INTRODUCTION
1. INTRODUCTION

Diversity in living organisms has been observed across all existing ecological niches, including extreme conditions, such as high and low temperatures (thermophiles vs. psychrophiles), acidic and alkaline pH (acidophiles vs. alkaliophiles), high pressure (barophiles), and high salt concentrations (halophiles). Among these so-called extremophiles, the organisms that have been isolated most frequently and studied extensively over the last two decades are the thermophilic microorganisms. Thermophiles have generally been found in the various geothermally heated regions of the earth such as the hot springs in Yellowstone National Park and in deep-sea hydrothermal vents, as well as in decaying plant matter like peat bogs and compost. A thermophile is a type of extremophile that is capable of existing at relatively high temperatures, above 45°C.

Thermophiles are classified into obligate and facultative types: obligate thermophiles (also called extreme thermophiles) require high temperatures for growth, while facultative thermophiles (also called moderate thermophiles) can survive at higher and at lower temperatures (below 50°C). Hyperthermophiles are particularly extreme thermophiles for which optimal temperatures are above 80°C. For a long time the subdivision of organisms was based on their cellular architecture, and resulted in the establishment of the prokarya and eukarya domains. Based on a comparison of the 16S/18S rRNA sequences that existed in all living organisms the prokaryotes were further divided into bacteria and archaea (Woese et al., 1990). With the exception of the bacterial orders of Thermotogales,
and *Aquifilales*, all thermophilic and hyperthermophilic microorganisms belong to the *Archaea*. Together they formed the deepest branches in the phylogenetic tree of all the three domains (Woese et al., 1990). Such a division had led to the hypothesis that a heat loving organism stood at the root of origin and early evolution of life (Di Giulio, 2003; Di Giulio, 2001; Stetter, 1996; Stetter, 1994). Currently, the only hyperthermophilic organisms within the bacterial domain are members of the genus *Thermotoga* and *Aquifex* (Stetter, 1996). No hyperthermophilic microorganisms in the eukarya domain have been reported till date (Carolina, 1999).

**Table (1.a) The classification of thermophiles based on optimal growth conditions are as follows:**

<table>
<thead>
<tr>
<th>Classification</th>
<th>Conditions</th>
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<tbody>
<tr>
<td>Hyperthermophiles</td>
<td>80-121°C</td>
</tr>
<tr>
<td>Thermophiles</td>
<td>55-80°C</td>
</tr>
<tr>
<td>Mesophiles</td>
<td>20-55°C</td>
</tr>
<tr>
<td>Psychrophiles</td>
<td>2-20°C</td>
</tr>
<tr>
<td>Halophiles</td>
<td>2-5 M NaCl</td>
</tr>
<tr>
<td>Acidophiles</td>
<td>pH &lt; 4</td>
</tr>
<tr>
<td>Alkaliphiles</td>
<td>pH &gt; 9</td>
</tr>
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</table>

*Adapted from Hough & Danson (Hough and Danson, 1999) and Stetter (Stetter, 1999)*

Thermophiles and hyperthermophiles have been isolated from many natural habitats, including continental mud-pools (Solfatara), hot springs and geysers (Yellowstone National Park), and deep sea sediments or vents such as black smokers (Mid-Atlantic Ridge). They have also been isolated from heated industrial
environments, like geothermal power plants and outflows of sewage sludge systems (Vieille and Zeikus, 2001). Most of the thermophiles are well equipped to degrade peptides, and many are even capable of growing on a variety of simple and complex carbohydrates (de Vos et al., 1998; Kelly and Adams, 1994). Many hyperthermophiles grow well on α and β linked carbohydrates (Stetter, 1999; Stetter, 1996), present in plant materials (starch, cellulose, xylan, pectin) or in animal cells or in microorganisms (glycogen). Carbohydrates are preferably used by the thermophiles as di- or polysaccharides due to the thermostability of the monosaccharide species (Driskill, 1999). However, as a prerequisite for their survival, thermophiles contain enzymes that can function at high temperature.

1.1 Thermostable Enzymes

Thermostability is the quality of a substance to resist irreversible changes in its chemical or physical structure at high temperature. Thermostable enzymes are proteins, that are resistant to irreversible change in protein structure when subjected to heat. Thermophilic membranes and proteins are found to be unusually stable at extremely high temperatures. Some thermophilic enzymes are used in molecular biology work and in commercial detergents. While there is always a demand for thermophilic proteins for biotechnological applications; the efficiency of many industrial processes had been improved by the use of different types of thermostable enzymes.

Thermophilic and hyperthermophilic enzymes are intrinsically stable and active at high temperatures and offer major biotechnological advantages over mesophilic or psychrophilic enzymes. Once expressed in mesophilic hosts thermophilic and hyperthermophilic enzymes are isolated in larger amounts for
purification by heat treatment. Thermostability is generally found associated with a higher resistance to chemical denaturants such as solvents or guanidinium hydrochloride. Isolating purified thermostable enzymes from newer sources therefore continues to remain an exciting challenge (Godfroy et al., 1997).

1.1.2 Factors affecting protein thermostability

Enzymes that demonstrate extreme thermal stability accomplished the metabolism in hyperthermophiles. Enzymatic reactions at higher temperatures allowed for the use of higher substrate concentrations, provided lower viscosity and fewer risks of microbial contamination and offered higher reaction rates. The protein amino acid composition is correlated to an enzyme's thermal stability. Comparison of the amino acid composition of thermostable proteins with their homologs from mesophiles reveals higher contents of Ala, Arg and Tyr (Kumar et al., 2000; Vieille and Zeikus, 2001). The occurrence of Asn, Gln and Cys are less frequent in the sequences of enzymes from thermophiles, due to their sensitivity to chemical deamination or oxidation at higher temperatures (Jaenicke, 2000; Kumar et al., 2000). Structural factors such as surface loop deletion, increased occurrence of hydrophobic amino acid residues with branched side chains, and a greater proportion of charged residues also contributed to higher intrinsic thermostability of proteins (Kumar, 2001). Folding of thermophilic proteins and enzymes is facilitated by their amino acid sequence (primary structure) and mutually attracting and repulsing forces that contributed to their energetically most favorable, native structure (secondary, tertiary and quaternary) (Radford, 2000). Other mechanisms for stabilization include an increased number of ion-pair networks and salt bridges in the thermostable proteins when compared to their mesophilic counterparts (Aguilar,
1997; Lebbink, 1999; Yip, 1998). Earlier it was believed that protein thermostability was a result of the additional hydrogen bonds and salt bridges alone (Perutz, 1975). Detailed comparisons of homologs from mesophilic and thermophilic microorganisms have recognized thermostability is due to a combination of several stabilizing features (Kumar, 2001; Voorhorst, 1997).

An increased number of aromatic-aromatic interactions have also been observed in hyperthermophilic enzymes, compared to their mesophilic homologs (Machius et al., 2003). An abundance of disulfide bonds in the intracellular proteins of thermophiles, particularly the archaeal Pyrobaculum aerophilum and Aeropyrum pernix, underlines its role in protein thermostability (Mallick et al., 2002, Toth et al., (Giorgi Kvesitadze et al., 2007) 2000). In case of enzymes from hyperthermophiles, the presence of an increased number of subunits has been observed, compared to their mesophile counterparts. Additionally multimerization is also believed to be a thermostabilizing factor (Vieille and Zeikus, 2001). Even though homologs exhibit similarity at the amino acid level, the gain in stabilization is believed to be achieved in regions that remain less conserved (Kumar et al., 2000; Vieille and Zeikus, 2001). Besides enhancing enzyme activity, metal ions, like Co$^{2+}$, Mg$^{2+}$, Ca$^{2+}$ and Mn$^{2+}$, have their share of a role to play in protein stabilization (Chang, 1999; Vieille and Zeikus, 2001). Thermostabilization can also be influenced by external factors. Thermophiles are known to accumulate compatible solutes, such as mannosylglycerate and di-myo-inositol-phosphate, which are believed to contribute a stabilizing role intracellularly (Santos and da Costa 2002). Similarly, chaperone proteins assist in the folding of other proteins, preventing them from aggregation. Thermosomes as they are called have been observed in increased quantities in heat-shocked thermophiles (Ladenstein and Antranikian, 1998; Shockley et al., 2003). Thermostability of
proteins thus is not realized by a single factor, but by a combination of the mechanisms as described above.

1.2 Thermophilic Enzymes as biocatalysts in industries

The use of enzymes as biocatalysts in the industry has increased enormously over the last few decades, and nearly all classes of enzymes are represented in industrial processes. Due to their thermostability, a simple heat incubation step is found sufficient to remove the large part of protein contamination that accompanied when these proteins were expressed in mesophilic hosts, like *E. coli*. With the establishment of genome sequencing projects, the complete array of genes of a number of organisms has been identified and mapped. Due to their key position in the phylogeny, thermophilic and hyperthermophilic microorganisms have been included in such projects.

Following is a list of thermophilic enzymes, their source and their industrial applications for a comparative reference.

**Table (1.2 a) Thermophilic organisms, thermophilic enzymes and their applications**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Origin</th>
<th>Application</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endo-1,4-β-glucanase</td>
<td><em>T. maritima</em></td>
<td>Cellulose degradation</td>
<td>Liebl (1996)</td>
</tr>
<tr>
<td>Cellbiohydrolase</td>
<td><em>R. maritima</em></td>
<td>Cellulose degradation</td>
<td>Bronnenmeier (1995)</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Source</td>
<td>Function</td>
<td>Reference</td>
</tr>
<tr>
<td>-----------------------------</td>
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</tr>
<tr>
<td>Trehalose synthase</td>
<td><em>Thermus caldophilus</em></td>
<td>α,α-Trehalose production; used in food, cosmetics, medicine, and organ preservation</td>
<td>Koh (1998)</td>
</tr>
<tr>
<td>Hydantoinase</td>
<td><em>Bacillus stearothermophilus</em></td>
<td>Synthesis of D-amino acids as intermediates in the production of semi-synthetic antibiotics, peptide hormones, pyrethroids, and pesticides</td>
<td>Lee (1995)</td>
</tr>
<tr>
<td>Esterase</td>
<td><em>P. furiosus</em></td>
<td>Transesterification and ester synthesis</td>
<td>Ikeda (1998)</td>
</tr>
<tr>
<td>Aldolase</td>
<td><em>S. solfataricus</em></td>
<td>Synthetic chemistry; C-C bond synthesis</td>
<td>Buchanan (1999)</td>
</tr>
<tr>
<td>Pectin methylesterase</td>
<td><em>T. thermosulfurigenes</em></td>
<td>Fruit juice clarification, wine making</td>
<td>Schink (1983)</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td><em>T. aquaticus</em></td>
<td>PCR technologies</td>
<td>Bergquist (1992)</td>
</tr>
<tr>
<td>Carboxypeptidase</td>
<td><em>S. solfataricus</em></td>
<td>C-terminal sequencing</td>
<td>Colombo (1992)</td>
</tr>
<tr>
<td>α – Amylase</td>
<td><em>Desulfurococcus mucosus</em></td>
<td>Starch processing</td>
<td>Canganella (1994)</td>
</tr>
<tr>
<td>Pullulanase</td>
<td><em>Bacillus flavocaldarius</em></td>
<td>Starch processing</td>
<td>Kashiwabara (1999)</td>
</tr>
<tr>
<td>Amylopullulanase</td>
<td><em>Besulfurococcus mucosus ES4</em></td>
<td>Starch processing</td>
<td>Canganella (1994)</td>
</tr>
<tr>
<td>Glucoamylase</td>
<td><em>Clostridium thermosaccharolyticum</em></td>
<td>Starch processing</td>
<td>Specka (1991)</td>
</tr>
<tr>
<td>α – Glucosidase</td>
<td><em>Thermoanaerobacter ethanolicus</em></td>
<td>Starch processing</td>
<td>Saha (1991)</td>
</tr>
<tr>
<td>β- Amylase</td>
<td><em>Thermotoga maritima</em></td>
<td>Starch processing</td>
<td>Schumann (1991)</td>
</tr>
<tr>
<td>CGTase</td>
<td><em>Thermococcus sp.</em></td>
<td>Starch processing</td>
<td>Tachibana (1999)</td>
</tr>
<tr>
<td>Xylose isomerase</td>
<td><em>Thermoanaerobacterium thermosulfurigenes</em></td>
<td>Starch processing</td>
<td>Lee (1991)</td>
</tr>
<tr>
<td>Enzyme Type</td>
<td>Organism</td>
<td>Activity</td>
<td>Reference</td>
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</tr>
<tr>
<td>Polygalacturonate hydrolase</td>
<td><em>T. thermosulfurigenes</em></td>
<td>Fruit juice clarification, wine making</td>
<td>Schink (1983)</td>
</tr>
<tr>
<td>Pectate lyase</td>
<td><em>Thermoanaerobacter italicus</em></td>
<td>Fruit juice clarification, wine making, fruit and vegetable maceration</td>
<td>Kozianowski (1997)</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td><em>T. maritima</em></td>
<td>Production of lactose-free dietary milk products</td>
<td>Gabelsberger (1993)</td>
</tr>
<tr>
<td>β-Fructosidase</td>
<td><em>T. maritima</em></td>
<td>Confectionery industry; production of invert sugar; hydrolysis of inulin to produce HFCS</td>
<td>Liebl (1998)</td>
</tr>
<tr>
<td>Phytase</td>
<td><em>Bacillus sp. Strain DS11</em></td>
<td>Phytate degradation in animal feed</td>
<td>Kim (1998)</td>
</tr>
<tr>
<td>Keratinase</td>
<td><em>Fervidobacterium pennavorans</em></td>
<td>Degradation of poultry feathers and production of rare amino acids (i.e., serine and proline)</td>
<td>Friedrich (1996)</td>
</tr>
<tr>
<td>Chitinase</td>
<td><em>Streptomyces thermoviolaceus</em></td>
<td>Chitin utilization as a renewable resource; production of biologically active oligosaccharides</td>
<td>Tsujibo (1993)</td>
</tr>
<tr>
<td>α-Galactosidase</td>
<td><em>T. maritima</em></td>
<td>Sugar beet processing removal of raffinose from sucrosesyrups; oligosaccharide synthesis through glycosyl transfer reactions</td>
<td>Liebl (1998)</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td><em>T. neapolitana</em></td>
<td>Diagnosis enzyme labeling applications where high stability is required</td>
<td>Dong (1997)</td>
</tr>
</tbody>
</table>

The discovery and the use of thermostable enzymes in industrial processes involved biocatalytic conversions at high temperature either as a specific need for product generation or as a specific tool for reducing operational costs. The identification of a thermostable endo-1, 4-β-D-glucanase reported here, in two eukaryotic xerophytic plant (cacti) species *Opuntia vulgaris* and *Cereus pterogonus* therefore becomes a subject of significant interest.
1.3 Xerophytic plants - Cactus

The Cactaceae are an exciting and challenging group of plants because of their varied morphology and succulence, their showy flowers, their adaptations to the environment, and their reproductive strategies. The family has 1600 species and 115 genera (Barthlott & Hunt, 1993). Cenozoic cooling that occurred nearly 50 million years ago was thought to result in the formation of geographically distinct arid regions, and contributed to the evolution of a group of plants that came to be known as xerophytes (Fensome, 2001). Scientists believe that cacti developed their physiological traits in response to changing climatic conditions that occurred several million years ago (Jacobsen, 1954c). Among those, a specific type of jungle thorn bush exhibited seven characteristics by which all cacti were later defined (Chidamian, 1958). The thorn bush was established as the ancestor of all cacti. A Lemon Vine (Pereskia aculeatavar.godsefiana) that was a hardy succulent, tropical, leafy and woody shrub produced the delicious "Barbados Gooseberry" and resembled the ancestor very closely.

The Opuntia species of plants used for animal feeding are abundant, easy to grow, palatable varieties that can withstand prolonged droughts (Shoop et al., 1977). Such characteristics make these species a potentially important feed supplement for livestock, particularly during periods of drought and seasons of low feed availability. The plant has become important fodder in many parts of the world, based on natural and cultivated populations. It is cultivated in Africa, Argentina, Bolivia, Brazil, Chile, Colombia, Israel, Italy, Mexico, Spain, USA and Peru (Barbera et al., 1992; Le Houérou, 1979; Brutsch, 1984; Clovis de Andrade, 1990; Curtis, 1979; Pimienta, 1990; Russell and Felker, 1987a; Saenz, 1985).
In North Africa and from the start of the century several projects were undertaken to reduce water and wind erosion and range land degradation by using shrubs and cacti (*Opuntia ficus-indica* mainly). This variety of plants is used all year around or as emergency feedstock during drought. In many arid areas (Tunisia, Mexico, South Texas, South Africa, etc.) the farmers use cactus extensively as emergency forage that is harvested from both wild and cultivated populations to prevent the disastrous consequences of frequent and severe droughts (Le Houérou, 1992). Large areas under cultivation of this species are encountered in South Africa, Algeria, Mexico and Brazil. Therefore, from the 50th century until now large areas were planted in Algeria, Tunisia and Morocco. They are estimated to be 700,000 to 1 million ha and are located mainly in low rainfall areas and aiming to combat erosion and desertification and to provide feed for livestock during frequent drought periods.

There are three centers of cactus diversity: 1) central Mexico, from where the North American cacti have evolved, 2) the Andean region, and 3) Brazil. The dispersal of species radiating from these three centers has overlapped very little, except for human introductions and there is a subcenter extending from northern South America, northward through the Antilles and West Indies to Florida. The origin of all cacti is probably in South America, perhaps 90-100 million years ago (Gibson and Nobel, 1986). However, there are no fossil records known beyond the desert (Mc-Carten, 1981).

Cacti occur naturally from just south of the Arctic Circle in Canada to the tip of Patagonia in South America. Native cacti are restricted to the new world, except for one species, *Rhipsalis baccifera*, and mistletoe cactus of tropical Americas, which prehistorically migrated to Africa, Madagascar and Ceylon (Barthlott 1983).
The sticky small fruits of *R. baccifera* were presumably carried across the Atlantic Ocean by birds. Cacti grow at altitudes from below sea level to over 4,500 m in the Andes; and in climates having no measurable rainfall to more than 500 cm of annual precipitation. Cacti vary in size from that of a large marble to as tall as 20+ m and weighing several tons.

Companies processing cladodes into various foods are exclusively found in Mexico. Consequently, the greatest product variety is found in Mexico and the USA. The small percentage of exported products mainly comprise of vegetable preserves (Saenz-Hernandez et al., 2002 and Saenz et al., 2002). The cactus hydrocolloids are used as fat replacers and adsorbers for unpleasant smells (Saenz-Hernandez et al., 2002; Saenz et al., 2002). Cacti have antiviral properties, anti-atherogenic effect, antispermatic properties, diuretic, hypoglycemic, antidiabetic and impact on uric acid metabolism. Cladode powder was also proposed as ingredient of drinks based on milk, whey, and water with up to 10% nopalito (Saenz-Hernandez et al., 2002 and Saenz et al., 2002). Cladodes are powdered and sold in capsules. They are used to regulate weight, blood sugar, or proliferate the general fibre intake. After hydration, the resulting gel exerts a cooling effect, will ease the skin and thus contribute to accelerated wound healing similar to Aloe vera preparations. Cladode flour is composed of 52–53% carbohydrates, 20–22% ash, 15–16% proteins, 9.75% water, 9.5% fibers, and 0.25% lipids, respectively (Mulas 1993) and (Saenz et al., 2002). Although the amount applied in cosmetics is less important, the range of potential products is large. Juice from cladodes may be found in shampooing, conditioners, lotions, soaps, and sun protectors (Saenz-Hernandez et al., 2002, Saenz et al., 2002) and was also claimed to improve hair growth (Warschkow, 1994).
1.4 Desert Plant Adaptations

Cacti have no escapability and therefore are subject completely to their environs. Two major problems exist for cacti, particularly those in arid climates: temperature extremes and lack of available water. However, several adaptations allow them to cope with these difficulties. Freezing temperatures in the United States limit distributions by destroying the growing apical meristems (stem tips) of many cacti at the northern edges of their ranges and on northern north-facing slopes. High temperature effects may be reduced by evasion from direct insolation by tilting toward the sun such that only the smaller top surface gets direct sun and not the sides, e.g., barrel cacti (Ferocactus species); or by an orientation of pads of prickly-pears such that minimal surface area (at edges of, not faces of pads) is exposed to direct sunlight during the hottest periods of the day. The characteristics of cacti fit most of the requirements of a drought-resistant fodder crop. During drought, cacti remain succulent. They possess a specialized photosynthetic mechanism, which leads to a more efficient production of dry matter per unit water consumed than that of grasses or legumes. Most of the plants grow in drought-prone, nearly arid climates or physiologically dry soil (e.g., frequently frozen or periodically salty soils). This allowed them to adapt with minimal utilization of available water, and survive long periods without rainfall. In botanical ecology, plants with these qualities are called xerophytes. Cacti are succulent xerophytes, capable of storing large quantities of water in their leaves, stems, or roots, giving them a fleshy (succulent) character. Xerophytic plants have adapted to the extremes of heat and aridity by altering their physical and behavioral mechanisms.

Some plants avoid drought by allowing as seed or tuber survival and later regrow when water is available some (Ocotillo) shed their leaves when water is
scarce and then sprout a new set. Some are adapted to sustain through the drought while they have adaptations like being able to extract water from soil (by having very salty cell sap and therefore a very low water potential in the roots / by having very extensive or deep roots or very shallow roots which pick up the slightest dew and survive on that), exhibit different shape or structure (xeromorphs), have hairy surfaces (Edelweiss), dense packing of leaves, reduced leaf size (Cupressus), etc. Others have special features about their thick leathery cuticle (Aloe), reduced density of stomata (Cactus), pitted and grooved position of stomata (Ammophila), water storage in stem and tubers, etc. (Baobab), protection (by spines and chemicals) of water store, C4 / CAM and nocturnal opening of stomata for carbon fixation. The shape of cacti generally reflected the methods of adaptation. Cacti may have a barrel or candle like shape. This allowed for maximum internal volume and minimum surface area (a smart adaptation), and allowed storing more water combined with reduced water loss.

The plant surface appears tough leathery, with the presence of ribs and spines and sometimes even fur. These are considered very smart adaptations, that helped the plant survive the heat and perhaps used sometimes even for defense. Small, thick leaves minimized water loss by limiting surface area, and the presence of thick cuticle limited water loss. The tough leathery skin is impermeable to water, and therefore reduced evaporation from the surface of the plant. This skin often had a layer of plant wax on it, that is often lightly colored (Pilosocereus azures is an example of a plant with such wax), white or blue. The wax coating reflected light and also reduced the evaporation from the inside of the plant body. This is called a 'glaucus bloom' and helped to reduce evaporation by the plant, by holding in more of the precious moisture so rare in the desert (Benson, 1982; Cullmann, 1986). The ribs
are special structures that are also used for enduring extreme heat. The ribs (and spines) trap wind so that the plant is enveloped in a layer of extremely still air, and this is a very important factor in reducing evaporation. On very windy days even the ribs don't help and cacti sometimes wilt because of high water loss.

Succulents have adapted such that their stomata are closed during the day and are opened at night. This mechanism allowed them to store carbon dioxide in their tissues as crassulean acid that is converted to carbon dioxide during the daytime. The CAM plants absorbed carbon dioxide during the night, when the temperature was lower and reduced water vapour loss (Russell and Felker, 1987a; Nobel, 1989a). They converted carbon dioxide into organic acids and used them in photosynthesis during the day. This process is called crassulean acid metabolism or CAM and is considered a very smart way of respiring in the desert (Cullmann, 1986). The retention of water within the cactus is enhanced by lowering transpiration rates via: 1) having leaves reduced to spines which lowers surface area; 2) having a heavy wax coating (cuticle) on surfaces, impeding direct water loss to the atmosphere; 3) having daytime closure of stomata; and 4) being succulent wherein water adheres to complex carbohydrates called mucilage. Mucilage holds water very tightly, requiring energy to free the water. In some cacti, ribs allow the stems to expand and contract like the pleated part both daily and seasonally, allowing for gains and losses of water. Cacti also conserve water by quickly sealing any breaks in the stem’s surface caused by animals, humans, or weather. *Cylindropuntia bigelovii* survived three years without any contact with the soil (Gibson and Nobel, 1986). The scar tissue that formed where the plant was cut quickly cut off the loss of water.

Cacti however depend upon chlorophyll in the outer tissue of their skin and stems, to conduct photosynthesis for the manufacture of food. Spines protect the
plant from animals, shade it from the sun and also collect moisture. C4 plants did likewise, but formed different acids to store carbon dioxide. Some xerophytes even lost their leaves during dry months, and others had their leaves rolled up to prevent water loss (with stomata in pits and are surrounded by hairs to prevent water loss). Xerophytes have however an extensive and deep root system to extract maximum amount of water from the soil. Some of them also exhibited short life cycles that coordinated with the rainy season.

The juicy, slimy tissues in xerophytes contained significant amount of moisture for use during the dry seasons. Water and food is stored between the pith and the palisade parenchyma and occupies 85% of the plant's volume. This is a major adaptation found in desert plants. The plants remain completely alive during the dry season and continued to grow large sizes. The plants also retained supplies (in the form of starch) for the winter that allowed them to flower instantly during spring without the need for further supplies (unlike most other plants that accumulated supplies during spring). The stored supplies facilitated cacti to energize flowering during spring, and grow much earlier (Schwantes, 1957).

Cactus can be used in combination with cement barriers or cut palm leaves to stop wind erosion and sand movement. It will fix the soil and enhance the restoration of the vegetative plant cover. Root behavior is another adaptation that cacti have made for survival; Extensive shallow root systems are usually radial, allowing for quick acquisition of large quantities of water when it rained. As they stored water in the core of their stems and roots, cacti are well suited to dry climates and they survived years of drought using water collected from a single rainfall. The roots that spread laterally may exist in a suspended state until the rains activated them, and a small amount of moisture caused the feeding roots to grow quickly out of the main
roots. They acted as feeders that brought water and dissolved minerals and nutrients. Following the rain, the feeder roots died in the dry soil and disappeared enabling the plant to live on stored water. There is no loss of energy and moisture for the upkeep of these roots alive, as they may not be required for several months. Some species (plants of very dry deserts) have very shallow root systems that spread far from the plant (Benson, 1982; Cullmann, 1986) to assimilate tiny amount of available moisture from dew or light rain. In contrast, some cacti (Echinocacti) develop deep roots to reach ground water. Rainforest cacti often have aerial roots that collected water all the time during rains as in South American forests.

Some desert plants used behavioral adaptations, to developed lifestyles in conformance with the seasons of greatest moisture and/or coolest temperatures. These types of plants are usually referred to as perennials. They lived for several years, whereas annuals lived for only a single season. Desert trees and shrubs adapt by eliminating their leaves, replacing them with thorns (Riha, 1981). They bear greatly reduced leaf size to eliminate transpiration (loss of water to the air). Such plants usually have smooth, green bark on stems and trunks serving to produce food on the one hand and seal in the moisture on the other hand. During stress, plants will often bend over as the 'hygroscopic' pressure inside the plant diminished. The hygroscopic pressure held the plant erect, and as it reduced the plant experienced reduced 'turgidity' and the consequent in ability to stand erect (Barthlott, 1977). When the plant bends over, its exposure to the sunlight is reduced, and much of the plant remained shaded by the overhang portion, reducing further moisture loss.
1.5 Polysaccharide degradation

Among the various nutrients available to plants, cellulose is one of the major form of energy. Cellulose is the most abundant and renewable biopolymer on the earth. An estimated synthesis rate of cellulose is approximately $4 \times 10^{10}$ tonnes per year. Although it is the most promising renewable energy source to overcome the problems of energy resource, chemicals and food in the future, its enormous potential was recognized only after the cellulose degrading enzymes or cellulases had been identified. Cellulose and hemicellulose, that constitutes the bulk of most plants, are carbohydrates. Polysaccharides are chains of carbohydrate moieties, which can be either $\alpha$ or $\beta$-linked. Both types of linkages require distinct glycolytic enzymes for their cleavage. The breakdown of these linkages can be achieved via a $\beta$-elimination reaction performed by lyases, or by hydrolysis, carried out by glycoside hydrolases. Like for polypeptides, endo- or exo-active enzymes can degrade polysaccharides, the latter generally attacking from the non-reducing end. The presence of this range of enzymes allows an organism to completely degrade carbohydrate polymers into oligomers and, eventually, monosaccharide units. Based on their amino acid sequence homologies, the glycoside hydrolases have been classified into over 90 different families (Henrissat, 1991; Henrissat and Bairoch, 1996) (URL server: http://afmb.cnrs-mrs.fr/~cazy/CAZY/), of which many contain glycosidase of thermophilic and hyperthermophilic origin. They degrade a range of polymeric substrates, which are abundantly present in nature, such as starch, (hemi) cellulose and pectin (Bauer et al., 1998; Niehaus et al., 1999). These can be grouped according to their substrate preference, and are discussed below.

Cellulose is a member of the complex polysaccharide family found in plants as microfibrils (2-20nm diameter, 100-40,000nm long) forming the structurally
strong framework of the plant cell wall. It is the skeletal basis of plant cell walls. Cellulose is a linear polysaccharide of glucose residues connected by β-1, 4 linkages. Like chitin, it is not cross-linked. Native crystalline cellulose is insoluble and occurs as fibers of densely packed, hydrogen bonded, anhydro glucose chains of 15 to 10,000 glucose units. Its density and complexity make it very resistant to hydrolysis without preliminary chemical or mechanical degradation or swelling. In nature cellulose is usually associated with other polysaccharides such as xylan or lignin.

Xylans are polysaccharides (hemicellulose) composed of β-1, 4-linked xylopyranose units. They are mostly highly branched and are in tight association with other biopolymers. Xylans belong as a major constituent of the plant cell wall. As the most abundant hemicellulose, they account for more than 30% of the dry weight of terrestrial plants.

Glycogen, the animal polysaccharide is a polymer of α (1-4) linked D-glucose with α (1-6) linked branches for every 8-14 residues. Glycogen occurs as intracellular granules of 100-400 Å diameter spheroidal molecules that each contains up to 120,000 glucose units (Lomako et al., 1993; Chen et al., 1993). Glycogen granules also contained enzymes that catalyze glycogen synthesis and degradation as well as many of the proteins that regulate the process.

Among the variety of complex polysaccharides in nature, starch is also a polysaccharide usually deposited as large granules in the cytoplasm. Starch is composed exclusively of α-glucose units that are linked by α-1, 4- or α-1, 6-glycosidic bonds. The two high-molecular-weight components of starch are amylose (15-25%), a linear polymer consisting of α-1, 4-linked glucopyranose residues (Rundle, 1943; Hybl, 1965), and amylopectin (75-85%), a branched polymer containing, in addition to α-1, 4 glycosidic linkages, α-1, 6- linked branch points.
occurring at every 17-26 glucose units (French, 1975; Rao; 1972; French, 1973; Madeleine et al., 1994).

1.6 Cellulose-degrading enzymes

Cellulose is an abundant and renewable biopolymer known to be present in the plants, bacteria and fungi (Coughlan, 1990). Cellulose consists of glucose units linked via β-1, 4-glycosidic bonds and is nature's most abundant plant polymer. Complete hydrolysis of cellulose entails the synergistic action of endo- and exoglucanases, and β-glucosidases. Considerable effort has been spent to create an economically feasible ethanol production method from cellulose, but without much success. Typically embedded in a network of hemicellulose and lignin, cellulose requires an alkaline pretreatment to become accessible to enzyme action. One of the main limitations to this process is the low activity and high cost of the cellulases used. Since cellulose's alkaline pretreatment is performed at high temperature, hyperthermophilic cellulases should be the best candidate catalysts for cellulose degradation. Consequently, thermostable cellulases have gained considerable interest. The production of cellulases by hyperthermophiles is rare, however only recently endoglucanases and cellobiohydrolases have been characterized in the thermotogales Thermotoga sp. strain FjSS3-B.1. (Ruttersmith, 1991). In recent years, the possibilities have been explored to produce thermostable cellulases in plants for an efficient degradation of endosperm cell walls of barley. Examples for these transgenic plants is the availability of a thermostable (1,3-1,4)-β-glucanase, a hybrid of parental enzymes from Bacillus macerans and B. amyloliquefaciens produced in barley, and a 1,4-β-glucanase from Acidothermus cellulolyticus produced in chloroplasts of tobacco plants (Jensen et al., 1996; Jin et al., 2003).
In the paper production process, pulping is a step during which wood fibers are broken apart and most of the lignin is removed. Pulping often corresponds to a chemical hot-alkali treatment of the wood fibers. The remaining lignin is removed by a multistep bleaching process. Performing with chlorine and/or chlorine dioxide at high temperatures, pulp bleaching generates high volumes of polluting waste (Tolan, 1996). The amount of chemicals used and the resulting pollution can be reduced however if the paper pulp was pretreated with hemicellulases. Since pulping and bleaching are both performed at high temperatures, the paper industry preferentially required and used thermophilic hemicellulases, preferably those active above pH 6.5 or pH 7.0 (Wakarchuk et al., 1994).

Many of the several enzymes produced were chiefly by fungi, bacteria, and protozoans that catalyzed the hydrolysis of cellulose. Fungi are the main cellulase-producing microorganisms, though a few bacteria and actinomycetes have also been recently reported to yield cellulase activity as described in the table (1.6a).

Microorganisms of the genera *Trichoderma* and *Aspergillus* are believed to be cellulase producers, and crude enzyme preparations from these microorganisms are commercially available for agricultural use. Microorganisms of the genus *Trichoderma* produce relatively large quantities of endo-β-glucanase and exo-β-glucanase, but only low levels of β-glucosidase, while those of the genus *Aspergillus* produce relatively large quantities of endo-β-glucanase and β-glucosidase with low levels of exo-β-glucanase production. The thermostable endo-1, 4-β-D-glucanase in the eukaryotic xerophytic plant species *Opuntia vulgaris* and *Cereus pterogonus* reported here serves to accomplish all requirements for the industrial purposes.
### Table (1.6 a) Cellulase producing Microorganisms

<table>
<thead>
<tr>
<th>Fungi:</th>
<th>Bacteria:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus acculeatus</td>
<td>Clostridium thermocellum</td>
</tr>
<tr>
<td>Aspergillus fumigatus</td>
<td>Ruminococcus albus</td>
</tr>
<tr>
<td>Aspergillus niger</td>
<td>Streptomyces sp.</td>
</tr>
<tr>
<td>Fusarium solani</td>
<td></td>
</tr>
<tr>
<td>Irpex lacteus</td>
<td></td>
</tr>
<tr>
<td>Penicillium funiculosum</td>
<td></td>
</tr>
<tr>
<td>Schizophyllum commune</td>
<td></td>
</tr>
<tr>
<td>Sclerotium rolfsii</td>
<td></td>
</tr>
<tr>
<td>Sporotrichum cellulophilum</td>
<td></td>
</tr>
<tr>
<td>Talaromyces emersonii</td>
<td></td>
</tr>
<tr>
<td>Thielavia terrestris</td>
<td></td>
</tr>
<tr>
<td>Trichoderma koningii</td>
<td></td>
</tr>
<tr>
<td>Trichoderma reesei</td>
<td></td>
</tr>
<tr>
<td>Trichoderma viride</td>
<td></td>
</tr>
</tbody>
</table>

### 1.7 Structure of cellulose

Cellulose is one of the most abundant linear biopolymer in nature, consisting of flat chains of D-glucose linked by $\beta$-1, 4-glucosyl linkages. All the links connect $C_1$ of one glucose to $C_4$ of the other glucose. Cellulose molecules are strongly associated through inter and intra molecular hydrogen bonding and vanderwaal forces that result in the formation of microfibrils, which in turn forms fibers. Cellulose molecules are oriented in parallel, with reducing ends of the adjacent glucan chains positioned at the same end of the mirofibril. Most of the cellulose in nature is found in the cell wall of plants, where it is the major component. Despite great differences in composition of the cell wall, cellulose content ranges from
approximately 35 to 50% of plant dry weight (Lynd et al., 1999). It is highly stable and insoluble in water (Delmer et al., 1995; Richmond et al., 1991). In cotton bolls, cellulose is present in a nearly pure state. The cellulose fibers are embedded in a matrix of other structural biopolymers, such as hemicelluloses (20-35%) and lignin (5-30%) comprises the plant dry weight (Lynd et al, 1999; Marchessault, 1993; Van Soest, 1994) The cell wall being a very complex structure also contains xyloglucans, hemicellulose, pectin and proteins (Carpita et al, 1993; Vian et al., 1991).

**Fig. (1.7a) Structure of Cellulose**

![Structural Formula](image)

**A STRUCTURAL FORMULA**

![Conformational Formula](image)

**B CONFORMATIONAL FORMULA**
An important feature of cellulose is its crystalline structure. Cellulose is synthesized as individual molecules that undergo self-assembly at the site of biosynthesis (Brown et al., 2000). Approximately 30 individual cellulose molecules are assembled to form larger units called elementary fibrils (protofibrils), that are then packed into still larger units called microfibrils. (Delmer et al., 1995; Brown et al., 2000). Microfibril size can vary from approximately 36 chains elementary fibril to more than 1200 chains large microfibrils of cellulosic algae (Brown et al., 1996). Microfibrils are 5/15 nm wide and are spaced 20/ 40 nm from each other (McCann, 1990). And these are in turn assembled into the familiar cellulose fibers. The polymeric glucose chains are attached by the network of hydrogen and van der waals bonds, eventually leading to crystallization of the cellulose in certain areas in the microfibril.

Fig. (1.7 b) Linkages in cellulose microfibril: (picture adapted from Daniel J. Cosgrove, 2005)
**Linkages in cellulose microfibril:** Hemicelluloses such as xyloglucans (1) and arabinoxylans (2) may form a primary network with cellulose and may also be linked to acidic pectins (3). Additionally, neutral pectin polysaccharide such as arabinans (4), are able to bind the cellulose surfaces as well (Zykwinska, 2005).

**Structure of primary cell wall:** Cellulose microfibrils (purple rods) are synthesized by large hexameric complexes in plasma membrane, whereas hemicelluloses and pectins, which compose the matrix polysaccharides, are synthesized in the golgi apparatus and are deposited to the wall surfaces by vesicles. For clarity, the hemicellulose- cellulose network is shown on the left part of the cell wall without pectins, which are emphasized on the right part of the Fig (1.7c).

**Fig. (1.7c) Structure of primary cell wall (picture adapted from Daniel J. Cosgrove, 2005)**
In most plant species the main hemicellulose is xyloglucan (blue), while hemicelluloses such as arabinoxylans (grey) and mannans (not shown) are found in lesser amounts. The main pectin polysaccharides include rhamnogalacturonan I and homogalacturonan, with smaller amounts of xylogalacturonan, arabinan, arabinogalactan I (Not shown) and rhamnogalacturonan II. Pectin domains are believed to be covalently linked together and to bind to xyloglucan by covalent and non-covalent bonds (Cumming, 2005; Rizk, 2000). Neutral pectin polysaccharides (green) are also able to bind to cellulose surface (Zykwinska, 2005).

1.7.1 Synthesis of Cellulose:

Structures responsible for cellulose synthesis have been identified by electron microscopy in freeze-fractured plasma membranes of many organisms (Brown, 1996; Kimura et al., 1999a). Linearly arranged terminal complexes (TCs) in single or multiple rows may be observed in bacteria, D. discoideum and some algae, or hexagonal structures with six-fold symmetry termed rosettes, are observed in mosses, ferns, algae and vascular plants (Brown, 1996; Delmer, 1999; Tsekos, 1999). Although terminal complex and rosettes reside in the plasma membrane, during synthesis, rosettes are assembled in the golgi and then transported to the plasma membrane (Haigler and Brown, 1986). Each cellulose microfibril is formed from the spontaneous 'bundling' and crystallization of dozens of (1, 4)-linked β-D-glucan chains, each made by a CESA (cellulose synthase) protein. CESA polypeptides interact to form a rosette subunit. Plant cellulose synthase (CESA) genes were identified in the late 1990s through molecular and genetic studies (Pear et al., 1996 and Arioli et al., 1998). In the model plant Arabidopsis thaliana, the CESA family contains ten genes, which are expressed in different tissues and cell types. The CESA
family belongs to a larger superfamily of genes called *CELLULOSE SYNTHASE-LIKE*, which includes eight other gene families (Richmond and Somerville, 2000), named *CSLA, CSLB* and so on, up to *CSLH*. CESA catalytic subunits utilize UDP-Glc as substrate for glucan chain elongation, as well as other components that may be involved either in providing the substrate or in initiating or terminating chain elongation or that may be involved in regulating the activity of the complex. Based on our current understanding of synthesis, UDP-Glc is thought to bind to an active site on the cytoplasmic face of the plasma membrane with the polysaccharide being extruded through the membrane, presumably through a pore-type structure, into the wall (Delmer, 1999; Brown and Saxena, 2000). Each rosette (~25 nm in diameter comprising six subunits) is believed to contain a number of synthetic units, possibly six per subunit, each of which polymerizes a glucan chain that associates with adjacent chains of the same rosette to form elementary microfibrils (Fig. 1.7.1).

A rosette model (1.7.1 A) possibly containing six CESA polypeptides, interact to form a rosette. Each CesA polypeptide is shown to be involved in the synthesis of one β-1, 4- glucan chain and (1.7.1 B) In this modified rosette structure model of (Scheible et al., 2001) at least two types of CESA polypeptides, α and β, are required for spontaneous rosette assembly. Two different types of α isoforms can be distinguished, α₁ which interacts with two β isoforms only, and α₂ interacting with another α₂ isoform and two β isoforms.
The highly organized crystalline domains are spaced by less crystalline ('amorphous') regions. An important feature of the crystalline array is that the component molecules of individual microfibrils are packed sufficiently tight to prevent penetration not only by enzymes but also even by small molecules such as water. The crystalline areas form tight arrays, which shield many of the glycoside bonds from enzymatic attack (Brett, 2000; Young, 1986). In addition, the presence of other components (i.e. hemicelluloses, pectin and xylloglucans) along with the cellulose makes the cell wall a very compact and inaccessible substrate (Brett, 2000; Rose, 1999). This highly complex structure has challenged microorganisms throughout evolution to develop systems that could handle cell walls efficiently. The complete degradation of cellulose to glucose requires the action of at least three different types of enzymes: endo-1, 4-β-glucanase (Endoglucanase. EC 3.2.1.4), exo-1, 4-β-glucanase (cellobiohydrolase. CBH. EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Organisms capable of modulating cellulose-containing materials usually produce complex extracellular or membrane-bound cellulase systems comprised of α
combination of several enzymes (Beguin, 1996; Leschine, 1995) to degrade the cell wall. Both plants and microorganisms evolved simultaneously and use structurally similar enzymes in order to degrade polysaccharides. In some cases, the proteins that metabolize glucan chains exhibit similar mechanisms and structure (Brummell et al., 1994), whereas in others, the structures and mechanisms are different (Beguin et al., 1998). Considering the fact that cellulose is one of the most abundant polymers in nature, it makes sense that enzyme and organisms that modify and degrade cellulose would be spread across kingdoms and environments.

Although cellulose forms a distinct crystalline structure, cellulose fibers in nature are not purely crystalline. In addition to the crystalline and amorphous regions, cellulose fibers contain various types of irregularities, such as kinks or twists of the microfibrils, or voids such as surface micropores, large pits, and capillaries (Blouin et al., 1970; Cowling, 1975; Fan et al., 1980; Marchessault et al., 1993). The total surface area of a cellulose fiber is thus much greater than the surface area of an ideally smooth fiber of the same dimension. The net effect of structural heterogeneity within the fiber is that the fibers are at least partially hydrated by water when immersed in aqueous media, and some micropores and capillaries are sufficiently spacious to permit penetration by relatively large molecules including, in some cases, even cellulolytic enzymes (Stone and Scallan, 1968; Stone et al., 1969).

Purified cellulosates used for studies of hydrolysis and microbial utilization vary considerably in fine structural features, and the choice of substrate for such studies undoubtedly affects the results obtained. 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.

1.7.2 Carboxymethyl cellulose (CMC)

Carboxymethyl cellulose (CMC; E466) is a derivative of cellulose formed by its reaction with alkali and chloroacetic acid. The CMC structure is based on the β-1, 4-D-glucopyranose polymer of cellulose. Carboxymethyl cellulose, or CMC, is formed by replacing one or more of three reactive hydroxyl groups present in each glucose unit of cellulose. Soluble cellulose substrates exist such as carboxymethylcellulose or barley β-glucan (Schwarz, 2001). Different preparations have different degrees of substitution, and are generally in the range 0.6 - 0.95 derivatives per monomer unit.

CMC molecules are reportedly shorter, than native cellulose molecules and have uneven derivatization yielding areas of high and low substitution. The substitution is mostly 2-O- and 6-O-linked, followed in order of importance by 2, 6-di-O-, 3-O-, 3, 6-di-O-, 2, 3-di-O- and 2, 3, 6-tri-O-linked. CMC molecules remain extended (rod-like) at low concentrations while at higher concentrations these molecules overlap and coil up and entangle to become a thermo reversible gel. Increasing ionic strength and reducing pH, decrease the viscosity as they cause the polymer to become more coiled. CMC dissolves rapidly in cold water and is mainly used for controlling viscosity without gelling (CMC, at typical concentrations, does not gel even in the presence of calcium ions). As its viscosity reduces during heating, it is considered for use to improve the volume yield during baking, by encouraging gas bubble formation. It is therefore used as a thickener, phase and emulsion stabilizer (e.g. with milk casein), and as a suspending agent.
1.8 CELLULASE:

Cellulases are enzymes that are capable of hydrolyzing cellulose. The products of the reaction include cellobiose and glucose, which can be used for a variety of applications. Cellulase is the class of enzymes produced chiefly by fungi, bacteria, and protozoans that catalyze the hydrolysis of cellulose. However, there are also cellulases produced by other types of organisms as plants and animals. Several different kinds of cellulases are known, which differ structurally and mechanistically. The Enzyme Commission number for this group of enzymes is E.C.3.2.1.4. The main reaction of cellulase is the hydrolysis of 1, 4-β-D-glycosidic linkages in cellulose, lichenin and cereal β-D-glucans. Other names of endoglucanase are
endo-1,4-β-glucanase, carboxymethyl cellulase, endo-1,4-β-D-glucanase, β-1, 4-glucanase, β-1, 4-endoglucan hydrolase, celludextrinase and avicelase.

1.8.1 Types of Cellulase

The cellulase exists in multiple forms. The occurrence of multiple forms of cellulase components has complicated the study of cellulases. This multiplicity is due to several factors including (i) multiple genes (ii) macroheterogenity based on formation of multienzyme aggregates (iii) microheterogenity due to complexing of cellulases with proteins, glycoproteins or polysaccharides (iv) synthesis of variants of a single gene product via infidelity of translation, proteolysis, variable glycosylation or interaction with components of the culture broth. Karl-Erik Eriksson and Bert Pettersson, 1975 have identified five endoglucanases in Chrysosporium lignorum with different molecular weights.

Table (1.8.1 a) below show the physical properties and molecular weight of multiple forms of endoglucanase from Aspergillus species.

<table>
<thead>
<tr>
<th>Species and enzyme type</th>
<th>Enzyme</th>
<th>Mol mass (kDa)</th>
<th>pHopt</th>
<th>Topt(°C)</th>
<th>pI</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. aculeatus</td>
<td>XEG</td>
<td>23.6</td>
<td>3.4</td>
<td>4.8</td>
<td></td>
<td>Pauly et al., (1999)</td>
</tr>
<tr>
<td>A. aculeatus</td>
<td>FI-CMCase</td>
<td>25</td>
<td>4.5</td>
<td>50</td>
<td>4.0</td>
<td>Murao et al., (1988)</td>
</tr>
<tr>
<td>A. aculeatus</td>
<td>FII-CMCase</td>
<td>66</td>
<td>5.0</td>
<td>70</td>
<td>4.0</td>
<td>Takada et al., (1999)</td>
</tr>
<tr>
<td>A. aculeatus</td>
<td>FV-CMCase</td>
<td>38</td>
<td>4.0</td>
<td>65</td>
<td>3.4</td>
<td>Takada et al., (1999)</td>
</tr>
<tr>
<td>A. aculeatus</td>
<td>hydrocellulase</td>
<td>68</td>
<td>2.5</td>
<td>60</td>
<td>3.5</td>
<td>Takada et al., (1999)</td>
</tr>
<tr>
<td>A. aculeatus</td>
<td>FI-Avicelase</td>
<td>109</td>
<td>5.5</td>
<td>65</td>
<td>4.7</td>
<td>Takada et al., (1999)</td>
</tr>
<tr>
<td>A. aculeatus</td>
<td>FIII-Avicelase</td>
<td>112</td>
<td>2.5</td>
<td>65</td>
<td>4.0</td>
<td>Takada et al., (1999)</td>
</tr>
<tr>
<td>A. nidulans</td>
<td>Endo-I</td>
<td>25</td>
<td>6.0</td>
<td>65</td>
<td></td>
<td>Bagga et al., (1990)</td>
</tr>
<tr>
<td>A. nidulans</td>
<td>Endo-II</td>
<td>32.5</td>
<td>5.0</td>
<td>50</td>
<td></td>
<td>Bagga et al., (1990)</td>
</tr>
<tr>
<td>A. nidulans</td>
<td>EG A</td>
<td>35</td>
<td>6.5</td>
<td>50</td>
<td></td>
<td>Chikamatsu et al.,</td>
</tr>
</tbody>
</table>
<pre><code>                                                           |                |    |         |   | (1999)                   |
</code></pre>
The relative susceptibilities of various substrates to enzyme hydrolysis have been used to characterize these enzymes and to aid in identification of the components of enzyme complexes. Five general types of cellulases have been recognized based on the type of reaction catalyzed:

- **Endo-cellulase** breaks internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains.

- **Exo-cellulase** cleaves 2-4 units from the ends of the exposed chains produced by endocellulase, resulting in the tetrasaccharides or disaccharide such as cellobiose. There are two main types of exo-cellulases (or cellobiohydrolases) - one type working processively from the reducing end, and one type working processively from the non-reducing end of cellulose.

- **Cellobiase** or beta-glucosidase hydrolyses the endo-cellulase product into individual monosaccharides.

- **Oxidative cellulases** that depolymerize cellulose by radical reactions, as for instance cellobiose dehydrogenase.

- **Cellulose phosphorylases** that depolymerize cellulose using phosphates instead of water.

### 1.8.2 Mechanism of action of Cellulase on Cellulose

Three types of reactions are catalyzed by cellulases. They are:-

1. **Breakage of the non-covalent interactions** present in the crystalline structure of cellulose (endo-cellulase)

2. **Hydrolysis of the individual cellulose fibers** to break it into smaller sugars (exo-cellulase)

3. **Hydrolysis of disaccharides and tetrasaccharides** into glucose (beta-glucosidase).
Most cellulases comprise of three types of catalytic function that works together to catalyze the biological conversion processes such as cellulose hydrolysis. First, an endoglucanase attacks one of the cellulose chains within the crystal structure, breaking it via hydrolysis, and creating new chain ends. During this hydrolysis, a molecule of water is consumed, and one of the chain ends becomes "reducing" and the other "non-reducing." An exoglucanase then attaches to a loose end, pulls the cellulose chain out of the crystal structure, and then works its way down the chain, breaking of cellobiose (dimers of two glucose molecules) as it goes. Actually, there are two types of exoglucanase to match the two types of loose chain ends. A cellobiohydrolase I (CBH I) attaches to the "reducing" end, and a cellobiohydrolase II (CBH II) attaches to the "non-reducing" end. CBH I catalytic domain contains ten active subsites that physically adjoin to the cellulose and initiate the chemical reactions that break the chains apart into cellobiose. Finally, a beta-glucosidase splits cellobiose into two separate glucose molecules, making them available for processing into chemicals or fuels. (NREL, 2003).
Schematic representation of the morphology and organisation of crystalline cellulose as presented in Fig. (1.8.2 b). Amorphous regions occur in particular near the crystal surfaces but many also transverse the entire width of the crystalline cellulose. Different exoglucanases or cellobiohydrolases (CBH) are currently thought to attack the crystalline areas at the opposite chain ends and the endoglucanases (EG) in the middle of the more disordered regions of cellulose. The filled circles, denoted R, represent the reducing ends and the open circles, denoted NR, represent the nonreducing ends, C defines the highly crystalline regions (Teeri, 1997).

1.8.3 Cellulases and their modes of action

Cellulases include three main types of enzyme activities namely, endoglucanases (EC 3.2.1.4), cellobiohydrolases or exoglucanases (EC 3.2.1.91) and $\beta$-glucosidases (EC 3.2.1.21). These enzymes can either be free (mostly in aerobic microbes) or grouped together in a multicomponent enzyme complex (cellulosome) generally found in anaerobic cellulolytic bacteria (Bayer et al., 1998). Cellulases from different sources have been reported to show similar modes of action (Mosier
The enzymatic hydrolysis of the glycosidic bonds takes place through general acid catalysis involving two carboxylic acids (glutamate or aspartate) (Mosier et al., 1999).

(Fig. 1.8.3 a) The two mechanisms of enzymatic hydrolysis of the glycosidic bonds (Schülein, 2000).

This hydrolysis occurs through two major pathways which give rise to either retention or inversion of the anomeric configuration of the substrate (Fig 1.8.3a). Whereas Inversion is simple single displacement reactions while the retention mechanism involves two steps. During inversion mechanism, an acidic amino acid residue donates a proton while another charged acidic amino acid residue in the neighborhood “activates” water molecule for a nucleophilic attack. The proton donor thus becomes the charged residue while the charged residue gets protonated. Due to this alternation of the protonation of the catalytic residues, this mechanism is called
as inversion. The retention mechanism involves the formation of a covalent glycosyl-enzyme intermediate that is subsequently hydrolysed through an oxocarbonium ion-like transition state (Fig 1.8.3(a B) (Schülein, 2000).

(1) Endoglucanases play an important role in cellulose hydrolysis by cleaving the substrate chains randomly and offering strong degradation (Cao and Huimin, 2002). The indiscriminate action of endoglucanases generally increases the accessibility of cellulose chain ends, for exocellulase activity (Ramos et al., 1999). Endoglucanase attacks the β-1, 4 glycosidic bonds within the amorphous regions of cellulose chains (Abdelnasser et al., 2007) (Mosier et al., 1999). The products of this attack are oligosaccharides of various chain lengths and newer reducing ends (Lynd et al., 2002).

(2) Exoglucanases degrade crystalline cellulose most efficiently and acts on the reducing or non-reducing ends of the cellulose polysaccharide chains, releasing either glucose (glucohydrolases) or cellobiose (cellobiohydrolases) as major products (Bhat and Bhat, 1997; Lynd et al., 2002).

(3) β-glucosidases complete the hydrolysis of cellulose. They hydrolyse cellobiose, a potential inhibitor of cellobiohydrolases (Lemos et al., 2003). The catalytic activity of β-glucosidase is inversely proportional to the degree of substrate polymerisation. Though a cellulase, β-glucosidase does not attack cellulose. These enzymes can be grouped as aryl β-D-glucosidases (hydrolysing exclusively aryl-β-glycosides), cellobiases (hydrolysing diglycosides and cellooligosaccharides) or β-glucosidases with wide range of substrate specificities (Bhat and Hazlewood, 2001).
1.8.4 Synergism

Hydrolysis of cellulose requires interaction of numerous cellulase constituents in a mixed reaction system (Gan et al., 2003). Cellulase systems (complex) comprising of the three cellulase activities (endoglucanases, exoglucanases and \( \beta \)-glucosidases) act in concert to efficiently degrade cellulose. The cellulase complexes demonstrate better joint activity than when individual activities, and contributes to the phenomenon known as synergism (Lynd et al., 2002). Four forms of synergism have been reported.

1. Endo–exo synergism: Synergism between exoglucanases and endoglucanases, as a result of generation of more chain ends on the cellulose surface by endoglucanases for cellobiohydrolases to attack (Lemos et al., 2003).

2. Exo-exo synergism: Synergism between exoglucanases processing from the reducing ends and from the non-reducing ends of cellulose chains.

3. Synergism between exoglucanases and \( \beta \)-glucosidases to get rid of cellobiose (and cellodextrins) as end product of the first two enzymes (Lynd et al., 2002).

4. Intramolecular synergism between catalytic domains and cellulose binding domains (Which effects binding of the enzymes to cellulose surface) dependent upon the enzyme structure (Mosier et al., 1999).

1.8.5 Processes preceding enzymatic hydrolysis of cellulose

There are three processes that reportedly occur before the initiation of the enzymatic hydrolysis of cellulose, they are: (a) diffusion of cellulase in the aqueous medium, (b) movement of cellulase from the liquid to the surface of the substrate and (c) surface assimilation of cellulose to the enzyme resulting in an enzyme-
substrate complex. At high substrate concentration, the transfer of cellulase to the substrate surface is impeded (Cao and Huimin, 2002).

1.8.6 Rate limiting factors in enzymatic hydrolysis

The rate of conversion of cellulose fibers to individual, easily hydrolysable shorter chains is dependent upon a number of determining factors of hydrolysis rate (Lynd et al., 2002) such as:

1. Crystallinity: generally regarded as a key factor influencing cellulose hydrolysis at both enzymatic and microbial levels. The highly crystalline regions of cellulose chains are recalcitrant to hydrolysis, as a result of their tightly packed nature, which prevents accessibility of the enzymes. The more of these regions present the slower the rate of hydrolysis.

2. Degree of polymerisation: The longer the cellulose chain, the lower the rate of hydrolysis (Walker et al., 1990).

3. Particle size: Within a given cellulose sample, there is a great degree of unevenness in the size and shape of individual particles, thus affecting the rate of hydrolysis.

4. Pore volume: The pore structure of cellulosic materials should be able to accommodate particles of the size of a cellulolytic enzyme. The greater the availability of such pore volumes, the more will be the enzyme particles that get adsorbed (Mosier et al., 1999).

5. Accessible surface area: Most cellulose chains are hidden within the microfibrils and prevent exposure to enzymes, and thus limit the rate of hydrolysis.
1.9 Endo-1, 4-β-glucanase belongs to glycosyl hydrolase family

Glycosyl hydrolyzing enzymes contain more than 300 members that are classified into more than 50 families. The classification is based on biochemical characteristics, amino acid sequence similarities and hydrophobic cluster analysis. According to these definitions, glycosyl hydrolases that can degrade β-1, 4-glucosidic bonds are denominated cellulases and endoglucanases is such an enzyme. Today, endoglucanases are classified into 12 families (Knowles et al., 1987; Henrissat and Bairoch, 1996). Endoglucanases are assigned mainly to families 5 and 12, with the exception of CelB (cellulase) from A. oryzae. Microbial endoglucanases are found in all the 12 families whereas all plant endoglucanases are placed in family 9 which also includes several bacterial cellulases and one from a unicellular eukaryotic slime mold (Brummell et al., 1994; Gilkes et al., 1991).

1.10 Endo-1, 4-β D-glucanase and cellulose-binding domains /distribution in nature

Endo-1, 4-β D-glucanase is a part of the enzymatic system that enables the modification or complete degradation of cell walls into basic building units. The variety of organisms capable of producing this catalytic activity includes bacteria, slime mold, fungi, nematodes, algae and higher plants. Most likely it is possible that many more organisms that have not yet been identified may also exhibit such a feature. During the growth process, plants respond to many different, internal (metabolites, hormones) and external (light, water) signals. In many instances, the response is by way of loosening the cell wall integrity in order to enable a turgor driven cell expansion (Rose, 1999; Cosgrove, 1999). In order to enable cell wall loosening, the polysaccharide matrix in the cell wall need to be metabolized for
which endoglucanase is one of the enzymes for the coordinated and monitored activity (Rose, 1999; Fry, 1995). In nature, many microorganisms can degrade cell walls and since both plants and microorganisms had evolved simultaneously, they used structurally similar enzymes in order to degrade polysaccharides. In some cases, the proteins that metabolized glucan chains exhibited similar mechanisms and structure (Brummell, 1994), and in others, they were different (Beguin, 1998).

1.11 Plant endo-1, 4-β-glucanase

Plant endoglucanases are found in a wide range of species including tomatoes, melon, bean, pepper, and peach, orange and avocado trees (Tucker, 1987; del Campillo 1999). The genes that have been identified to date are usually associated with different developmental stages of the plant such as elongation (Shani, 1997; Hayashi, 1994), ripening (Tucker, 1987; Lashbrook, 1994; Cass, 1990; Fischer, 1991) and abscission (Tucker, 1987; Tucker, 1991; Kemmerer, 1994). The plant endoglucanases are of approximately 50/70 kDa and possess discrete amino acid sequences that are conserved alongside microbial cellulases of the E2 subgroup. Another common feature in all plant endoglucanases is the absence of a cellulose binding domain (CBD) (Brummell et al., 1994; Henrissat, 1996; Kemmerer, 1994) however, it must be noted that putative CBD in plant endoglucanases have been reported (Catala, 1998; Trainotti, 1999). Little is known however about the true in vivo substrate as also about the biochemical properties of these enzymes. Thus all endoglucanases are characterized by the presence of the same active site that can also be found in the microbial endoglucanase genes from family 9 (Henrissat, 1996).

Evidence gathered to date suggests that the membrane anchored endoglucanase plays a role in cell wall biosynthesis (editing cellulose synthesis)
while the extracellular enzyme play specific roles in cell wall catabolic processes, eventually leading to cell death (del Campillo, 1999). A structure (a long cleft and presence of conserved catalytic amino acids) similar to that in microbes has been detected in a family of plant glucanases that degrade fungal 1,3-β-glucan and grass cell wall 1,3:1,4-β-glucans (Hoj 1995). Further the plant endoglucanase sequences reveal similarity to microbial endoglucanases. They contained two conserved catalytic regions, suggestive of similar catalytic mechanism (Beguin, 1994; Tomme, 1992). Nevertheless the three dimensional structure of plant 1, 4-β-glucanase has not yet been determined however.

In several plant species, endoglucanases are encoded by tightly regulated multigene families (Brummell et al., 1994; Nicol, 1998) with different expression patterns in tomato plants, for example, Seven endoglucanase cDNAs have been characterized (Lashbrook et al., 1994; Catala et al., 1997; Brummell et al., 1997; del Campillo, 1996). The Arabidopsis gene family, one of the most extensively studied, has more than 12 members. Not all enzymes encoded by them are structurally similar while the enzymes can be located variously within the cell, in the plasma membrane, or some as secreted (Arabidopsis gene family, review del Campillo, 1999).

Plant endoglucanases belong to family 9 and they do not appear to contain a CBD, generally seen attached to the catalytic core of several microbial cellulases employing a peptide linker (Gilkes et al., 1991; Hayashi, 1989). Phylogenetic analysis of plant endoglucanase has revealed the existent polymorphism and indicates its origins to an early phase in plant evolution, against that preceded to the divergence of monocotyledons and dicotyledons (Brummell et al., 1994).
1.12 Structure of Endoglucanase

The first 3-D structure of a cellulase, the catalytic core, that of *Trichoderma reesei* Cel6A cellobiohydrolases (CBH II) was published in 1990 (Rouvinen et al., 1990). Thereafter, more than 20 different cellulase structures have been recognized covering 8 different family folds. Even though they all cleaved the $\beta$-1, 4-glycosidic bonds, they displayed a variety of topologies ranging from all $\beta$-sheet proteins, through $\beta/\alpha$ barrel to all $\alpha$-helical proteins. This included the structure of three different family representatives of 'cellobiohydrolases'. A notable feature of cellulases and many glycoside hydrolases is their modularity (Coutinho and Henrissat, 1999) structure with one (occasionally more) catalytic domain linked to one or several non-catalytic modules. The non-catalytic modules are known to be involved in protein-carbohydrate and protein-protein interactions and contained carbohydrate-binding domains, (Tomme et al., 1995; Coutinho and Henrissat, 1999). Based on the catalytic core sequence, cellulases have been classified into sequence related families (Henrissat and Bairoch, 1993).

Endoglucanases in general contain three major functional units, catalytic, anchoring and the linker units. The linker unit connects the catalytic unit to the anchoring unit. Microbial endoglucanases, exhibit an anchoring unit that usually a cellulose-binding unit (Gilkes et al., 1991), whereas in plant endoglucanases the anchoring unit can either be a membrane-anchoring domain or a lack of it in the secreted form of the enzyme (Brummell et al., 1994). Recently, several genes have been cloned from bacteria that lacked a CBD but consist only the catalytic domain (Covert, 1992; Takashima et al., 1998).

Database sequence analysis has revealed that catalytic cores of several bacterial, fungal and plant endoglucanases shared many characteristics (Gilkes et al.,
1991; Henrissat et al., 1989). Gilkes et al. (Gilkes et al., 1991) demonstrated that catalytic domains from different families are associated with the same type of CBD. Thus, a given organism could possess enzymes from several families but would contain only one type of CBD. Furthermore, the linker domain showed sequence identity between enzymes within the same organism, but exhibited no such identity between linkers from different organisms. Biochemical studies have revealed that some enzymes may exhibit mixed specificity: that is enzymes that hydrolyzed β-1, 4 bonds in cellulosic substrates may also hydrolyze xylan or chitin at significant rates (Davis, 1998; Gilkes et al., 1984). Thus considering all available information, it seems that endoglucanases appeared to have resulted from a few progenitor sequences through mutation and domain shuffling during evolution. Sequence based comparison and determination of 3D structures of other cellulases have suggested that the mode of action of cellulases on polymeric substrates were dictated by the shape of their active sites. Endoglucanases with open active sites would bind and act in the middle of the glucan chains, while exoglucanases with the tunnel-shaped active site are directed to chain ends for their action.
Fig 1.12a Model of cellulase enzyme (Endoglucanase A (EgL A) of Bacillus pumillus (Q5YLG1_BACPU) (Hitomi et al., 1997). EgIA modular domain organization predicted by pfam. EgIA 3-dimentional structure predicted by Swiss-Model.

A

B
Fig 1.12a Model of cellulase enzyme (Endoglucanase A (EgL) of Bacillus pumilus (Q5YL1_BACPU))

Model of endoglucanase enzyme, produced by Bacillus pumilus, based on Protein Data Bank (PDB) structure (Q5YL1_BACPU). Modular and structural organization predicted for EgIA. (Fig 1.12a A) EgIA modular domain organization predicted by pfam. The numbers indicate the protein residues. The thin bar underneath the numbers represents the deduced amino acid sequence for EgIA. The green and red bars indicate the relative position and the size of the glycosyl hydrolase family 9 (GH9) domains and the carbohydrate-binding module (CBM3), respectively. EgIA 3-di- mensional structure (Fig 1.12a B) predicted by Swiss-Model (Schwede et al., 2003) and visualized in Swiss-Pdb Viewer (Guex and Peitsch, 1997). The GH9 module, shown in green, is rich in α-helix motif and discrete in relation to the red module, which represents the CBM3 domain folded in a β-sandwich fashion. The ▼ represent the pfam predicted active site D101, H419, D457.

EgIA encodes a protein of 659 amino acids (EgL), with a predicted molecular mass of 74.98 kDa and pI 5.5. The N-terminal amino acid sequence shows features of a typical Bacillus signal peptide with a predicted cleavage site between Glu-37 and Gly-38 residues (Nielsen et al., 1997). Highest identity at the protein level was observed with endoglucanases from a strain of B. pumilus (NCBI, gi: 6525242) and Bacillus sp. KSM-522 (Hitomi et al., 1997), with identities of 88.3% and 85.3%, respectively. However, when considering the amino acid sequence of the nineteen most similar bacterial cellulases, high identity was detected for other genera, including Clostridium, Myxobacter, Thermohifida and Paenibacillus.
That endoglucanase A is a modular protein with two discrete domains. The first one, at the N-terminal is the catalytic domain, is composed of the glycosyl hydrolase family 9 (GH9) sequence and covers a total of 334 residues, from amino acid 48-482. The second domain, at the C-terminal is shorter and related to the function of binding to the cellulose surface. It is composed of the type 3 carbohydrate-binding module (CBM3) (Coutinho and Henrissat, 1999). This domain consists of 85 residues starting at amino acid 506 and ending at residue 591. The 3-dimensional structure of EgIA predicted by Swiss-Model (Schwede et al., 2003) showed that the modular feature described by the primary amino acid sequence is also observed at the tertiary level. Two completely distinct structures were detected, with one corresponding to the catalytic domain (folds [α/α]_6) and the other to the CBM3 domain, which folds into a β-sandwich (Fig 1.12a B). This modular organization (GH9/CBM3) is rare among Bacillus endoglucanases, having been reported only for Bacillus pumilus (gi: 6525242) and Bacillus sp. KSM-522 (Hitomi et al., 1997).

1.13 Applications of Cellulase

Owing to its biodegradability and physical strength, cellulose has been used as starting materials in many industries (Teeri, 1997). The usage of cellulose actually depends on the products of its degradation and this is done under mild, easily controlled and specific conditions by the cellulases. Recently, cellulases are used in many industrial applications and the demand for more thermostable, highly
active and specific enzymes is on the increase (Bhat, 2000). Some of the uses of cellulases are discussed below:

1.13.1 Ethanol Production: Ethanol is a very promising replacement for fossil fuels due to its low net emission of carbon dioxide upon combustion. Cellulose is one of the most abundant sources of glucose, which can be converted into ethanol. The attention of the scientific community is aimed at transforming this process into economically feasible operation (Bin Yang et al., 2007). The conversion of cellulose into ethanol involves the production of glucose from cellulose and its fermentation to ethanol by yeast.

1.13.2 Pulp and Paper Industry: Cellulases have been used in many processes in the paper industry, as the raw materials used are lignocellulosics (Tengerdy and Szakacs, 2003) in which cellulose is sequestered within a matrix of other components. Cellulases therefore are used at both the pre-production and post-production stages in pulp and paper making, and these include:

1. Treatment of waste paper: Detachment of ink (bio-deinking) is improved by using cellulases in waste paper treatment and this waste paper can be subsequently biotransformed to fermentable sugars (van Wyk and Mohulatsi, 2003). Glucose released from cellulose is fermented into ethanol, which shows a potential alternative for fossil fuels due to its low net emission of CO$_2$ upon combustion (Levy et al., 2002). The main advantage of enzymatic deinking is the prevention of the use of an alkali; it is therefore environmentally friendly (Bhat, 2000). Microbial enzymes can prove valuable alternative in order to reduce the use of toxic chemicals. Microbial cellulases (Jeffries et al., 1994; Vyas and Lachke, 2003) hemicellulases (Morkbak and Zimmermann, 1998a and
1998b), amylases (Zollner et al., 1998; Elegir et al., 2000), lipases (Morkbak et al., 1999) have shown promising results in increasing brightness of the fibres when used in combination with flotation deinking. Now plant thermostable cellulase is added to the list in the industrial application for deinking. Based on enzyme trials and microscopic observations, Dinus and Welt (Dinus et al., 1998) concluded that the primary role of cellulases in deinking involves separating ink-fiber agglomerates and dislodging or separating ink particles and fibrous materials in response to mechanical action during disintegration.

2. Treatment of recycled pulps: Endoglucanase facilitates pulp drainage by removing amorphous cellulosic materials such as fines and surface elements (peeling off of individual fibrils) (Ramos et al., 1999).

1.13.3 **Feed and Food Industry**: Cellulases hydrolyse plant cell wall, thus finding usage in animal feeds and the food industry. Applications of cellulases in this area are:

1. Increase in juice production by decreasing the viscosity of the raw juice slurry from fruits during the production of fruit drinks (Levy et al., 2002).

2. Cellulases in combination with hemicellulases are added to animal feeds to supplement the animal’s own digestive enzymes, thus increasing the feed digestibility and vitamin assimilation in the animal’s gut as a result of the partial hydrolysis of the lignocellulosic materials (Gilbert and Hazlewood, 1993).

1.13.4 **Textile and Laundry Industry**: Cellulases have been widely used in textile and laundry due to their ability to alter cellulosic fibres in a controlled and mild manner, thus increasing the quality of the fabrics (Bhat, 2000). Cellulases are also
used in biofinishing for the removal of surface hairs from garments for increased comfort and fashion. The textile industry uses cellulases for finishing textiles during manufacture and washing (Teeri, 1997). Of recent, the application of these enzymes in the textile industry represents the major market for cellulase enzymes (Cavaco-Paulo, 1998).

1. Cellulases are used for stone washing by removing excessive dye without damaging the fibre and defuzzing (depiling) fabrics (Levy et al., 2002). The softness caused by cellulase treatment also reduces the need for cationic fabric softeners (Galante et al., 1998).

2. Cellulase preparations are used as additives to laundry detergents to improve the colour brightness, texture and dirt removal from cotton and cotton garments (Bhat, 2000).

1.13.5 Research, Development in Agriculture: Applications of cellulases in these aspects include:

1. Cellulases and related enzymes (hemicellulases and pectinases) are used as biological control of plant pathogens and infections as a result of their ability to degrade the cell wall of plant pathogens, inhibit spore germination, germ tube elongation, and fungal growth (Bhat, 2000).

2. Cellulases and related enzymes can be used in biotechnology for developing new breeds of plant or fungal protoplasts with desired characteristics by solubilising the plant or fungal cell walls which can be merged to form hybrids (Bhat, 2000).

3. Recently methods have been developed for attacking and removing microbial slime in covered surfaces and maintaining a slime-free surface as in exposed
cooling tower surfaces and in waste water treatment and paper making. This method comprises utilizing an enzyme blend in 2 to 100 parts per million (ppm) of cellulase, alpha-amylase and protease. Such enzyme blends have been found specifically to digest microbial slime and reduce microbial attachment and biofilm (Gretty et al., 2007).

The application of specialized thermostable cellulases in various industrial processes such as textile finishing, biodeinking of waste paper and detergent ingredient is the recent trend. For the successful application of cellulases in these processes understanding of structural and functional relationship of individual enzyme with its substrate becomes important.

1.14 Scope of the present investigation

The present study aimed at identification, purification and characterization of thermophilic endo-1, 4-β-D-glucanase (cellulase) from the xerophytic plant species *Opuntia vulgaris* and *Cereus pterogonus* found commonly growing in the coastal areas of Puducherry and compare their physico chemical properties to establish a database for these eukaryotic enzymes. Multiplicity and presence of different isozymes of endoglucanase have been are the major difficulties in purification of these enzymes. Specific objectives of the study included investigations on the Biochemistry, Biophysics, Immunology, Molecular Biology and Bioinformatics of the enzyme protein from these two species. Critically, they are existed as follows –

- Isolate, purify and characterize thermophilic endoglucanase enzyme activity from the xerophytic eukaryotes, *Opuntia vulgaris* and *Cereus pterogonus* plant species.
➢ Biochemical characterization through, pH and temperature studies, normal and denaturation kinetics, Gel filtration, SDS-PAGE, Activity gel assay.

➢ Estimate catalytic rate constant, reaction free energy, activation energy, enthalpy and entropy.

➢ Investigate the purified enzyme for presence of endogenous cations employing Electron Paramagnetic Resonance Spectrometry. Thermal denaturation/aggregation using Spectrofluorimetry, and Tm using Differential Scanning Calorimetry.

➢ Generate polyclonal antibodies to thermophilic endoglucanase isoforms and study cross-reactivity with other thermophilic endoglucanase.

➢ Isolation of genomic DNA, mRNA, synthesis of cDNA, PCR amplification of the endoglucanase gene.

➢ Use of bioinformatics tools for comparative analysis with eukaryotic mesophilic plant endoglucanase enzymes, and for use in primer design.
Cereus pterogonus

Opuntia vulgaris