The plant cell wall is a complex structure which constitutes a formidable barrier to microbial invasion of cells. Majority of plant pathogens have evolved enzymes capable of degrading the various wall constituents. Pathogens capable of elaborating appropriate enzymes alone can be expected to dismantle the various components.

Pectolytic and cellulolytic enzymes are the major chemical weapons of microbial pathogens in colonizing host tissues. Even biotrophs like rusts, of late, have been found to involve these enzymes in making inroads into the host tissues. These enzymes bring about tissue disintegration and maceration leading to cell death. In absence of a direct target on the membranes, these enzymes cause death of cells by indirect and physical action (Hall and Wood, 1973). Leaf-blight, fruit-rot, and wilts are the major groups of plant diseases where these enzymes find their maximum involvement.
Studies on physiology of parasitism in fact started with the study of these enzymes by Anton de Bary, H.M. Ward and William Brown. Brown's serial publications entitled "Physiology of Parasitism" gave enzymology a firm footing in plant pathology. Happily, the lead was pursued with zeal and much informations is now available on the enzymes that are involved in tissue disintegration. In the recent years much new information has been obtained on the nature of pectic substances and cellulose and more importantly about their orientation in the primary cellwall. Albersheim and his associates (Keegstra et al., 1973) gave a model of plant cellwall (Fig. 1) based on their studies with the walls of suspension-cultured Sycamore cells. The model shows specific covalent-connections between the various polysaccharide constituents of the cellwall. Plant cellwall, as envisaged in this model is no more an amorphous mixture of various polysaccharides. This important contribution has made a great impact in understanding the role of cellwall degrading enzymes in plant pathogenesis. This contribution is rated as a "Citation Classic" (Current Contents: 48, Dec. 1982)
Proposed model of primary cell walls (from Keegstra et. al., 1973).

Fig. 1.1

Rhamnogalacturonan
Xyloglucan
4 Linked (arabino) galactan
3,6 Linked (arabino) galactan
THE PLANT CELLWALL:

Understanding the structure of plant cellwall is prerequisite in elucidating the enzymes that attack them. The publications of Talmadge et al. (1973), Bauer et al. (1973) and Keegstra et al. (1973) stimulated more work in this field and it came to light that the model proposed by Keegstra et al. (loc. cit.) represents the cell walls of only dicots. The model represents the plant cellwall as a giant molecule in which the pectic fraction (Rhamnogalacturonan, to be more accurate) and cellulose are united by hemicellulosic fractions. Each cellulose fiber is completely coated with a layer of xyloglucan, one molecule thick. The multiple glucose units of the xyloglucan lie parallel to the axis of the fiber, and they are hydrogen-bonded to the glucan chains in the fiber. Hydrogen bonding appears to be feasible because the glucose portions of xyloglucan are structurally identical with the glucan chains of xylose. Unlike the glucan, however, xyloglucan can form hydrogen bonds on one side only; bonding is impeded on the opposite side by the protruding fucose and galactose units. At the reducing end each xyloglucan is glycosidically bonded to
an arabinogalactan molecule (Fig. 1.2). The exact geometric relations are not yet known, nevertheless, the arabinogalactan chains are thought of as running radially away from the cellulose fiber, in the same way as spokes radiate from a hub of a wheel. The galactose at the reducing end of the arabinogalactan is glycosidically linked to a single rhamnogalacturonan (Fig. 1.3), molecule (unit of pectic polymers).

Thus, each cellulose fiber is coated with several xyloglucan molecules which are bonded to arabinogalactans. Each arabinogalactan in turn binds covalently to a single rhamnogalacturonan chain. Each rhamnogalacturonan chain receives several arabinogalactan molecules radiating from several cellulose fibers. By the same token each cellulose fiber can be connected to several rhamnogalacturonan chains. This extensive cross-linking of the fibers results in a more or less rigid, immobilizable matrix.

The existence of a structural protein in the plant cell wall, which was suggested since long, has been a subject of much controversy. Most experts in the field now agree that a hydroxyproline-rich glycoprotein is
ARABINOGLACTAN is believed to consist of a chain of arabinose (ARA) appended to another chain of galactose (GAL). The relatively few side chains are known to have only a single sugar unit each but their exact location has not been ascertained. Xyloglucan bonds to an interior galactose unit, and the reducing end of the arabino- galactan molecule is attached to rhamnogalacturonan.

RHAMNOSITURONAN is a straight polysaccharide interrupted by kinks. Straight sequence consist of galacturonic acid (GUA); the kinks are produced by rhamnose (RHM). Half of the rhamnose units have an arabinogalactan molecule bonded to them.
present in the primary cell wall as a structural element. The carbohydrate moieties are arabinose and galactose; the arabinose is glycosidically linked to the hydroxyl of the hydroxyproline residues while galactose is believed to be covalently linked to serine residues (Lamport, 1970; Lamport et al., 1973). In their model discussed above, Keegstra et al., (1973) proposed that the glycoprotein is covalently linked through a minor arabinogalactan component to the pectic fraction. However, Albersheim (1975) has preferred to ignore the protein fraction from the model of the cell wall as the structural role of the protein has not been established and that a coherent model could be formulated without it. According to Bateman (1976) "The Albersheim wall model provides a useful context from which to examine our knowledge of the effects of polysaccharide-degrading enzymes on plant tissues and cell walls".

The model discussed above, however, does not hold good for monocotyledons (Albersheim, 1975). The composition of the walls and the pattern of bonding in six grasses (wheat, oats, rice, sugarcane, brome grass and rye grass) are quite unlike those of dicotyledons, even though they
follow the same architectural principles. It has been suggested by these workers that the cellulose fibers are cross-linked by polysaccharides with different compositions but with functions similar to those of xyloglucan, arabinogalactan and rhamnogalacturonan.

**PECTIC SUBSTANCES:**

In spite of forming not more than 1% of the fresh weight, the pectic polymers provide integrity and coherence to the tissues (Doesburg, 1965).

The structural concept of pectic substances as unbranched chains consisting of 1,4 linked 1,4-β-D-galacturonic acid units with carboxyl groups methylated to varying degree is now definitely outdated (Rombouts, 1972, 1981).

Pectic substances are composed primarily of rhamnogalacturonans, galactans and arabinans (Aspinall, 1970a); they are extracted with cold and hot water and solutions of chelating agents. These are the primary constituents of the middle lamella and are structural elements in the primary wall (Mc Clendon, 1964; Talmadge et al., 1973). The major component is the high molecular
weight polymer consisting of a backbone of \( \alpha-1,4 \)
linked D-galacturonic pyranose interspersed by links
produced by 1,2-linked rhamnopyranose. The uronic acid
carboxyls may be methylated and the uronide moieties
may be acetylated at positions 2 and 3. Half of the
rhamnose units have an arabinogalactan molecule bonded
to them, which serve as bridge between the rhamnogalacturonan
and the hemicellulosic wall components (Talmadge et al.,
1973). Arabinogalactan is believed to consists of a chain
of arabinose (ARA) appended to another chain of galactose
(GAL). The relatively few side chains are known to have
only a single sugar unit each but their exact location has
not been ascertained. Xyloglucan binds to an interior
galactose unit, and the reducing end of the arabinogalactan
molecule is attached to rhamnogalacturonan.

At one end of the polymer there is always a single
monosaccharide unit that can unfold into the open-chain
form exposing its reducing aldehyde group. That end of
the chain is called the reducing end. The reducing power,
which is a function of the numerical value of the aldehyde
groups is used as measure of the average degree of
polymerization. The carboxyl groups of \( C_6 \) of the galacturonic
acids may be esterified (methylated) and, depending on the degree of methylation, the pectic substances are designated by different names viz. pectinic acid, pectin and pectic acid. Rhamnogalacturonans with up to 75 percent of the uronic acid carboxyls methyl esterified are known as pectinic acids, those with a higher degree of esterification are referred to as pectins, while the unesterified molecules are known as pectic acids. The measure of esterification can be indicated by their methoxyl content or by the degree of esterification which is the number of carboxyl groups esterified with methanol calculated as a percentage of the total number of the anhydrogalacturonic acid units. This is found to vary in nature from 0-85 per cent. The molecular weight of pectic substances varies from about 10,000 to 400,000 depending on the source.

HEMICELLULOSES:

The hemicelluloses are composed mainly of neutral hexose and pentose sugars, (Aspinall, 1970b; Bauer et al., 1973; Northcote, 1972), among which two are more widespread; xyl glucan (β-1,4 linked glucopyranose chain with terminal branches of 1,6 linked D-xylopyranose), and xylans
(β-1,4 linked xylopyranose), which commonly have side branches of 1,3 linked arabinofuranose and αC-1,2 linked glucuronopyranose (or its 4-methyl ester). Other hemi-celluloses are glucomannan, galactoglucomannan, mannan, and galactomannan.

**CELLULOSE:**

Cellulose, the most abundant organic substance on earth, is a glucose polymer (poly alcohol) made up of β-1,4 linked anhydro D-glucopyranose units. The D.P. (degree of polymerization) value varies between 15 and 14,000. Native cellulose has a D.P. value of about 10,000 (Sihtola and Neimo, 1975) which corresponds to a molecular weight of 1.5 million. The total length of a cellulose molecule is above 5 μm calculated on the basis of the length of 1 anhydroglucose unit as 0.515 nm (Goksøyr and Eriksen, 1981). The individual chains are ribbon-like flat molecules stabilized by hydrogen bonds between adjacent glucose residues. These chains are associated to form fibrils by inter-chain hydrogen bonds between hydroxyl groups at carbon 6 and the glycosidic oxygens of adjacent chains (Northcote, 1972). These fibrils show crystalline and amorphous areas due to more
compact orientation of molecules in the former and comparatively loose arrangement in the latter region (Fig. 1.4). The crystalline areas do not yield to enzymatic degradation easily. There are two opinions regarding the arrangement of crystalline and amorphous areas. One view holds that the fibrils have a crystalline core surrounded by amorphous cellulose, while the other view holds that the amorphous regions are random in their distribution. There is difference of opinion regarding the diameter of the fibrils also. The crystalline areas also called micelles are opaque to ordinary analysis. X-ray crystallography, including the X-ray diffraction patterns are employed in the investigations related to its structure. The crystalline regions merge with the amorphous areas through mesomorphous or paracrystalline regions, where the arrangement of cellulose chains shows imperfect or loose interlocking. A single chain passes through several crystalline and amorphous regions in the fibrils. In the primary cell wall of plants, the cellulose fibrils have little or no crystalline regions but the secondary walls are completely crystalline.
GLYCOPROTEINS:

The structural glyco-protein in the cellwall consists of arabinose and galactose as carbohydrate moieties. Tetraarabinosides, which are linked glycosidically to the hydroxyl group of the hydroxyproline residues, serve to stabilize the polypeptide backbone. An arabinogalactan (3,6 linked) joined with-OH of serine in the protein, connects the protein with the pectic fraction.

PECTOLYTIC ENZYMES:

Plant pathogenic microorganisms have evolved a variety of enzymes that can be divided into two categories: pectinmethylesterase and chain-splitting enzymes.

Pectinmethylesterase (FME)

Pectin methyl esterases, FME (syn. pectin esterase PE, pectase), are saponifying enzymes i.e. split the methyl ester group of pectin or pectinic acids hydrolytically to produce rhamnogalacturonide of lesser methoxyl content and methyl alcohol. The de-esterification is never complete and about 10 per cent of the methyl esters are always left unhydrolysed. Thus, true pectic acid is not formed from pectin or pectinic acids. This is said to be due to
interruptions of the chains by the neutral sugars which serve as 'kinks'. The amount of enzyme that is free to react with its substrate appears to be regulated by the ionic strength of the environment and pH. The proposed site of binding of the enzyme to the cell wall is the free uronic carboxyl groups of the pectic fraction (Jansen et al., 1960). According to the rules of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry, the enzyme is named and numbered as pectin-pectyl hydrolase (3.1.1.11). Since these enzymes are specific for methyl esters of pectic materials, with no or feeble activity on ethyl ester (6-16% of the methyl ester), the name PME (pectin methyl-esterase) is preferred over PE (pectin esterase). The methyl esters of mono-, di-, or trigalacturonic acids are not saponified by PE. According to Schultz et al., (1945) PME starts the saponifying activity on methyl ester next to a free carboxyl group and then continues to act along the molecule in either direction. Free carboxyl groups are necessary for the formation of complex between enzyme and substrate (Solms and Deuel, 1955). Lee and Mac Millan (1970) and Miller and Mac Millan (1971) reported that with highly methylated pectin about half of the PME activity
always initiated at the reducing end. The ME of *Clostridium multifermentans* (which is complexed with exopectate lyase) invariably attacks the substrate at the reducing end only (Sheiman et al., 1976). ME activity is inhibited by the acid groups formed. Although action of these enzymes has nothing to do with chain splitting; nevertheless, they modify the substrate which accelerates or inhibits the activity of the chain-splitting enzymes. The production of ME augments action of depolymerizing pectolytic enzymes preferably active at low methoxyl pectinic acids or pectic acids. But the activity of enzymes reactive with pectin is inhibited.

MEs of plant origin lose their activity upon dialysis which, however, can be restored by addition of salts to the dialysed solution. In contrast, the MEs of fungi are not readily adsorbed by plant cell walls and they do not respond to dialysis or salt activation (Mc Colloch and Kertesz, 1947; Bateman, 1963). Mixtures of MEs formed by the host and pathogen are freely encountered in vivo. The optimum pH values of MEs from different sources also vary. The fungal MEs have pH optima between 4-5 i.e. in acid range whereas, those of bacterial origin have it in the alkaline range 7.5-8.0. Plant MEs (from tomato and orange) have
been found to be most active at 7.5 pH. Mayorga and Rolz (1971) gave mathematical equations that predict the activity of plant PME as a function of cation (Ca^{2+}, Na^+), enzyme concentration and pH. They also demonstrated kinetic differences between the two PMEs. End-product inhibition of plant PMEs by pectate oligogalacturonides, with a degree of polymerization of 8 is reported (Termote et al., 1977). Without holding time a temperature of 96°C denatures the enzyme (Pilnik, 1958).

**Chain-splitting enzymes**

The more important pectolytic enzymes in plant pathogenesis are those that attack the integrity of the pectic chain by snapping off the C–1,4 bonds linking the galacturonosyl moieties. These enzymes do it by two mechanisms: hydrolytic or trans-eliminative attack (β-elimination). The former enzymes are referred to as polygalacturonases and the latter as trans-eliminases or preferably, as lyases. The trans-elimination reaction in pectic degradation was reported by Albersheim et al., (1969). It involves shifting of the hydrogen from C_5 of one unit to C_4 of the adjacent unit. It results in cleavage of the glycosidic bond and formation of a double bond between C_4 and C_5 at the non-
reducing end of the first uronide moiety. A similar trans-eliminative reaction occurs when pectinic acid is heated in neutral or alkaline environment. The unsaturated products of lyase action absorb maximally at 235 nm and react with thiobarbituric acid (TBA) to form a chromogen which displays an absorption maximum in the visible range (510 nm). The unsaturated monogalacturonic acid, however, goes undetected in the UV absorption as it readily converts into 4-deoxy-CC-L-threo-5 hexoseulose uronic acid which does not absorb at the above wavelength. The TBA reaction however, detects this compound also and, therefore, TBA test is more useful and also more reliable. The TBA test has the additional advantage that it reacts also with galacturonic acid, formed by hydrolytic cleavage to form a chromogen which gives peak of absorption at 510 nm. The two modes of cleavages (hydrolytic and lytic) of the pectic substrate can be detected simultaneously as the two reaction products do not interfere in the assay.

The chain-splitting pectolytic enzymes have been grouped according to the following criteria (Bateman and Miller, 1966; Rombouts and Pilnic, 1972, 1981; Bateman,
1976):

i) mechanism by which the CC-1,4 glycosidic bond is split (i.e. hydrolytic or lytic cleavage). In trans-eliminases the split is between C₄ and the bridge oxygen whereas in hydrolytic enzymes it is between C₄ and oxygen.

ii) enzyme specificity for substrate (i.e. pectin or pectic acid), and

iii) position in the pectic chain at which the cleavage occurs (i.e. random or terminal).

Hydrolases that show distinct specificity for pectin over pectic acid as substrate, are referred to as pectin methylgalacturonase (PMG), whereas those that prefer pectic acid over pectin are called polygalacturonase (PG).

Depending on the terminal or random cleavage, these enzymes may be further differentiated as exo- or endo- enzymes. The PMG may be exo-PMG or endo-PMG, whereas the polygalacturonase may be an exo-, or endo-PG. Polygalacturonases of bacteria show alkaline pH optima (ranging from 7-8) as compared to fungi and higher plants in which these values always lie in the acidic range (2.4-5.5 for PGs and 5.5-6.0 for PMGs).
Likewise, the lyase enzymes are also designated as PAL (pectic acid lyase, pectate lyase or polygalacturonate lyase) or PL (pectin lyase) which, in turn, may be exo-, or endo-PAL or PL. Unlike the exo-hydrolases, which release monomeric reaction products, exo-lyases normally release unsaturated dimers (Okamoto et al., 1964). In recent years the lyase enzymes have been reported for a broad range of phytopathogens.

Exoenzymes are claimed to be more active on oligomers (Voragen and Pilnik, 1970). Also, the rate of depolymerization by the endo-enzymes decreases with the progress of enzymatic reaction.

The activity of polygalacturonases is often reduced by Ca\(^{2+}\) (Bateman, 1964; Rombouts and Pilnik, 1972). Calcium is supposed to form insoluble calcium pectate which is resistant to PG action. On the other hand, lyases are potentiated by Ca\(^{2+}\); their activity is lost in presence of chelating agents which make Ca\(^{2+}\) unavailable to the enzymes (Bateman, 1966; Rombouts and Pilnik, 1972). Another important difference lies in the pH optima of these enzymes. For pectic hydrolases it is in the acidic range whereas for lyases (excepting some PLs) the optimum pH invariably
lies in the alkaline range, sometimes as high as 9 or 10 which makes it difficult to visualize its action in vivo (Hall and Wood, 1973). The technical difficulties in determining pH conditions at the site of enzyme action in vivo prevent information about the role of these enzymes in tissue maceration. Nevertheless, the alkaline pH optima is a characteristic of lyases (Albersheim et al., 1960; Bateman, 1966; Hall and Wood, 1970; Rombouts and Pilnik, 1972). Since the bacterial pectic enzymes have alkaline pH optima and are stimulated by Ca$^{2+}$, it is possible that the earlier reports of bacterial PCs and PMGs were in fact lyases (MacMillan and Vaughn, 1964; Voragen and Pilnik, 1970 and Rombouts and Pilnik, 1972).

The various chain-splitting, depolymerizing pectolytic enzymes are given in Table 1.1 with their numbers based on enzyme nomenclature (1973). As is evident from the Table 1.1 the enzymes endo-FMG, exo-FMG and exo-PL are not recognized. Seegmiller and Jansen, (1952); Deuel and Stutdz, (1958), have however, reported endo-FMG activity.

It is evident from above that plant pathogens have evolved a variety of enzymes to cleave a single type of glycosidic bond. Some attack only at the ends while others
Table 1.1: Characteristics of depolymerizing pectic enzymes (Recommendations of International Union of Pure and Applied Chemistry and International Union of Biochemistry, 1972).

<table>
<thead>
<tr>
<th>Pectic enzymes mainly acting on pectic acid</th>
<th>Pectic enzymes mainly acting on pectin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polygalacturonase</td>
<td>Pectate lyase</td>
</tr>
<tr>
<td>FG (3.2.1.15)</td>
<td>PAL (4.2.2.2)</td>
</tr>
<tr>
<td>exo-FG (3.2.1.67)</td>
<td>exo-PAL (4.2.2.9)</td>
</tr>
</tbody>
</table>

* never reported
** after the discovery of PL, endo-FMG has not been reported
(PG = polygalacturonase; PAL = pectic acid lyase; PMG = polymethylgalacturonase; PL = pectin lyase).
cleave at random, internally. There are enzymes which attack only methyl esterified galacturonosyl linkages, and enzymes attacking galacturonosyl residues with free carboxyl residues. The bonds are broken hydrolytically by some and trans-eliminatively by others. The isozymes further expand organisms' versatility towards pectic degradation. The one obvious advantage is that the production of each enzyme can be controlled individually. The pathogen can withstand changing environments with respect to its pectolytic activity. If one or some particular type of enzymes are repressed under one set of conditions, other ones may be pressed into operation. Stimuli disfavouring lyase production or action would be unable to stop the pathogen from employing the hydrolases, and vice-versa. Thus, the pathogens have equipped themselves to withstand diverse ecological conditions. The capability of plants' saccharides to thwart the production of these enzymes plays a key role in host resistance.

Pectolytic enzymes and Pathogenesis

Pectolytic enzymes play key role in soft-rot diseases. That they are just as important in other diseases needs more experimental data. The soft-rot pathogens bring about
the characteristic symptom by tissue maceration which is more neatly described as "cell-separation". Though the enzymic basis of this was clearly established in early studies of de Bary, (1886); Jones, (1909); Brown, (1915), who attributed it to the digestion of the 'intercellular cement' it was only in 1960's that the precise mechanism was deciphered. With the better knowledge of the chemical makeup of the middle lamella (Joslyn, 1962) and use of purified enzymes for experimental work, it is now known that the endopectolytic enzymes alone can bring about cell-separation. No other enzymes can do it (Zaitlin and Coltrin, 1964; Dean and Wood, 1967; Bateman, 1968; Bush and Codner, 1966; Byrde and Fielding, 1968; Hall and Wood, 1970; Mount et al., 1970; Garibaldi and Bateman, 1971; Bateman, 1972; Bateman, 1976). Reports that Cc-L arabinofuranosidase (arabanase) macerates plant tissues has been refuted (Bush and Codner, 1968; Byrde and Fielding, 1968; Cole and Bateman, 1969; Cole and Wood, 1970). However, recently Cole and Strudy (1973) have claimed that endo-C-1,4 galactanases from Fusarium caeruleum and Phytophthora erythroseptica are capable of causing tissue maceration and host cell death similar to endo-pectolytic enzymes. This is yet to be confirmed. Tissue maceration continues to be a rich hunting ground for plant pathologists working
with pectolytic enzymes. 'Phytolysin', a factor distinct from pectolytic enzymes has been attributed for the macerating action of Diplodia ribesia (Kern and Neef-Roth, 1971). But more has to be done with phytolysin to accept it as equal to endo-pectolytic enzymes, which till date are the only enzymes that can cause tissue maceration. It must be mentioned here that all endo-pectolytic enzymes are not capable of causing maceration. A polygalacturonase from Clostridium felsineum (Kaji, 1958) and endo-polygalacturonase from Sclerotinia fructigena (Byrde and Fielding, 1962) failed to show macerating activity. The PAL-complex produced by Erwinia chrysanthemi has 4 isozymes of which one (with a PI of 4.6) failed to cause maceration (Garibaldi and Bateman, 1971).

All pectolytic enzymes that cause tissue maceration simultaneously cause cell death (Basham and Bateman, 1975 a,b). The role of pectolytic enzymes does not end with tissue maceration. The association of these enzymes with killing of protoplasts has added a novel dimension to the study of these enzymes, which is receiving attention of leading workers in this field at present (Wood, 1973). Injury of tissues treated with purified pectolytic enzymes causes irreversible increase in permeability of affected
cells as evidenced by leakage of ions. This amounts to damage to the plasma membrane. How these enzymes can do it in the absence of any substrate target in the membranes has necessitated the search of its modus operandi in some feature other than the catalytic activity of these enzymes. This killing property does not reside in such specific properties as the net ionic charge, pH, pH optima and mode of substrate cleavage. Nonetheless, the enzymes that have lost the catalytic property also fail to cause membrane damage or cell death (Fushtey, 1957; Basham and Bateman, 1975). One hypothesis suggests that soluble reaction products from tissue macerated by the enzymes are responsible for cellular injury, but no such product is detectable in the maceration of susceptible tissue which can alter membrane permeability (Basham and Bateman loc cit). Hydrogen peroxide, which is known to increase membrane permeability is a byproduct of pectolytic enzyme activity in cauliflower floret tissue (Lund, 1973) and cotton leaf (Russell, 1973), but the role of H$_2$O$_2$ in tissue maceration has been excluded by the studies of Basham and Bateman (1975 a, b). This problem is difficult to resolve but with the present tempo of research in this area, it may soon be overcome.
HEMICELLULASES:

The degradation of hemicelluloses, known for a relatively insignificant number of plant pathogens, is a hitherto neglected field of research in this area. Substantial hemicellulolytic activity has been reported for the pathogens examine viz., *Sclerotium rolfsii* (Van Etten and Bateman, 1969); *Rhizoctonia solani* (Bateman et al., 1969); *Sclerotinia sclerotiorum* (Hancock, 1967); *Fusarium roseum* 'Avenaceum' (Mullen, 1974) and *Diplodia viticola* (Strobel, 1963).

CELLULASES:

The microorganisms that can bring about cellulose degradation are many and these can be placed in two categories. Those that can degrade native cellulose like the raw cotton, are termed cellulolytic. These are believed to act enzymatically on the crystalline areas. On the other hand, there are organisms which fail to degrade native cellulose but can hydrolyse soluble, substituted forms of cellulose, like carboxymethyl cellulose (CMC). Two groups of enzymes called the $C_\upalpha$ and $C_\beta$ are involved in cellulose break-down. This is the $C_\upalpha - C_\beta$ concept given by Reese (1956). The $C_\upalpha$ enzyme was postulated to
act on native cellulose by destroying its crystalline structure and exposing the glucan chains to $\beta-1,4$ endo-glucanases, a member of $C_x$ group of enzymes. These split the glucosidic bonds hydrolytically, and degrade the glucan chains to cellobiose. Conversion of cellobiose to glucose also requires a cellobiase or $\beta$-glucosidase. Thus, the cellulase system is made up to $C_1$, $C_x$ ($\beta-1,4$ glucanase) and $\beta$-glucosidase. This is also called the multienzyme hypothesis (Reese, 1965; Gould, 1969).

Several workers have found an exo-enzyme in the $C_1$ fractions (from DEAE-Sephadex) which removes cellobiose units from the chain end to which is given the trivial name cellobiohydrolase (CBH). Most workers of cellulolytic enzymes believe that this is $C_1$. Reese (1975), however, doesn't agree to this. Since there are few ends in crystalline cotton, this interpretation has necessitated a change in our thinking concerning the sequence with which the two types of enzymes act. One of the endo-$\beta-1,4$ glucanase components is considered to act first, to form the free ends required for CBH activity. This is just the reverse of the original hypothesis. These developments led Reese (1975) to modify the $C_1-C_x$ concept as given below.
Originally (Reese, 1956), only a disruption of the hydrogen bonds was claimed for C₁, but now in the revised scheme (Reese, 1975), it is believed to split a covalent linkage accompanied by splitting of hydrogen bonds, as happens in the case of collagenase, the enzyme that splits collagen. Collagen is a triple-stranded helix, a structure of degree of order in which hydrogen plays an important role. Most proteases fail to attack collagen, except a specific protease, collagenase. It acts at a specific site yielding two fragments which spontaneously denature. The denatured products are easily degraded by many proteases, but not by collagenase. Thus, the requirement of collagenase is for a highly ordered, constrained substrate.

Thus, C₁ in the revised C₁-Cₓ concept becomes a member of Cₓ group, the endo-β-glucanase (Fig. 1.5). But it is a very special member characterized by properties not possessed by other endoglucanases. These properties are: (i) activity on crystalline cellulose, (ii) disruption of H-bonds, (iii) lack of action on CMC, and (iv) inability to act on products of its own action (since it produces no soluble products from crystalline cellulose). Reese
Fig. 1.4
Alignment and composition of elementary fibril of cellulose.
A: Bundle of parallel fibrils (held together crosswise by hydrogen bonds).
B: Lateral section view of one fibril. From Sihtola and Neimo (1975).

Fig. 1.5
Model of a crystalline being opened up by C1. Shaded areas crystalline. R, reducing end; NR, non-reducing end of liberated chain.
(1975) prefers to maintain the special C₁ designation for this component, as the other random acting components lack the special properties required for initiation of the reaction, but are, nevertheless, capable of causing hydrolytic breakdown of products of C₁ action. They act on the partially-liberated chains.

The systemic name of the C₂ group of enzymes is 1,4 (1,3;1,4)-β-D-glucan-4-glucanohydrolase (EC 3.2.1.4) which can hydrolyse 1,4 linkages in β-glucans that also contain β-1,3 linkages. The exo-enzymes, glucohydrolase (usually called as endo glucanase) and cellobiohydrolase (EC 3.2.1.91, abbrivated as exogluconase) are limited in their action to the non-reducing ends of the cellulose molecules. Endoglucanases are required for the complete removal of the liberated chains, a removal which is necessary if C₁ is to act on the underlying material. The concept of successive actions by various cellulase components is given below:

Cotton

\[ \xrightarrow{C_1} \]

Amorphous cellulose

\[ \xrightarrow{β-1,4-glucan} \text{glucanohydrolase} \rightarrow \text{oligomers} \]

\[ \xrightarrow{β-1,4-glucan} \text{glucohydrolase} \rightarrow \text{glucose} \]

\[ \xrightarrow{β-1,4-glucan} \text{cellobiohydrolase} \rightarrow \text{cellobiose} \]
Thus, the crystallinity of the cellulose fibrils has to be under some sort of constraint if enzyme action is to occur and this is the limiting factor in the degradation of crystalline polymers. It is most pertinent to quote Whittaker (1963) that "The physical structure of cellulose has discouraged many microorganisms from careers in cellulose chemistry".

Halliwell (1966) working with *Trichoderma koningii* observed a new enzyme which he termed C₂. It fragments native cellulose fibers into "short fibers". Thus, it resembles C₁. Nevertheless, it differs from C₁ in acting synergistically with Cₓ component to promote production of "short fibers". Thus, the cellulase complex of *T. koningii* has four pure components: (a) cellobiase, (b) a CM-cellulase (Cₓ), (c) a "short fiber"-forming component designated as C₂ and (d) a C₁ component. According to model (Fig. 1.6) given by Montecourutt *et al*., (1979), based on the observations of several independent workers (Emert *et al*., 1974; Eriksson and Pettersson, 1975; Halliwell and Griffin, 1973; Nisizawa *et al*., 1972; Wood and Mc Care, 1975) the endo-glucanase (EC 3.2.1.4, carboxymethyl cellulase, Cₓ) initially attacks cellulose randomly and this is followed by exo-glucanase
Fig. 1.6

Schematic representation of sequential stages in cellulolysis (After Montenecourt et. al., 1979).
(EC 3.2.1.91, C1) which cuts cellobiose units terminally. β-glucosidase (EC 3.2.1.21), which may not be specific for cellobiose, releases the glucose units. In this sequential co-operative action by three different enzymes, the efficient hydrolysis of cellulose is brought about.

REGULATION OF PRODUCTION AND ACTIVITY OF ENZYMES:

Several reports suggest that the cellwall polysaccharide-degrading enzymes of pathogenic microorganisms are subject to catabolite repression (Horton and Keen, 1966 a,b; Keen and Horton, 1966; Patil and Dimond, 1968; Moran and Starr, 1969; Mussell and Green, 1968; Biehn and Dimond, 1971 a,b; Mullen and Batesman, 1971; Zueker and Hankin, 1971; Spalding et al., 1973; Weinhold and Bowman, 1974). Catabolite repression of pectolytic and cellulolytic enzyme synthesis by sugars is a common phenomenon and there is irrefutable evidence that this factor is important in pathogenesis. Horsfall and Dimond (1957) even classified the wilt diseases into "low-sugar diseases" and "high-sugar diseases" on the basis of differential effects of high and low sugar levels on disease predisposition. Glucose, when introduced into
vascular bundles of *Fusarium*-infected tomato plants, reduced the wilt symptom by repressing enzyme production (Patil and Dimond, 1968). Less amount of polygalacturonase was recorded in the glucose-injected diseased tissues.

There are relatively few detailed studies on factors controlling the synthesis of these enzymes. The increased production of the enzymes by a pathogen in the presence of their substrates has usually been termed induction and such enzymes are often referred to as inducible. How does the organism recognize the substrate so as to produce the proper enzyme? Probably all inducible solubilizing enzymes are always produced but in minute quantities, also in the absence of the inducer because the repressor system will not function as an absolute block. Those which are secreted and find no substrate send back no message while those which do find a substrate produce soluble products that enter the cell notifying it to turn the proper synthetic machinery for more production. There are usually dimers or trimers produced by the enzyme action.

The best candidate for inducer of this kind is cellobiose but Mandels et al. (1975) doubt whether
cellobiose is true inducer as believed earlier (Mandels and Reese, 1960). However, sophorose, also a dimer, frequently accompanying glucose a contaminant, is a specific inducer of cellulase, especially for *Trichoderma viride* (Mandels et al., 1962; Nisizawa et al., 1971 a,b). Sophorose does not stimulate α-amylase production. This strongly indicates that sophorose is a specific inducer of cellulase system.

In a favourable environment, the organism consumes the sugar products as rapidly as they are produced without letting them to accumulate. The organism then runs into the problem of catabolite repression of enzyme synthesis. This was demonstrated nicely in the experiments of Horton and Keen (1966 a,b) and Keen and Horton (1966), working with *Pyrenochaeta terrestris* with respect to polygalacturonase and cellulase synthesis. They observed that cellulase synthesis in this fungus is controlled by a combination of induction and repression, a mechanism which provides a unique sensitivity to environmental changes. The cellulase synthesis in this fungus is induced by cellulose, but there is an optimal concentration above which cellulase synthesis is repressed. When the cellulase concentration exceeds this optimal level, the
rate of glucose release from cellulose degradation exceeds the rate of glucose utilization by the fungus. This results in building up of glucose concentration and when it exceeds $5 \times 10^{-4} M$ value, the cellulase synthesis gets into catabolite repression. Studies on replacement media showed that cellulase synthesis was repressed to the basal level by glucose concentrations of $5 \times 10^{-4} M$ or above. Krebs' cycle intermediates had the same effect. By dilution of the medium, reformation of a maximum cellulase level followed identical course as obtained before sugar repression had occurred. This had been taken as evidence for the regulation of the cellulase synthesis by cellulolytic products through a repressor-inducer mechanism. Thus, for phytopathogenic microorganisms in general, it may be said that high sugar level in the plants delays development of disease through repression of cellulase synthesis whereas a low sugar level would promote pathogenic invasion, as a result of decreased repression of enzyme synthesis. Since the repression to basal level occurs at such a low concentration as $5 \times 10^{-4} M$, in the case of P. terrestris, cellulolytic enzymes are considered unimportant in pathogenesis. Sugar concentration below this low level
will exist only in few localized areas in the plants.

Endo-polygalacturonase synthesis was found to be induced by galacturonic acid, pectin, polygalacturonic acid, mucic acid, tartonic acid and dulcitol. In pectin containing medium P. terrestris shows accelerated endo-polygalacturonase synthesis by hexose supplements up to as high a concentration as 0.005 M or above (Horton and Keen, 1966 a,b). The rate of endo-polygalacturonase synthesis is diminished only by hexose concentrations of 0.05 M or above. Thus, this enzyme may be of greater importance in pathogenic invasion of plants, as repression occurs at 100 times higher sugar concentration. It is for this reason that cellulolytic enzymes have been found to be less important than pectolytic ones in pathogenesis. The obligate parasites seem to fall among "high-sugar organisms" which keep these tissue-degrading enzymes in check.

Similarly, peptic acid lyase (=Polygalacturonate trans-eliminase) is not inducible in Aeromonas liquefaciens (Hsu and Vaughn, 1969) and certain species of Erwinia (Moran and Starr, 1969). It is constitutive, subject to severe catabolite repression. This is proved by the fact
that slow feeding of *A. liquefaciens* with glucose, glycerol, or polygalacturonic acid when incubated in a carbon-deficient medium permitted up to 500 times more enzyme synthesis than when the bacterium was incubated in a carbon-sufficient medium. It seems, then, probable that in many cases where enzyme induction has been claimed, the actual mechanism involved may be one of derepression or relief of catabolite repression. Repression may not be specific and sugars released from different polymers by various enzymes may function to repress the synthesis of other polysaccharide-degrading enzymes. The extent of the catabolite repression may depend upon both the specific enzymes and the particular catabolite (Cooper and Wood, 1975).

In some cases, on the other hand, these enzymes are of course inducible. *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lucopersici* were cultured by Cooper and Wood (1975) in a carbon-deficient medium and various monomeric sugars or dimers of cell wall polymers were fed at a rate which prevented accumulation in the fungal cells. Specific sugars served to induce synthesis of specific enzymes. D-xylose induced synthesis of xylanase; D-galacturonic acid induced synthesis of endo-polygalacturonase and endo-pectin lyase; cellulbiose induced synthesis
of cellulase etc. In V. albo-strum, these very sugars which serve as specific inducers, also act as non-specific catabolite repressors when critical sugar levels were exceeded.

Then, there are also pathogens which produce the cellwall degrading enzymes constitutively, which are not subject to catabolite repression. The examples include polygalacturonases produced by V. albo-strum (Mussell and Strouse, 1972) and Anamnomyces euteiches (Ayers et al., 1969); the polygalacturonase and xylanase of Helminthosporium maydis (Bateman et al., 1973) and the cellulase produced by Pseudomonas solanacearum (Kelman and Cowling, 1965).

More importantly, many pathogens appear to have different systems for controlling degradative-enzyme synthesis. β-glucosidase appears to be produced constitutively in the C-strain of Colletotrichum lindemuthianum, while β-xylosidase is induced when either the C or λ strain of the fungus is grown in presence of xylose as the sole carbon source.

Fusarium oxysporum f.sp. lycopersici secretes both PME and polygalacturonase into the culture medium when
pectin serves as the carbon source, but it produces only EME when the pectin is replaced by glucose (Waggoner and Dimond, 1955). Data of this type suggest operation of any of the following three possible mechanisms: (i) both enzymes are produced without specific induction and polygalacturonase synthesis is repressed by glucose, (ii) EME is secreted without induction and PG is induced by pectin but not by glucose; (iii) synthesis of both enzymes is induced by pectin but only EME synthesis is induced by glucose. This amply proves that each enzyme production is controlled in a different way. Similar differential control of synthesis of endo- and exo-polygalacturonases has been reported for three species of Botrytis (Hancock et al., 1964). Later, these authors (Hancock and Miller, 1965) established differential control of PAL and exo-PG synthesis in Colletotrichum trifolii. Bateman (1966) reported differential control mechanisms of pectolytic enzyme-production in Fusarium solani f. sp. phascolii.

Studies using isolated plant cell walls as carbon sources have indicated that the cell wall-degrading enzymes are produced in a definite temporal sequence. F. oxysporum f. sp. lycopersici grown on isolated tomato
cells (Jones et al., 1972); *Colletotrichum lindemuthianum*, grown on bean walls (English et al., 1971); *Fusarium roseum* "avenaceum", grown on potato walls (Mullen and Bateman, 1975a), and *V. albo-atrum*, grown on tomato walls (Cooper and Wood, 1975), all produce enzymes that degrade the pectic polymers first, followed by those which hydrolyse the hemicelluloses and finally cellulase, which appears to be last in the sequence. This shows the relative accessibility of the various components of the cellwalls to enzyme action.

Our knowledge of the factors that regulate polysaccharidase production is limited to facultative pathogens. Histological evidences suggest involvement of enzyme action during penetration of host cellwalls by biotrophic parasites (Braker and Littlefield, 1973; Mc Keen and Rimmer, 1973; Sargent, 1973; Aist, 1976). If cellwall-degrading enzymes are involved in the infection processes of obligate parasites then it must be under high regulation to avoid host cell death (Basham and Bateman, 1975a,b; Stephens and Wood, 1975). To quote Bateman (1976) "Further elucidation of the systems regulating production and excretion of cell wall-degrading enzymes by plant pathogens
should add considerably to our understanding of host parasite relationships.

**SYNTHESIS AND RELEASE OF EXTRA-CELLULAR ENZYMES:**

In the earlier days of research with pectolytic enzymes, the enzyme samples were obtained by extracting ground-up mycelium of young cultures (Brown, 1915). The major portion of our knowledge, however, is derived from extra-cellular enzymes that are secreted into the culture medium. Since *in vivo* the degradation of tissues is affected by extra-cellular enzymes, intracellular enzymes are important only in so far as the later become extra-cellular.

An organism having the genetic capability when suitably induced, turns the synthesizing machinery 'on' and packages of the enzymes in minute vesicles are transported to the cytoplasmic membrane (Lampen et al., 1972; Chapman and Loewenberg, 1976). The vesicles fuse with the plasma membrane and liberate the enzymes outside the protoplast. If the cellwall is freely penetrable to large molecules, this is equivalent to liberating enzymes into surrounding environment. If it is not, it is cell-bound. Certain organisms tend to retain an appreciable
part of the enzyme between the protoplast and the cell wall. Before their release into the medium, the extracellular enzymes are bound to the surface layers. The adsorption is affected to a great extent by pH, and ionic concentration, both of which are highly variable factors during growth of the pathogen in host tissue.

According to Berg and Peterson (1977) cellulase is first bound to the cell wall and is released in an autocatalytic process. The binding of cellulase can have a rate-limiting effect on cellulase production. This has been demonstrated by addition of Tween-80 to the medium which by enhancing release of wall-bound cellulase augments cellulase synthesis (Mandels et al., 1975; Ghose et al., 1975). It is generally agreed that the most active sites of enzyme production are the vigorously growing cells of plants or the hyphal tips of fungi.

THE ORGANISMS:

Two organisms, one fungus (R. nigricans) and one bacterium (X. campestris pv. vesicatoria), were used in the study, with a view to elucidate their enzyme production and also to note the differences in bacterial and fungal pectolytic and cellulolytic enzymes.
Rhizopus-rot is a serious problem in storage and marketing of such commodities as strawberries, peaches and sweet potatoes, besides papaya. It is a cosmopolitan fungus which has assumed great economic importance because of the damage it causes to several harvested crops (Cappellini, 1966). Harter and Weimer (1923) first showed that \textit{R. stolonifer} (= \textit{R. nigricans}) produced a tissue macerating enzyme (pectinase). Information on the pectolytic and cellulolytic enzymes of this fungus is very little. Phaff (1959) reported polygalacturonase production by this organism. Srivastava \textit{et al.}, (1959) reported polygalacturonase and pectinmethylesterase production. Sommer \textit{et al.}, (1963) also reported polygalacturonase production by this organism. Unless overlooked, there are no other reports on the pectolytic enzymes of this important fungus.

\textit{Rhizopus nigricans} Ehrenberg causes soft-rot (watery-rot) of papaya fruits. Irregular water-soaked lesions develop which gradually enlarge and subsequently get covered by sporangiophores that are white when young but later turn-brown. The fruit tissues collapse in 4-5 days with lot of exudation of watery sap. Inspite of its ubiquitous nature and involvement with several soft rots,
very little is known about the enzymes it involves in pathogenesis.

Not much is known about the enzymology of pectin degradation by Xanthomonas, when compared to the knowledge available for other groups of microbes (Starr and Nasuno, 1967). This is possibly because the symptoms caused by Xanthomonas are unlike those induced by soft-rot bacterial species belonging to genera Erwinia, Bacillus and Pseudomonas. This gives the impression that Xanthomonas lacks pectolytic activity. Nevertheless, several reports contradict this false impression.

Burkholder and Starr (1948) first reported that 38 out of the 77 xanthomonad cultures (14 out of 25 Xanthomonas species) liquified pectate gel, though compared to soft-rot erwinias, the action was slow and less vigorous. Similar observations were made by some other workers (Sabet and Dowson, 1951; Smith, 1958 a, b; Dye, 1960; Porwal and Chakravarti, 1972; Caribaldi, 1972 and Knosel and Lange, 1972).

Starr and Nasuno's (1967) is the major work with pectolytic enzymes of xanthomonads in which they examined 29 nomen species of Xanthomonas. Ten out of nineteen species
liquified a nutrient pectate gel. None of the 19 nomen-species produced PG or PL. Seven of the ten pectate-liquifying nomen species produced PAL in presence of pectin in the medium. \textit{X. campestris} cultures produced PAL and PME inducibly in presence of pectin. Some strains of this pathogen formed PAL constitutively on glucose, but PME was never constitutive. The PAL degraded NaPP randomly releasing di- and tri-galacturonic acids as the major end products accompanied with some saturated mono-, di- and tri-galacturonic acids. Three xanthomonads which liquified the nutrient pectate gel did not show any pectolytic enzyme activity in their culture filtrates. The enzymological basis, if any, was not put forward by the authors (Starr and Nasuno, 1967).

\textit{Xanthomonas campestris pv. vesicatoria} (Doidge) Dowson was isolated from leaf-spots of chillies. Patel \textit{et al.}, (1950) first reported this disease from India. In fields the disease is characterized by a number of small, round to irregular, raised water-soaked spots, on the lower surface of the leaves, having pale-white centre with a slight depression on the upper surface. Leaves curl when edges and veins are infected. The spots gradually turn to brown in colour on both the surfaces. Severely-affected leaves become yellow and drop off.
The diagnostic staining and biochemical reactions of the organism showed that the bacterium was Gram -ve, rod-shaped, motile, capsulated, non-spore forming, and appeared yellow in mass on agar. The organism liquified gelatin, hydrolysed starch, showed catalase positive reaction, produced NH$_3$ and H$_2$S. It was strongly lipolytic, utilized citrate and produced acids from carbohydrates but no gas. The organism grew well in media containing glucose, galactose, fructose, mannose, xylose and lactose. The growth was poor on sorbose, mannitol and maltose. It failed to grow on salicin. It showed negative reactions in the following tests: nitrate reduction, production of indole and urease.