellulose as substrate using supernatant fluids from cultures grown with CMC, the cellulolytic activity of all strains but one was 30-75% less than when CMC was the substrate (the activity of Clostridium remained about the same). Although these bacteria were grown with CMC and the presence of a C₅₇-cellulase would be expected, the activity seen with ball milled cellulose indicates that C₈7-cellulase activity may also have been present. Alternatively, it could indicate that the ball-milled cellulose also contained polymeric degradation by the C₅₇ enzyme. Bignell (1977) showed that [¹⁴C] cellulose is degraded in the hindgut of Periplaneta americana. Feeding of antibiotics sharply reduced the ¹⁴CO₂ evolution. This indicates that the hindgut bacteria had a role in degradation of this polysaccharide. Breznak (1982, 1984) found no convincing evidence implicating bacteria in cellulose degradation in the higher termites that possess cellulolytic protozoa.

A 100KDa protein with endoglucanase activity was purified from Triton X-100 extract of cells of the thermoacidophilic Gram-positive bacterium Alicyclobacillus acidocaldarius. The pH and temperature optimum was found to be 4 and 80°C respectively (Eckert & Schneider, 2003). The enzyme has been sequenced to the gene level. Bacteroides succinogenes, because of its high cell numbers and ability to ferment cellulose rapidly, is one of the more important cellulolytic organisms in the rumen. It is the only cellulolytic organism able to actively digest undegraded cotton fiber (Halliwell & Bryant, 1963). The cellulase of these organisms appears to be associated with the outer surface of the cell in that the
cellulose being degraded must be in close proximity to the bacterial cells (Macy & Probst, 1979). Carboxymethylcellulase activity of this bacterium was studied. The partially purified enzyme gave an optimum temperature of 50°C and optimum pH of 5.6-6.6.

Aerobic cellulose degraders both bacteria and fungi, utilize cellulose by producing extracellular cellulase enzymes that are freely recoverable from culture supernatants (Rapp & Beerman, 1991; Schwarz, 2001). They are occasionally present in complexes at the cell surface (Bond & Stutzenberger, 1989; Wachinger et al., 1989).

Four electrophoretically distinct fractions from Cellvibrio gilvus cleaved cellohexose, cellohexitol, and cellopentitol at the second and third linkage from the non-reducing end (Cole & King, 1964). Electrophoresis in a block of starch grains separated five components with activity toward carboxymethylcellulose from an enzyme preparation from Streptomyces gilvus; according to immuno diffusion tests, three of them were immunologically identical (Enger & Sleeper, 1965).

An alkaline cellulase produced by alkalophilic Bacillus spp. N6-27 was purified by ammonium sulphate fractionation, sepharose CL-4B hydrophobic interaction chromatography and Bio-gel P-150 chromatography (Tian & Wang, 1998). Its mol wt on SDS PAGE was 94 KDa and pl by PAGE - IEF was 4.2. The optimum temperature and pH for the enzyme on CMC was found to be 55°C and 8.5 respectively. The enzyme activity was stable under 50°C and in the pH ranges
6-11. Fe$^{2+}$, Cu$^{2+}$ and Hg$^{2+}$ were strongly inhibited by this enzyme. An endoglucanase was reported to be purified from Clostridium thermocellum by (Ng & Zeikus, 1981). The enzyme purified to 22 fold had isoelectric point, optimum temperature and optimum pH of 6.72, 62°C and 5.2 respectively. The enzyme lacked cysteine and was low in sulphur containing aminoacids. The enzyme had increased activity towards cello-oligosaccharides with increasing degree of polymerization. Several extra-cellular hydrolytic enzymes (xylanase, endoglucanase, β-xylosidase, β-glucosidase) were produced by a Clostridium strain PXYL1 isolated from cold- adapted cattle manure digester at 15°C (Akila & Chandra, 2003). An extracellular cellulase produced by Bacillus brevis, isolated from the soil was purified by ultrafiltration and Sephadex G-200 column chromatography. The production increased almost five times on addition of galactose in culture medium and was optimum at pH 5.5 and 37°C and 175 rpm speed using environmental orbital shaker (Singh & Kumar, 1998). The location of cellulase in Cellvibrio fulvus was studied by Berg (1975). It is reported that the enzyme was repressed by glucose and formed at a constant differential rate on cellobiose and amylose. An endoglucanase was purified from cellulolytic thermophilic anaerobic bacteria by Creuzet and Frixon, (1983). The optimal pH was 6.4 and the enzyme was isoelectric at pH 3.85. It was found highly thermostable; it retained 50% of its activity after 1 hour at 85°C. Hydrolysis of CMC was detected by a rapid decrease in viscosity but slow liberation of reducing sugars, indicating endo-enzyme activity. A 100KDa protein with endoglucanase activity was purified from Triton X-100 extract of cells of
thermoacidophilic gram-positive bacteria *Alicyclobacillus acidocaldarius* (Eckert & Schneider, 2003). The enzyme showed activity towards CMC with pH and temperature optima of 4 and 80°C respectively.

“Fungi-like” prokaryotes such as Actinomycetes and the related Corynebacteria (*Cellulomonas*) might degrade cellulose, according to a mechanism similar to that of fungi, with cellulolytic enzymes that can be found non-associated in the culture medium (Coughlan & Ljungdahl, 1988). Conversely, in many anaerobic bacteria (rumen bacteria, *Clostridium thermocellum*), the various components are found in tightly associated multimolecular complexes, whose quarternary structure seems to be a key feature responsible for the efficient degradation of crystalline cellulose (Lamed & Bayer, 1988). The cellulase complex of *Clostridium thermocellum* is found to contain at least 14-18 different polypeptides forming a very stable extracellular structure termed cellulosome (Coughlan et al., 1985; Lamed et al., 1983a,b). In a study on cellulose digestion of roe deer (*Capreolus capreolus*) it was found that cellulase activities were lowest in winter when the cellulose concentration in rumen was the highest (Deutsch, 1998).

The best-studied species of cellulolytic aerobic bacteria belong to the genera *Cellulomonas* and *Thermobifida* (formerly *Thermomonospora*). *Cellulomonas* species are coryneform bacteria that produce at least six endoglucanases and at least one exoglucanase (Chaudhary et al., 1997). The thermophilic filamentous bacterium *Thermobifida fusca* (formerly *Thermomonospora fusca*) is a major cellulose degrader in soil. Six cellulases, three endoglucanses (E1, E2 & E5),
two exoglucanases (E3 & E6) and an unusual cellulase with both exo and endoglucanase activity (E4) have been isolated. The latter enzyme has high activity on BMCC and also exhibits synergism with both the other Thermomonospora fusca endoglucanases and exoglucanases (Irwin et al., 1993). The thermophilic and hyperthermophilic prokaryotes represent a unique group of microorganisms that grows at temperatures that may exceed 100°C. Several cellulolytic hyperthermophiles have been isolated during the past decade (Bergquist et al., 1999). However, no cellulolytic thermophilic archaea have been described, although archaea that can grow on cellobiose and degrade other abundant polysaccharides such as starch, chitin and xylan, have been isolated (Driskill et al., 1999; Sunna et al., 1997). Only two aerobic thermophilic bacteria have been described that produce cellulases: Acidothermus cellulolyticus (an actinomycete) and Rhodothermus (Sakon et al., 1996). A thermostable alkaline cellulase activity was detected in a culture medium of a strictly alkaliphilic Bacillus (KSM- S 237) by Hakamada et al., (1997). The alkaline enzyme of optimum pH 8.6-9.0 and optimum temperature 45°C was stable upto 50°C and more than 30% of the original activity was detectable after heating at 100°C.

Endoglucanase and protease of Oerskavia xanthineolytica act together in the yeast cell lysis (Scott & Schekman, 1980). Cellulase has been used to inhibit biofilm formation by a pathogenic bacteria Pseudomonas aeruginosa, commonly found in Medicinal plants (Loiselle & Anderson, 2003). Microorganisms produce multiple enzymes to degrade plant cell materials, known as enzyme systems (Warren, 1996). Endoglucanases of Bacillus circulans and Bacillus subtilis are
used in food processing (Belitz & Grosch, 1999). Cellulase can be used instead of pumice stones for "stone washing" - a process that is used to give blue jeans an abraded and worn in look. For this purpose Genencor International has created a cellulase, called Indi Age Neutra L Enzymes obtained from Streptomyces lividans. It is known to work best within a specific temperature range of 45 - 55°C and pH range of 6-8 (Chen et al., 2000).

Prior reviews consider the complexed cellulases of anaerobic bacteria (Bayer et al., 1998; Bayer et al., 1994; Doi et al., 1994, 1998; Felix & Ljungdahl, 1993; Leschine, 1995; Robson & Chambliss, 1989; Schwarz, 2001; Shoham et al., 1999) noncomplexed fungal and bacterial cellulases (Coughlan, 1990; Knowles et al., 1987; Ryu & Mandels, 1980; Stutzenberger, 1990; Teeri, 1997, Teeri et al., 1998; Wood, 1992).

2.6 Plant endoglucanase

Endoglucanases are widely distributed in plant products namely seeds and malts. However, comparatively, less work has been reported. The plant endoglucanases are capable of hydrolyzing polysaccharides found in the plant and fungal cell walls, thus functioning in plant development and in defense of the plant against fungal diseases (Simmons, 1994). Two forms of endoglucanases of molecular weight approximately 20,000 and 70,000 have been identified in growing regions of Pisum sativum epicotyls treated with auxin. One cellulase is buffer soluble, the other buffer insoluble but extractable with high salt concentrations. They were purified by DEAE cellulose chromatography,
Sephadex gel filtration and ultrafiltration methods. Both the enzymes hydrolysed CMC with optimum pH 5.5 to 6.0. Aminoacid analysis revealed that one buffer soluble enzyme is relatively rich in Gly, Ala and Val and deficient in Cys, Tyr and Phe compared to the other buffer insoluble enzyme (Byrne et al., 1975). An endoglucanase was purified from ripe strawberry fruit using affinity chromatography. Its mol. wt on SDS PAGE was determined to be 54KDa. Its pH optimum and $K_m$ against CMC was determined as 5.0-7.0 and 1.3 mg/ml respectively (Woolley, 2001).

Laminarin hydrolyzing activity ($\beta$-1, 3-EG EC 3.2.1.39) was reported to be developed in the endosperm of tomato (*Locopersicon esculentum*) seeds following germination. The enzyme was basic with isoelectric point greater than 10 and the apparent molecular mass was estimated to be 35KDa by SDS-PAGE (Morohashi & Matsushima, 2000). Two endoglucanase genes had been isolated from *Pinus radiata* and expressed (Loopstra et al., 1998). Both proteins contained domains conserved in plant and bacterial endoglucanases. They showed strong similarity to each other and higher similarity to and endoglucanase cloned from tomato pistils. Studies have shown that a 66 KDa endo-1,4-$\beta$ glucanase is present in the endosperm of *Euphorbia heterophylla L*. The carboxymethylcellulase activity decreased approximately by 66% in extracts of endosperm containing isopropanol or ethanol. The endoglucanases were isolated from endosperm extracts using ammonium sulphate fractionation followed by Sephacryl S-100-HR chromatography resulting in two peaks. Peak I was purified about 15 fold by DEAE Sephadex A50 followed by affinity
chromatography (CF-11 cellulose) and peak II purified to 10 fold by CMC chromatography (Suda & Giorgini, 2003). Activity of cell wall hydrolases in the germinating seeds of *Euphorbia heterophylla* L has been studied. The activities were very low or not detected during pre-emergence period (time interval before 2.2 days from the start of imbibition) and increased in the post emergence period (time interval after 2.2 days from the start of imbibition). Figure 2.2 (I and II) shows the endoglucanase activity on CMC and avicel/lichen, obtained by the authors.

Plant endoglucanases in glycosyl hydrolase family 17 include endo-(1,3) : (1,4) -β-glucanase (E.C.3.2.1.73) and endo-1,3-β-glucanase (E.C.3.2.1.39) (Henrissat & Bairoch, 1993). These enzymes hydrolyze polysaccharides found in the plant and fungal cell walls, thus functioning in the plant development and in defense of the plant against fungal diseases (Simmons, 1994). Maize coleoptile endoglucanase has been purified and partially sequenced (Inouhe & Nevins, 1991). Endoglucanase has been isolated from the cell walls of *Zea mays* seedlings (Hatfield & Nevins, 1986). The activity of cell wall hydrolases such as endo-β-1, 3-glucanases during the radicle emergence stage of germination may be related to the softening of the tissue in the micropylar region, where the protrusion of the radicle occurs (Leubner-Metzger et al., 1995; Nonogaki & Morohashi, 1996; Sánchez & de Miguel, 1997; Nonogaki et al., 2000; de Miguel et al., 2000; Leubner-Metzger & Meins Jr., 2000).
Figure 2.2 Time course of endoglucanase from *Euphorbia heterophylla* endosperm

Graph I shows the activity of endoglucanase on CMC determined by viscometry (left scale) and by reducing power production (right scale). Graph II shows the activities of endoglucanase on Avicel and lichenan. The arrows indicate average germination time.
A wall bound endo-(1,4)-beta glucanase obtained from a preparation of the cell walls of suspension cultured poplar cells was purified to electrophoretic homogeneity by cation-exchange, hydrophobic and gel filtration chromatography. The enzyme gave a molecular mass of 47 KDa by SDS PAGE and 48 KDa by gel filtration on Superdex 200 pg with isoelectric point 5.6, optimal pH 6.5, $K_m$ 1.2 mg/ml and $V_{max}$ 280 units (Ohmiya et al., 1995).

Cellulase is abundantly present in the pulp of ripe bananas (Peumans et al., 2000). This 1,3-glucanase showed similarity with other plant $\beta$-glucanases with respect to aminoacid sequence, structure and biological activity. Fabin, a novel calcyon like and glucanase like protein with mitogenic, anti-fungal and translation inhibitory activity was isolated from broad beans *Vicia faba* and properties studied (Ng & Ye, 2003). Cellulase activity is found to be increased during ethylene induced abscission of leaves and ripening of fruits in pepper plants (Ferrarese et al., 1995). Endo-$\beta$-1,4-glucanase and $\beta$-galactosidase are important enzymes associated with the mobilization of xyloglucans (Edwards et al., 1985; Crombie et al., 1998; Tinè et al., 2000). Few plant cellulases are capable of degrading crystalline cellulose (Rose & Bennet, 1999). Cellulase of *Phaseolus vulgaris* has been studied by Durbin and Lewis (1988).

Cellulase is thought to be maximally active in vivo during spore germination (Cotter et al., 1969). During the swelling stage of germination extracellular cellulase having a high specific activity is first detected. The time of appearance
of this extracellular enzyme coincides with that of the dissolution of both the outermost mucopolysaccharide layer and the two middle cellulosic wall layer (Cotter et al., 1969; Hemmes et al., 1972). Cellulase activity in vitro during differentiation parallels the accumulation of its substrate in vivo (Rosness, 1968). However, it is undetectable in extracts prepared prior to sorocarp stage of development (Killick & Wright, 1974).

In the brewing process, β-glucans from barley increase wort viscosity and impede filtration. Enzymatic endo-hydrolysis reduces viscosity. Exocellobiohydrolase of Trichoderma reesei has been expressed and biochemical characteristics studied in transgenic tobacco (Dai et al., 1997; Dai et al., 1999).

2.7 Endoglucanase isolated from insect gut

Cellulases have been isolated from the gut of several insects. Endoglucanases are synthesized in the esophageal glands of the cyst nematodes Globodera rostochiensis and Heterodera glycines (Smant et al., 1998). Cyst nematodes are obligatory plant parasites and the identified endoglucanases facilitate intracellular migration through plant roots by partial cell wall degradation. It has been reported that termites can exist for long periods on a diet of pure cellulose when the normal flora are present (Breznak & Pankratz, 1977). No cellulytic bacteria were found in the larvae of Costelytra zealandica, the grass grub beetle a root feeder on pasture plants (Bauchop & Clarke, 1975). Cellulase of both insect and bacterial origin was identified in the alimentary tract of Perplaneta americana (Wharton et al., 1965). An endoglucanase with specific activity 150 μmol/ min/ mg protein against CMC and molecular weight 47 KDa have been isolated from
the gut of larvae of yellow spotted longicorn beetle, *Psacothea hilaris*. The optimal pH of this cellulase was 5.5, close to the pH in the midgut of *P. hilaris* larvae (Sugimura et al., 2003). The deduced aminoacid sequence of *P. hilaris* showed high homology to the members of glycosyl hydrolase family 5 subfamily 2. Watanabe et al., (2002) have purified an endoglucanase from the hindgut of an Australian mound-building termite, *Coptotermes lacteus*. The hindgut extract had a peak separate from those for extracts obtained from the salivary glands and the midgut based on Sephacryl S-200 gel chromatography demonstrated an origin different from the endogenous endoglucanases of the termite itself. It showed high homology to endoglucanases from glycoside hydrolase family 7. A multienzyme distribution of endoglucanase activity was found in the digestive system of a worker caste of the lower termited *Coptotermes formosanus* by zymogram analysis. Distribution analysis showed that 80% of its activity was localized in salivary glands from where only one component (Endoglucanase-E) was secreted into the digestive tract. Its molecular mass, optimal pH, temperature, isoelectric point and $K_m$ were reported as 48KDa, 6.0, 50°C, 4.2 and 3.8 (mg/ml on CMC) respectively (Nakashima and Azuma, 2000). The N-terminal aminoacid sequence of Endoglucanase-E showed similarity with fungal endoglucanase of glycosyl hydrolase family 7 rather than the other insect Endo-$\beta$-1,4 - glucanases of family 9.

2.8 Cellulases of mollusks

The first unequivocal action of cellulase observed was that the hepatopancreas of Weinberg snail, *Helix pomatia* exhibited a slow action on cotton cellulose and
a much more rapid reaction on regenerated cellulosates. Studies on snail cellulose have been carried out extensively by (Pigman, 1951). Table 2.7 shows the works done on cellulases from mollusks. Of the thirty of more enzymes associated with the digestive tract of Helix (Holden & Tracey, 1950; Myers & North-cote, 1958), more than twenty are carbohydrases which are reported to include cellulases (Myers & Northcote, 1958) as well as a variety of glycosides. A wide range of carbohydrases has also been demonstrated in the snail, Tegula funebralis and it has been suggested that Tegula owes its abundance in the upper littoral zone in part to this wide array of enzymes which enables it to digest a wide variety of algae (Galli & Giese, 1959). Apart from Tegula and Helix, cellulases have also been reported from many genera including Patella, Littorina and Aplysia (Stone & Morton, 1958), Dolabella (Hashimoto & Onoma, 1949), Melanoides (Fish, 1955), Oncomelania (Wrinkler & Wagner, 1959) and Strombus and Pterocera (Younge, 1932). Myres and Northcote (1959), have suggested that more than one enzyme may be involved in the digestion of cellulose in the gut of Helix and that, although one or more of these may be produced by the gut flora, it is possible that others are produced by the animal. (This view is based on the properties of three fractions obtained during an attempt to purify the cellulase of Helix pomatia. All three fractions possessed the same order of cellulase activity, but their proteins showed different chromatographic mobilities, suggesting that at least three cellulolytic enzymes are present). Florkin and Lozet (1949), however, have reported that extracts of the digestive diverticula or crop of Helix show no evidence of cellululolytic activity. But in contrast, Galli and
Table 2.7 Occurrence of cellulases in Molluscs.

<table>
<thead>
<tr>
<th>Molluscs</th>
<th>Reference</th>
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<tr>
<td>Snail <em>(Helix pomatia)</em></td>
<td>Pigman (1951)</td>
</tr>
<tr>
<td>Giant snail <em>(Achatina fulica)</em></td>
<td>Maeda et al., (1996)</td>
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<tr>
<td>Snail <em>(Levantina hierosolyma)</em></td>
<td>Parnas (1961)</td>
</tr>
<tr>
<td>Shipworm <em>(Bankia indica)</em></td>
<td>Nair (1955)</td>
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<tr>
<td>Clam (style)</td>
<td>Lavine (1946)</td>
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<tr>
<td>Blue mussel</td>
<td>Xu et al (2000)</td>
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Giese (1959) have concluded that the cellulolytic bacteria present in the gut of *Tegula* are relatively unimportant. Only four strains of the bacteria isolated from the gut of this animal were capable of breaking down structural carbohydrates more readily than extracts of the digestive diverticula and crop, and these were present in small numbers only. The extracts contained a certain amount of bacterial protoplasm along with the animal tissue, but tests on the main residual bacterial species present showed it to be incapable of attacking any of the algae or algal polysaccharides examined.

Parnas (1961) has investigated the source of cellulases in the snail *Levantina hierosolyma* by comparing the cellulolytic activity of normal snails with that of snails whose digestive tract had been sterilized with antibiotics. This was achieved by feeding the snails on a mixture of sterile flour and antibiotics and transferring them every 24 hours to fresh petri dishes. Only when culture test showed that the feces were sterile were extracts made of the salivary glands, but in snails treated with antibiotics the activity persisted only in the digestive diverticula. From this diverticula of *Levantina* is produced by the animal whereas the cellulolytic activity of the crop and salivary glands may result either from bacteria or from the enzymes from the digestive diverticula. Thus, there now seems to be good evidence that at least some gastropods possess the ability to secrete a cellulase.

About 50 ml of contents were isolated from the guts of Weinberg snails. When the 50ml was dried over calcium chloride, about 7.5g of dry powder was
obtained. Cellulase splitting enzymes of *Helix pomatia* was isolated in several steps: grinding the intestinal pieces with toluene and sand, filtering through asbestos, dialyzing this solution for several days, then treated with 20 ml of 0.2N acetate mixture at pH 3.5 and a suspension of aluminium metahydroxide (corresponding to 370mg of alumina) and water added to make a volume of 100ml. The residual solution was then treated with 10ml of alumina suspension (corresponding to 250mg of alumina) and centrifuged again. Residual solution contains high cellulase content (de Stevens, 1955). Cellulase has been purified and characterized from giant snail *Achatina fulica* by Maeda et al., (1996). Endogenous cellulases have been isolated from marine mollusc, *Littorina brevicula* (Purchon, 1977; Kiesov, 1982). The Antarctic krill has a very active system of digestive enzymes, capable of hydrolyzing a large variety of polysaccharidas. The activity of these enzymes is positively correlated with the feeding intensity of the crustacean and activities are comparatively high at low temperatures (Kolakowski & Sikorsi, 2000). High activity of carboxymethylcellulase was found in Antarctic krill, *Euphausia superba* (Chen & Gau, 1981). A 20 KDa endoglucanase of whole blue mussel was purified to homogeneity by a combination of acid precipitation, heat precipitation, immobilized metal ion affinity chromatography, size exclusion chromatography and ion exchange chromatography (Xu et al., 2000). This endoglucanase was identified as a single polypeptide chain with 181 aminoacids cross-linked with six disulfide bridges. Its isoelectric point as estimated by isoelectric focusing in a PAGE was 7.6. The pH optimum curve of blue mussel endoglucanase is shown
in Figure 2.3. The enzyme could withstand 10 minutes at 100°C without irreversible loss of enzymatic activity. Aminoacid sequence based classification groups it under glycoside hydrolase family 45, subfamily 2. The characteristics of the endoglucanase are shown in Table 2.5.

2.9 Cellulase digestion in mollusks

The crystalline style present in the digestive system of most bivalves is largely composed of mucoproteins, carbohydrates, inorganic compounds and water. More than 90% of the dry matter of the style is protein and carbohydrate (Bailey & Worboys, 1960; Kristensen, 1972; Shahul-Hameed, 1986). Irrespective of the species, the ratio between protein and carbohydrate is approximately 3:1 (Yellowlees, 1980). The proteins in the crystalline style are crystalline. Inside the stomach the crystalline style softens and dissolves slowly, releasing a variety of digestive enzymes into the stomach. The stomach wall and the other digestive glands may also release enzymes into the stomach. Crystalline style breaks down spontaneously under a variety of physico-chemical conditions. At the same time the dissolution of the styles acidifies and lowers the viscosity of the mucoid contents of the stomach. In all bivalves, the style is continually being used up and renewed (Kristensen, 1972). The enzymes of the style are secreted by the epithelium of the sac and the style can be reabsorbed when it is not needed. It is often absent in animals that have not fed recently.

The crystalline style revolves in the stomach to perform functions like mixing and food digestion and in the process it dissolves liberating several enzymes into the
Figure 2.3: Graph showing the pH optimum for the blue mussel Endoglucanase (Xu et al., 2000)
stomach. These enzymes initiate a preliminary phase of extracellular digestion in the stomach and are capable of liberating reducing sugars from phytoplankton as well as from natural particulate detritus (Lucas & Newell, 1984). The carbohydrates of the crystalline style not only catalyze extracellular digestion in the stomach, but also participate in intracellular digestion in the digestive diverticula (Brock & Kennedy, 1992). When the particles are broken down sufficiently, they are carried on ciliary tracts in the stomach to the digestive diverticulum for intracellular digestion. Digestive cells in the diverticulum take up small food particles together with digestive enzymes into food vacuoles within the cells where nutrients can be used directly. The undigested particles and waste products are carried and stored by the intestine prior to evacuation from the anus through the exhalant siphon.

Thus the review describes briefly the work carried out in cellulases and its applications. The fact that endoglucanases have been identified in molluscs and that no work has been reported in green mussel endoglucanase, show that study of this aspect is an interesting topic of research. The thesis describes a simple procedure for purification, isolation and study of characteristic properties of the enzyme endoglucanase from green mussel, *Perna viridis.*