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The Properties of Fungal and Bacterial Cellulases with Comment on their Production and Application

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Introduction

Even a cursory perusal of current scientific literature shows cellulose hydrolysis by cellulases (EC 3.2.1.4, 3.2.1.91 and other enzymes) to be among the most intensively studied of topics. Research and development on these enzymes, their production, properties and applications is important if their actions are to be controlled and better utilized. By catalysing the decay of forest and agricultural residues, these enzymes recycle nutrients and so serve to maintain soil fertility and mechanical properties. In this context one may consider the problem of straw stubble remaining in the field after cereal harvesting. This material must be removed by burning or burying if the productivity of subsequent crops is not to be impaired. Burning pollutes the environment, creates a hazard if smoke blows across motorways and in any event throws away a potentially important source of food and energy. Burying straw is the practice in some areas but the length of time taken for conversion to usable humus is too long. The benefits of being able to expedite the digestion of this material *in situ* by cellulases, or more accurately, by cellulolytic micro-organisms, is obvious. Indeed, Lynch and co-workers (*see* e.g. Veal and Lynch, 1984) suggest that co-culturing cellulolytic and nitrogen-fixing organisms on buried agricultural waste may not only expedite humification but may also reduce considerably the amount of fertilizer needed for subsequent crop growth.

In the preface to the proceedings of a symposium on cellulases held almost twenty years ago, Hajny and Reese (1969) stated clearly that the economic

Abbreviations: CBH, cellobiohydrolase; CMC, carboxymethyl cellulose; C,I, crystallinity index; DNS, dinitrosalicylate; EG, endoglucanase; HPLC, high-pressure liquid chromatography; M_r, relative molecular mass (a dimensionless number); p*I*, isoelectric point; SSA, specific surface area.

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importance of the promotion of cellulase activity was enormous, as was the protection against such enzymes in the case of textiles, paper and lumber. Indeed, Gascoigne and Gascoigne (1960) remind us that even in the 1920s in the US many millions of dollars were being spent and many millions of gallons of preservative were being used to protect timber alone. In the Second World War the US Army were extremely concerned at the rate at which uniforms and cellulosic material decayed in the tropics (Augustine, 1976). Indeed, *Trichoderma viride* QM6a, from which the most powerful cellulolytic mutants have since been derived, was first isolated from a rotting cartridge belt in the jungles of New Guinea (Augustine, 1976). Largely as a result of these experiences the US Army set up the Natick Research and Development Laboratories in Massachusetts. Personnel in these laboratories were charged with the responsibility of isolating cellulolytic micro-organisms, investigating the mechanism of action of cellulases and of devising ways of preventing cellulase action without having recourse to fungicides.

The vast potential of cellulose as a source of food, fuel and chemical feedstocks now began to be appreciated. Cellulose, the major structural component of higher plants, is the most abundant of organic molecules—about 7×10^{11} tonnes are in existence. Moreover, it is a renewable resource: about 4×10^{10} tonnes are synthesized annually as a result of photosynthesis (*see* Coughlan, 1985). Accordingly, investigations at Natick and elsewhere quickly turned to examination of the feasibility of exploiting the action of cellulases (to be discussed later).

Cellulose structure

Cellulose is a linear polymer of up to 14 000 anhydroglucose residues in the chair configuration held together by β-1,4 linkages. Each residue is rotated 180 degrees about the main axis with respect to its neighbouring residues (*Figure 1*). Thus, the basic recurring unit is cellobiose. Cellulose chains, orientated in parallel and staggered with respect to their partners, associate to form insoluble fibrils in which the chains are held together by hydrogen bonding. It has been deduced that the hydrogen-bonding network consists of inter- and intramolecular bonds between successive and adjacent dextrose* residues (*Figure 1*; Gardner and Blackwell, 1974; Winterburn, 1974; Rees *et al.*, 1982). The intramolecular bonds help to maintain the rigidity of the cellulose chain. Bundles of the above fibrils aggregate to form the inert, insoluble fibres of great strength characteristic of the primary and secondary cell walls of higher plants (Rees *et al.*, 1982; McNeil *et al.*, 1984; *Figure 2*). Within cellulose fibres there are areas of complete order, i.e. crystalline areas, and also less well-ordered or amorphous regions. The degree of crystallinity within fibres varies with the source of the cellulose and the treatment to which it has been subjected. In the native state, cellulose fibres are associated with other polysaccharides, viz. hemicellulose and pectin and also with lignin (Rees, 1977; Alberts *et al.*, 1983; *Figure 3*). The crystallinity

*The term 'dextrose' (i.e. α-D(+)glucose) has been used throughout the text because 'glucose' to many in industry implies a mixture of starch degradation products.

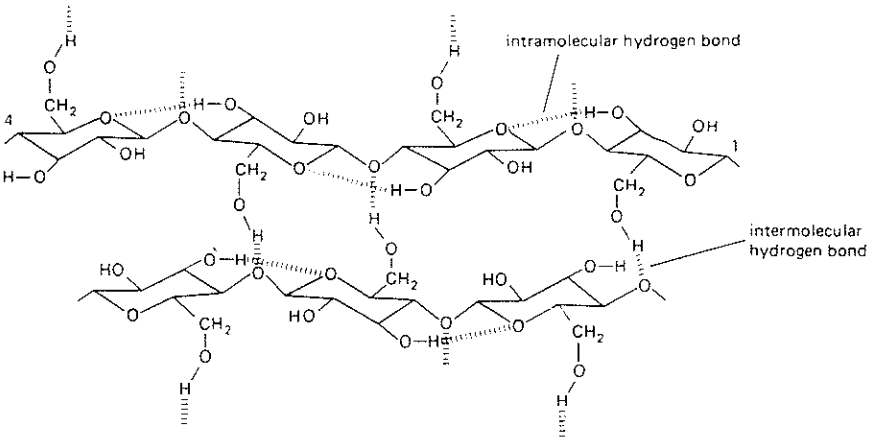

Figure 1. Cellulose chains showing the β-1, 4-linked residues rotated through 180 degrees with respect to their neighbours in the chain. Intermolecular hydrogen bonds tightly crosslink adjacent chains within a microfibril. Intramolecular hydrogen bonds stabilize each chain (from Alberts *et al.*, 1983, courtesy of Garland Publishing Inc.).

of the native material and its association with lignin are the major factors militating against the enzymic hydrolysis of cellulose.

Cellulase sources and production

Gascoigne and Gascoigne (1960) included molluscs, echinoderms, protozoa, nematodes, arthropods, annelids, plants, algae, basidiomycetes, ascomycetes, phycomycetes, fungi, actinomycetes and bacteria in a list of cellulase-producing organisms. In some instances it has since been shown that the enzyme(s) in question is synthesized by a symbiotic bacterium and not by the host organism originally thought to be responsible. This is certainly so in the case of wood-boring bivalves (shipworms) of the family Teredinidae. Waterbury, Calloway and Turner (1983) found that a cellulolytic nitrogen-fixing bacterium is present in 'enormous' numbers in the gland of Deshayes in the six species of shipworm examined, and concluded that this symbiosis accounts for the ability of shipworms to digest cellulose and so obtain their nitrogenous nitrogen requirements; furthermore, they suggested that the bacterium could be cultured to produce single-cell protein from cellulosic materials without the need for fixed nitrogen.

The cellulolytic systems of various bacteria and fungi have now been characterized (*Table 1*). *Trichoderma reesei* mutants are the most powerful producers of such enzyme systems. Others such as *Trichoderma koningii*, *Fusarium solani*, *Penicillium funiculosum*, *Sporotrichum pulverulentum* and *Clostridium thermocellum* are also being considered for commercial exploitation, while *Thermomonospora* spp., *Talaromyces emersonii* and others show promise in this regard.

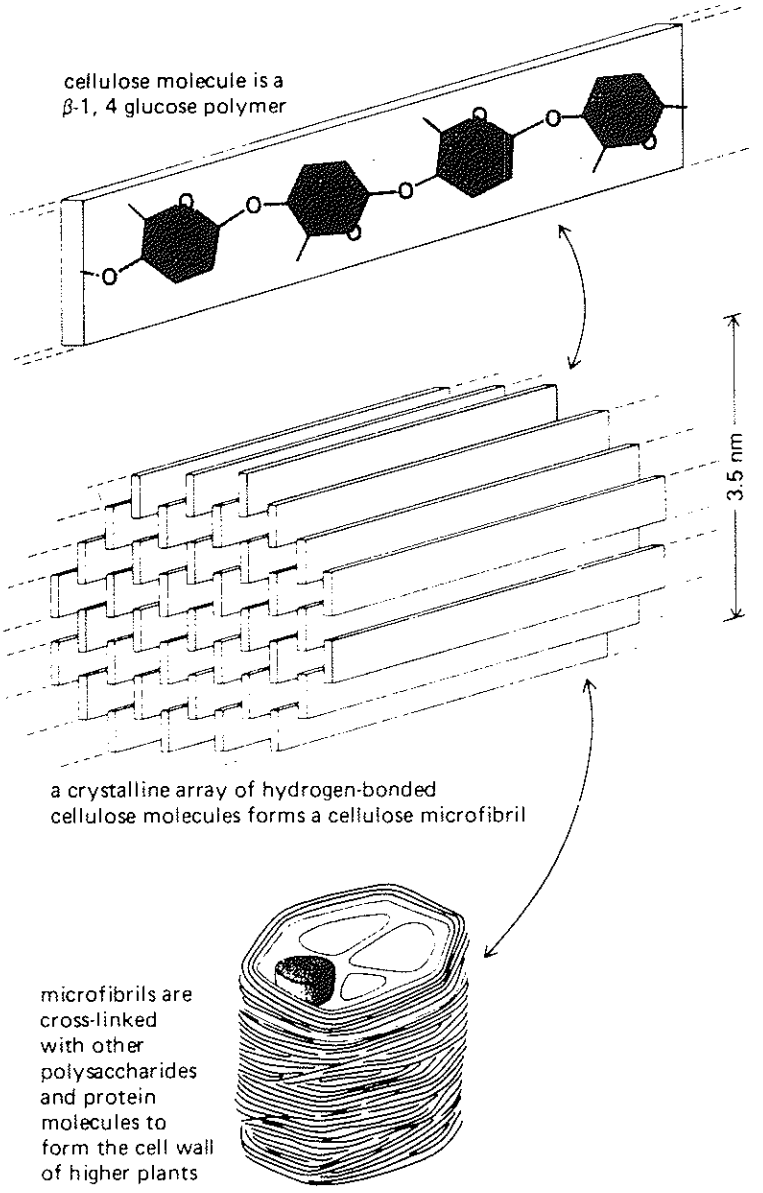

Figure 2. Organization of cellulose molecules in the cell wall of a higher plant (from Alberts *et al.*, 1983, courtesy of Garland Publishing Inc.).

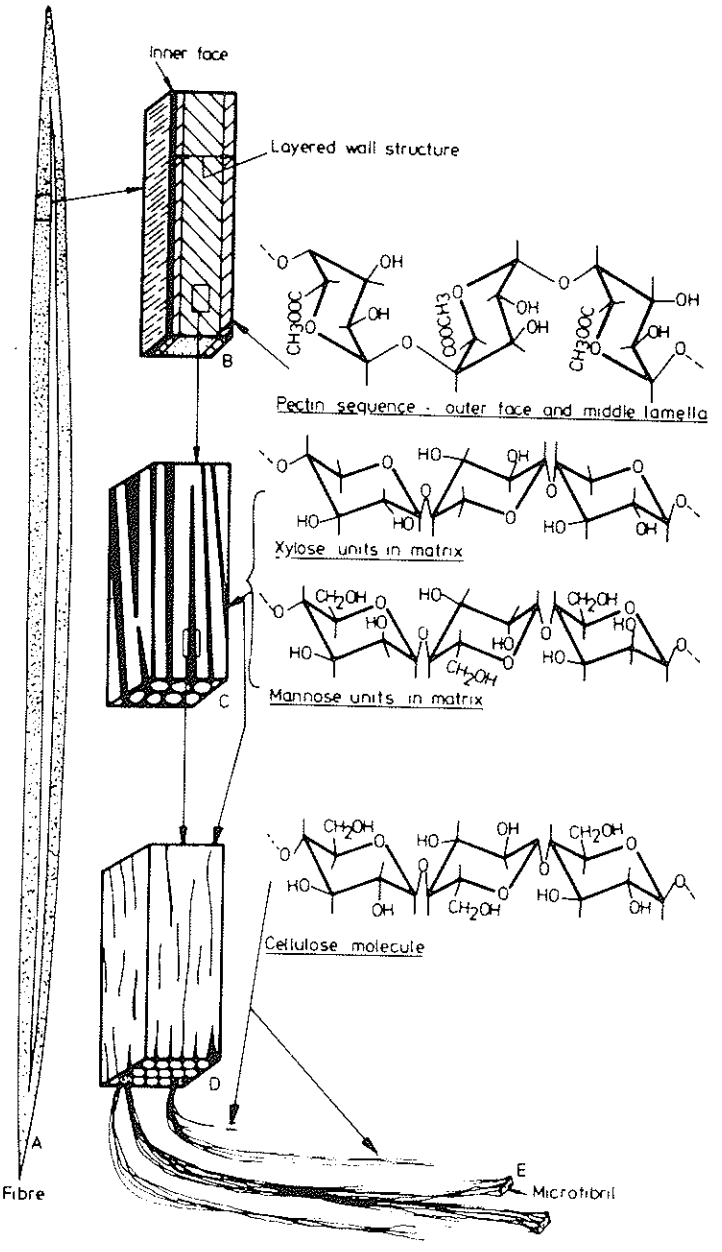

Figure 3. The cell-wall structure of a wood cell fibre (A). The fibre has a layered structure (B). In a fragment of the central layer of this wall (C), deposits of cellulose chains (white) are embedded in a matrix (black), of other polysaccharides and lignin. The cellulose deposits consist of many microfibrils (white in D) further embedded in matrix. Microfibrils consist of bundles of cellulose molecules in crystalline packing (E). Partial structures of some polysaccharide components of different wall regions are shown (from Rees, 1977, courtesy of Chapman and Hall).

Table 1. Cellulase-producing organisms of established or potential commercial use

Group	Organism	Group	Organism
Bacteria	*Acetivibrio cellulolyticus*	Fungi (continued)	*Myceliophthora thermophila*
	Bacteroides cellulosolvens		*Pellicularia filamentosa*
	Bacteroides succinogenes		*Penicillium citrinum*
	Cellulomonas spp.		*Penicillium funiculosum*
	Cellvibrio fulvus		*Penicillium irensis*
	Cellvibrio gilvus		*Penicillium janthinellum*
	Cellvibrio vulgaris		*Penicillium variabila*
	Clostridium thermocellulaseum		*Pestalotiopsis westerdijkii*
	Clostridium thermocellum		*Polyporus adustus*
	Clostridium thermomonospora		*Polyporus tulipiferae (Irpex lacteus)*
	Pseudomonas fluorescens		*Polyporus versicolor*
	Ruminococcus albus		*Poria* spp.
			Sporotrichum cellulophilum
Actinomycetes	*Streptomyces griseus*		*Sporotrichum dimorphosporum*
	Thermoactinomycete spp.		*Sporotrichum pulverulentum (Chrysosporium lignorum)*
	Thermomonospora curvata		*Sporotrichum pruinosum*
	Thermomonospora fusca		*Sporotrichum thermophile*
			Talaromyces emersonii
Fungi	*Agaricus bisporus*		*Thermoascus aurantiacus*
	Aspergillus fumigatus		*Thielavia terrestris*
	Aspergillus niger		*Tranetes sanguinea*
	Aspergillus terreus		*Trichoderma harzianum*
	Botryodiplodia theobromae		*Trichoderma koningii*
	Chaetomium thermophilum		*Trichoderma lignorum*
	Eupenicillium javenicum		*Trichoderma longibrachiatum*
	Fusarium solani		*Trichoderma pseudo-koningii*
	Humicola insolens		*Trichoderma reesei*
	Macrophomina phaseolina		*Trichosporon cutaneum*
	Myrothecium verrucaria		*Trichosporon pullulans*

The numerous published papers dealing with cellulase production have been reviewed by many authors, notably by Bailey, Enari and Linko (1975), Sternberg (1976), Gong and Tsao (1979), Ryu and Mandels (1980), Linko *et al.* (1983) and in the final report on the Natick programme (Anonymous, 1981). Celluloses are, generally speaking, the best substrates for enzyme production by micro-organisms but some such substrates are rapidly degraded and so yield only low levels of enzyme, while others, such as cotton, are not broken down readily enough to support adequate growth. Cellulases have been produced by cultivation of the appropriate organisms in shake flasks, batch reactors (Moreira, Phillips and Humphrey, 1981b), batch reactors with temperature and pH profiling (Tangnu, Blanch and Wilke, 1981), fed-batch fermentations (Allen and Mortensen, 1981; Hendy, Wilke and Blanch, 1984), continous cultivation on glucose (Brown and Zainudeen, 1977), two-stage (growth at 32°C, pH 4·5 in first stage and enzyme production at 28°C, pH 3·5 in second stage) continuous production on lactose (Ryu *et al.*, 1979), semicontinuous production in an aqueous two-phase system (Persson, Tjer-

neld and Hahn-Hägerdal, 1984), by immobilized *T. reesei* cells (Kumakura, Tamada and Kaetsu, 1984) and for many years in Japan by the solid-state Koji process. Under certain cultivation conditions *T. reesei* mutants produce extracellular protein in amounts up to 20 mg/ml, cellulase activities (as μmol of glucose released per minute from filter paper) of *c.* 25 IU/ml and productivities approaching 100–150 IU/ℓ/h (*see* e.g. Hendy, Wilke and Blanch, 1984). These are truly prodigious figures: indeed, *Trichoderma reesei* must be among the greatest known producers of extracellular protein.

INDUCTION, REPRESSION AND SECRETION

In virtually all micro-organisms examined to date, the synthesis of cellulases is induced by the presence of cellulose and repressed by the presence of dextrose or other readily metabolized sugars in the growth medium. Evidence supporting this conclusion has been obtained for bacteria, e.g. *Acetivibrio cellulolyticus* (Saddler, Khan and Martin, 1980) or *Cellulomonas uda* (Stoppok, Rapp and Wagner, 1982), actinomycetes, e.g. *Thermomonospora* (Moreira, Phillips and Humphrey, 1981a,b; Fennington, Neubauer and Stutzenberger, 1984) and fungi including *Penicillium janthinellum* (Rapp, Knobloch and Wagner, 1982), *Sporotrichum pulverulentum* (Eriksson and Hamp, 1978) and *Trichoderma reesei* (Sternberg, 1976). However, the nature of the actual inducer has yet to be determined. The most generally accepted view of the induction process, but one that remains to be tested experimentally, is that the organisms in question produce a low basal or constitutive amount of cellulase. This effects some breakdown of cellulose in the medium and soluble products including cellobiose are taken into the cell where they are converted, perhaps by β-glucosidase (EC 3.2.1.21), to the actual inducer of enzyme synthesis (*see* e.g. Gong and Tsao, 1979). Cellobiose is known to induce cellulase synthesis by *Trichoderma reesei* (Mandels and Reese, 1960) and by *Sporotrichum pulverulentum* (Eriksson and Hamp, 1978). However, it is effective only at high concentrations and, in the case of *T. reesei*, does not work with washed mycelia (Anonymous, 1981). Lactose is also an inducer in some species, including *T. reesei*, but again only at high concentrations (Gong, Ladisch and Tsao, 1979). By contrast, sophorose, i.e. 2-*O*-β-glucopyranosyl-D-glucose, at low concentrations is a powerful inducer of endo- and exocellulase synthesis by *Trichoderma reesei* (Mandels, Parrish and Reese, 1962; Gritzali and Brown, 1979; Sternberg and Mandels, 1979) and by *Sporotrichum pulverulentum* (Eriksson and Hamp, 1978). In a subsequent paper Sternberg and Mandels (1980) found that sophorose had two regulatory roles in *T. reesei*—on the one hand it induced the synthesis of endo- and exocellulases (induction being half maximal at 160 μmol/ℓ sophorose) and on the other it repressed the production of β-glucosidase (this being half maximal at 0·5 μmol/ℓ). Repression of β-glucosidase synthesis would decrease the rate of sophorose hydrolysis and effectively enhance its ability to induce cellulase formation. Sophorose could be produced by constitutive β-glucosidase within the cell, i.e. by glucosyl transfer to cellobiose and subsequent cleavage of the β-1,4 linkage (*see later*).

Arguing against sophorose being the 'true' inducer is the fact that the array of enzymes induced by the disaccharide is not as complete as that synthesized in the presence of cellulose (Sternberg and Mandels, 1979). Moreover, not all *Trichoderma* spp. respond to sophorose (Mandels, Parrish and Reese, 1962), nor does it act as an inducer of cellulase biosynthesis by *Acetivibrio cellulolyticus* (Saddler, Khan and Martin, 1980) or *Cellulomonas uda* (Stoppok, Rapp and Wagner, 1982).

In prokaryotic cells, genes subject to catabolite repression are expressed when two conditions are met. On the one hand dextrose concentration must be low, in which case cAMP concentration will be high. The complex between cAMP and catabolite-gene-activating protein then binds to the promoter site thereby 'preparing the way' for RNA polymerase to bind to the downstream region of the promoter site. Secondly, the appropriate inducer must be present so that it can combine with the repressor protein, causing it to dissociate from the operator. Only when these two conditions are met will RNA polymerase be able to transcribe the structural genes. The mechanism whereby transcription of fungal genes is regulated is, as yet, incompletely understood and may be more complicated than that obtaining in bacterial cells. Meanwhile, there is some disagreement as to whether cAMP is involved in controlling cellulase biosynthesis. The experimental results presented by Suzuki (1975) suggested that it was not thus involved in the case of *Pseudomonas fluorescens* var. *cellulosa*. Montenecourt and her colleagues claim that cAMP does not participate in regulating cellulase production by *Trichoderma reesei* (Montenecourt *et al.*, 1981; Montenecourt, 1983). Goksoyr and Eriksen (1980) mention the possibility that ATP rather than cAMP may be the small molecular regulator in some instances. By contrast, Stutzenberger and co-workers claim that cAMP is involved in regulating cellulase biosynthesis by *Thermomonospora* (Fennington, Neubauer and Stutzenberger, 1984; Stutzenberger, 1985).

Secretion is another possible point at which control can be exercised, although the mechanism whereby secretion is effected is still far from clear. Merivuori *et al.* (1985) have summarized the position as follows: the primary peptide with its core carbohydrate attached to asparagine residues is synthesized in the endoplasmic reticulum. In the Golgi, further carbohydrates are added to serine and threonine residues and the glycoprotein is then packaged into secretory vesicles and transported to the plasma membrane. The vesicles fuse with the membrane and the enzymes are released into the medium. Whatever the mechanism of secretion, both endo- and exocellulases appear in the medium concurrently rather than sequentially (Gong and Tsao, 1979). Moreover, induction, synthesis and secretion are considered to be closely co-ordinated, but it is uncertain whether induction of endo- and exoglucanase synthesis is subject to co-ordinate control.

MUTATION AND CLONING

Considerable enhancement of cellulase productivity, especially by *Trichoderma* sp., has been achieved in the last twenty years by optimization

of media and process engineering. Further improvements followed the identification of new species, isolation of hyperproducing mutants of existing strains and cloning of the appropriate genes. Screening for, and isolation of, mutants of *Trichoderma reesei* QM9414 or of the wild type QM6a has been carried out almost entirely at the US Army Natick Research Laboratories, Massachusetts, at Rutgers University by Eveleigh's group, and at the VTT Biotechnical Laboratory in Finland. Mutants of *Trichoderma* have been obtained by conventional techniques including treatment with mutagenic chemicals, UV- and γ-irradiation and linear accelerator; their genealogy has been summarized by Montenecourt (1983). Mutants exhibiting enhanced cellulase productivity have also been isolated from *Thermomonospora curvata* (Fennington, Lupo and Stutzenberger, 1982), *Sporotrichum pulverulentum* (Eriksson and Johnsrud, 1983), *Talaromyces emersonii* CBS 814.70 (Moloney *et al.*, 1983) and *Penicillium funiculosum* (Joglekar and Karanth, 1984).

The objectives of mutation programmes are to improve enzyme productivity and/or the intrinsic properties of the enzymes by, for instance, increasing the rate and extent of enzyme synthesis and secretion, removal of catabolite repression and perhaps isolation of constitutive mutants, as well as isolation of mutants producing enzymes with greater specific activity, increased operational stability and resistance to product inhibition.

Considerable success has been achieved in enhancing enzyme productivity especially with *Trichoderma reesei* mutants (*see* last section; Montenecourt, 1983; Linko, 1985). However, increased enzyme activities in most cases are matched by increases in protein synthesis and secretion: for instance, with the hypercellulolytic mutant *Trichoderma reesei* Rut-C30 there is a six- to sevenfold increase in endoplasmic reticulum compared with the wild type QM6a (Ghosh *et al.*, 1982). Only rarely, as with Rut-C30 and a closely related mutant RL-P37, has an increase (almost twofold) in cellulase specific activity been observed (Sheir-Neiss and Montenecourt, 1984). Catabolite repression has been largely overcome in the VTT series of *T. reesei* mutants (particularly strain VTT-D-79125) in that they produce considerable quantities of cellulase when grown on dextrose (Bailey and Oksanen, 1984).

The poor enzyme productivity by fungi, a result of the relatively prolonged fermentation times required, might be solved by cloning the cellulase genes in rapidly dividing bacteria. Montenecourt (1983) has, in fact, summarized the general strategies to be used in cloning the *Trichoderma* cellulase genes in *Escherichia coli*. Problems arise in that *E. coli* does not glycosylate proteins and there is ample evidence to indicate that most, if not all, fungal cellulases are glycoproteins (*see Tables 4 and 5*; Sheir-Neiss and Montenecourt, 1984) and that glycosylation is essential to secretion, activity and stability of these enzymes (*see* e.g. Merivuori *et al.*, 1985). This problem might, in part, be overcome if it were possible to locate the cellulases in the periplasmic space or the cell surface of *E. coli*, as in *C. thermocellum*. A second problem derives from the fact that fungal genes contain introns: for instance in *T. reesei* the gene coding for cellobiohydrolase I contains two such sequences (Shoemaker *et al.*, 1983; Teeri, Salovuori and Knowles, 1983). *E. coli* cannot process mRNA transcripts of such genes, but this difficulty may be overcome by

making complementary DNA copies of the mature fungal mRNA and cloning these in the bacterium. Teeri, Salovuori and Knowles (1983) and Knowles *et al.* (1983) made use of the fact that cellulases, and hence the corresponding mRNA, are present in cultures of *T. reesei* grown on cellulose but not in those grown on dextrose. They isolated polyadenylated mRNA from cultures grown on each medium and from these complementary DNA was made using reverse transcriptase. Differential hybridization of these cDNA probes to a λ phage gene bank permitted isolation of a number of genes that were expressed during cellulase synthesis. One of these clones contained the gene coding for the cellobiohydrolase CBH I, the product (M_r 67 000) being confirmed by immunoprecipitation with antiserum against CBH I.

The use of yeast rather than *E. coli* as a host for cloned genes may offer some advantages in that, being a eukaryote, the yeast presumably can process intron-containing mRNA transcripts and also can effect the glycosylation of protein (Montenecourt, 1983). Penttila *et al.* (1984) did make a gene bank of *Aspergillus niger* in *E. coli* using a yeast cosmid shuttle vector. Yeast transformants carrying *A. niger* genes were screened for several glycoproteins secreted by the fungus. Of these, only β-glucosidase was expressed in yeast and this only at a low level, thus indicating that there are differences between the expression of genes of yeast and of filamentous fungi. Sacco, Millet and Aubert (1984) cloned the *cel A* gene coding for endoglucanase A of *Clostridium thermocellum* (*see below*) in *Saccharomyces cerevisiae*. In the original micro-organism the enzyme is extracellular but all endoglucanase activity in the yeast was located in the cytoplasm. The specific activity in yeast carrying the *cel A* gene was 28% of that found in transformed *E. coli* and 11% of that in the *C. thermocellum* strain.

The two genes, *cel A* and *cel B*, coding for endoglucanases A and B in *C. thermocellum*, have recently been characterized (Beguin, Cornet and Millet, 1983; Cornet *et al.*, 1983a). Both enzymes were expressed in *E. coli* carrying the respective genes subcloned from cosmids of a gene bank into plasmid pBR322 (Cornet *et al.*, 1983b). Activity was found to be equally distributed between the cytoplasm and the periplasmic space. The nucleotide sequence of the *cel A* gene has now been determined and compared with the amino-terminal sequence of the purified enzyme (J. P. Aubert, personal communication). The mature protein is extended by a signal sequence of 32 residues (the enzyme is extracellular in *C. thermocellum*) and a 23-residue segment is duplicated at the carboxy-terminal end of the enzyme. Moreover, the GUG initiation codon is preceded by an AGGAGG sequence typical of prokaryotic ribosomal binding sites. Whittle *et al.* (1982) and Collmer and Wilson (1983) have cloned the genes coding for an endocellulase in *Cellulomonas fimi* and *Thermomonospora YX*, respectively, into *E. coli*. In each case, extracts of the *E. coli* transformed with the DNA plasmid carrying the endocellulase gene catalysed the hydrolysis of carboxymethyl cellulose (CMC). However, of greater interest was the finding that *E. coli* transformants containing plasmids pD316 and pD365 secreted 30% of their endoglucanase activity into the medium, the rest being distributed between the periplasmic space (30%) and the cytoplasm (40%) (Collmer and Wilson, 1983); the enzyme is, of course,

extracellular in *Thermomonospora*. In a subsequent paper, Gilkes *et al.* (1984) reported the isolation of a mutant of *E. coli* that leaks the cellulase activity encoded by cloned cellulase genes from *Cellulomonas fimi*. Leakage was maximal at 40°C, at which temperature 40% of the total CMCase activity was released to the medium. The genes for cellobiose utilization in *Escherichia adecarboxylata* have also been transferred to *E. coli* using recombinant DNA techniques (Armentrout and Brown, 1981). In this case all of the activity expressed was located in the membrane and allowed *E. coli* to grow efficiently on cellobiose.

From the above results it is clear, as pointed out by Cornet *et al.* (1983a), that genetic analysis of cellulose degradation at the molecular level is now possible. Further developments can confidently be expected.

Multiplicity of enzyme forms

It is evident from *Tables 4–6* (*see* pages 80–83, 86–87) that each of the major cellulolytic components synthesized by an individual organism may exist in a number of forms. The reason for this multiplicity has in the past given rise to much conjecture. Is each of the individual forms genetically determined, or do they arise from differential modification by proteolysis or glycosylation inside or outside the cell? Is the pattern of forms resulting from differential modification intentional or fortuitous? Do the individual forms have separate functions in cellulose hydrolysis?

Wood (1981) has argued that, for stereochemical reasons, at least two types of endoglucanase and two of exoglucanase should be required for hydrolysis (to be discussed later in the section on mechanism of action). In this context we note that two immunologically unrelated cellobiohydrolases, CBH I and II, have been isolated from filtrates of *Trichoderma reesei* (Fägerstam and Pettersson, 1979, 1980; Gritzali and Brown, 1979). Moreover, Pettersson *et al.* (1981) found no homology between the first 20 amino-acid residues of these enzymes. The information necessary for the synthesis of these forms would certainly seem to be encoded in different genes. The complete amino-acid sequence of CBH I has now been established by automated Edman liquid phase degradation of peptides obtained by chemical and enzymic cleavage of the reduced and *S*-carboxymethylated protein and shown to be in excellent agreement with that predicted by the nucleotide sequence of the CBH I gene (Shoemaker *et al.*, 1983; Fägerstam, Pettersson and Engstrom, 1984).

Several investigators favour the view that differential proteolysis gives rise to the chromatographically distinguishable forms observed. For instance Nakayama *et al.* (1976) found that treatment of an endoglucanase isolated from a commercial *Trichoderma viride* preparation with a protease from the same source yielded a number of forms with minor alterations in substrate specificity. Gritzali and Brown (1977) used sophorose to induce cellulase synthesis by *Trichoderma reesei* grown on dextrose. Under these conditions only one endoglucanase and two exoglucanases were produced. Gong and Tsao (1979) found that *T. reesei* produced only one type of cellobiohydrolase

when grown under 'controlled broth conditions'. Both groups of workers reported that multiple forms of each enzyme are produced under other growth conditions, the multiplicity increasing with age and protease activity of the filtrate. More recently, Eriksson (1982) showed that two different proteases, a carboxypeptidase and a chymotrypsin type, were present in culture filtrates of *Sporotrichum pulverulentum* following growth on cellulose. He suggested that these proteases effect the release of endoglucanases from the cell wall, possibly modify the cell wall itself and that they may be responsible for the finding of five endoglucanases in the culture broth. Kubicek (1981) had concluded earlier that proteolytic enzymes were instrumental in releasing endoglucanases from the cell walls of *Trichoderma reesei*. Recently, Sheir-Neiss and Montenecourt (1984) found that two acid proteases of different specificities were co-produced with cellulases during growth of *Trichoderma reesei* mutants, Rut-C30 and RL-P37, on Solka floc. Filtrates contained two cellobiohydrolases and six endocellulases and, again, the possibility that proteolysis was responsible for the multiplicity of forms of the latter was considered. Deshpande *et al.* (1984) showed that endoglucanase activity in 2-day-old filtrates of *Penicillium janthinellum* was enhanced fourfold on incubation with 10-day-old filtrates of *Penicillium funiculosum*. Trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1) and subtilisin (EC 3.4.21.14) were ineffective in this regard. The authors concluded that endocellulase is secreted as an inactive procellulase and subsequently activated by a specific protease. The isolation of an appropriate protease-negative mutant would, as pointed out by Sheir-Neiss and Montenecourt (1984), be instructive. Finally, Price *et al.* (1984) found that the only difference between endoglucanases (EG) I and II in filtrates of *Schizophyllum commune* is the presence of an alanine-rich sequence at the *N*-terminal end of the former, and concluded that this may be a leader sequence necessary for secretion and that EG II may be a proteolytic product of EG I, proteolysis taking place at, or outside, the cell wall.

Labudova and Farkas (1983) have concluded that the production of multiple forms of cellulase components by *Trichoderma reesei* QM9414 is an intrinsic property of the fungus and cannot be considered to result solely from proteolytic postsecretional modification. However, these workers assayed for protease activity in filtrates only at pH 7·5, whereas the pH of the medium in uncontrolled fermentations dropped steadily from pH 5 to pH 2 during growth and ranged between pH 5–5·7 under controlled conditions; thus, they would have overlooked the possible presence of acidic proteases. Dunne (1982) also argues against proteolysis as the sole arbiter of multiplicity, claiming that high-pressure liquid chromatography (HPLC) patterns of cellulase components from high-protease (QM9414) and low-protease (Rut-C30) strains of *T. reesei* are similar and that cellulase is resistant to acid proteases above pH 4. However, here again, without pH control the pH in fermentations of this sort drops to values of pH 3 or less.

Gum and Brown (1977) and Moloney *et al.* (1985) concluded that the chromatographically distinct but apparently functionally identical forms of endocellulase produced by *Trichoderma viride* and *Talaromyces emersonii*

respectively, differed from one another only in the content or composition of the carbohydrate portion of these glycoproteins. Consistent with this interpretation is the finding that the presence of tunicamycin, an antibiotic that impairs *N*-glycosylation of proteins, brought about a decrease in the number of very acidic cellobiohydrolases secreted by *Trichoderma reesei* (Sheir-Neiss and Montenecourt, 1984). Glycosylation may be essential for secretion of cellulases by *Trichoderma reesei* and by *Talaromyces emersonii* since all of the cellulolytic components in culture filtrates are glycosylated (McHale and Coughlan, 1981a; Sheir-Neiss and Montenecourt, 1984; Moloney *et al.*, 1985). Montenecourt's group (Merivuori *et al.*, 1985) used tunicamycin to block *N*-glycosylation (i.e. attachment of carbohydrate to asparagine side chains) and 2-deoxyglucose to impair *N*- and *O*-glycosylation (i.e. attachment of carbohydrate to serine and threonine side chains) and, from their observations, postulated that *N*-glycosylation is needed for thermal and pH stability of the secreted cellulases and for protection against proteolysis. By contrast, *O*-glycosylation, they concluded, is required for secretion and activity. By contrast with the above findings, Eveleigh's group (Murphy-Holland and Eveleigh, 1985) have observed that, while the yield of CMCase by *T. reesei* QM6a in the presence of 2-deoxyglucose is reduced, the specific activity is considerably less affected. This may suggest that prevention of glycosylation preferentially inhibited the secretion of proteins other than CMCase. They find, moreover, by using tunicamycin *in vivo* and by *in vitro* cleavage of glucosyl moieties with endoglucanase H, that *N*-glycosylation of the cellulase enzymes produced by QM6a is not essential to activity nor to pH and thermal stability. The contradictory findings by these two groups clearly shows that further investigations on the role of the carbohydrate moieties of these proteins is required. Finally, a note of caution: Alurralde and Ellenreider (1984) found that cellobiohydrolase from a commercial *T. viride* preparation, even after purification to electrophoretic homogeneity, contained non-covalently attached carbohydrate in addition to the glycoprotein constituents. The authors report that removal of this non-covalently bound carbohydrate is accompanied by a rapid increase in enzyme activity. It would be interesting to determine whether removal of non-covalently associated carbohydrate from other preparations would decrease the observed multiplicity of enzyme forms.

Cellulolytic enzyme systems

HYDROLYTIC ENZYMES

Many micro-organisms can grow on cellulose; many produce enzymes that can degrade soluble derivatives of cellulose or the amorphous regions of cellulose, but few synthesize the complete enzyme systems that can effect extensive hydrolysis of the crystalline material found in nature (Sternberg, 1976; *see also* Groleau and Forsberg, 1981). The best-known of the 'complete' cellulase systems are those produced by *Trichoderma* spp. and other fungi

(*Table 1*). Such systems comprise, minimally, endocellulases (EC 3.2.1.4), exocellulases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) (*Table 2*). The most commonly found exocellulase is that which removes cellobiose units from the non-reducing ends of cellulose chains. However, an endwise-acting enzyme (EC 3.2.1.74) that removes dextrose from cellulose and cello-oligosaccharides has been isolated from culture filtrates of *Penicillium funiculosum* (Wood and McCrae, 1982) and is also thought to be produced by *Trichoderma reesei* (Marsden, Gray and Dunn, 1982).

An enzyme, cellulose polysulphatase (EC 3.1.6.7), isolated from *Charonia lampas* liver removes the two- and three-sulphate esters from cellulose polysulphate (Takahashi and Egami, 1961). This enzyme does not seem to be very widespread nor is the substrate upon which it acts. However, the product of action of the above enzyme is then acted upon by polysaccharase, also present in *Charonia* liver, to provide dextrose and dextrose monosulphate (Takahashi and Egami, 1961). The polysaccharase does not seem to have been characterized as an endo- or exo-acting enzyme.

Cellobiose epimerase (EC 5.1.3.11), an enzyme that catalyses the conversion of cellobiose to glucosylmannose, has been isolated from culture filtrates of the rumen bacterium, *Ruminococcus albus* (Tyler and Leatherwood, 1967). The real role of this enzyme, whether synthetic or degradative, is not yet known. However, we note that glucosylmannose, the product, is a substrate for cellobiose oxidases and dehydrogenases (Ayers, Ayers and Eriksson, 1978; Dekker, 1980; *see below*).

As has been stated earlier, the actual inducer of cellulase synthesis by any organism is not yet known. Mandels and Reese (1960) found that during growth of *Trichoderma reesei* on cellobiose, products of glucosyl transfer were found in the growth medium and that these were more active than cellobiose as inducers of cellulase synthesis. Indeed, sophorose, a β-1,2-linked dimer of glucose, is the most potent inducer of cellulase synthesis by *T. reesei* (Mandels, Parrish and Reese, 1962). Apart from their hydrolytic activity, β-glucosidases are also usually found to catalyse glucosyl transfer under suitable conditions. Thus, for example, the intracellular β-glucosidase of *Talaromyces emersonii*, at high concentrations of both substrates, catalyses the transfer of dextrose to cellobiose to form a trimer of some sort (McHale and Coughlan, 1982). Sophorose could be synthesized by such a reaction followed by cleavage of the β-1,4 linkage. An enzyme, not a β-glucosidase, that catalyses cellobiosyl transfer has recently been isolated from culture filtrates of *Trichoderma reesei* (G. T. Tsao, personal communication). The reactions catalysed by these enzymes are as follows:

$$G_1 + G_2 \underset{\longleftarrow}{\xrightarrow{\text{glucosyl transfer}}} G_3$$

$$G_3 + G_3 \underset{\longleftarrow}{\xrightarrow{\text{cellobiosyl transfer}}} G_5 + G_1$$

Table 2. Enzymic and non-enzymic factors involved directly and indirectly in cellulose degradation

EC number	Systematic name	Recommended name	Reaction catalysed
1.1.5.1	Cellobiose:quinone 1-oxidoreductase	Cellobiose dehydrogenase (quinone)	Cellobiose + a quinone ⇌ cellobiono-1,5-lactone + a phenol
1.1.99.18	Cellobiose:acceptor 1-dehydrogenase	Cellobiose dehydrogenase (acceptor)	Cellobiose + A ⇌ cellobiono-1,5-lactone + AH_2
2.4.1.20	Cellobiose:orthophosphate α-D-glucosyltransferase	Cellobiose phosphorylase	Cellobiose + H_3PO_4 ⇌ α-D-glucose-1-P + D-glucose
2.4.1.49	1,4-β-D-Oligoglucan:orthophosphate α-D-glucosyl transferase	Cellodextrin phosphorylase	$(1,4\text{-}\beta\text{-D-glucosyl})_n + H_3PO_4 \rightleftharpoons (1,4\text{-}\beta\text{-D-glucosyl})_{n-1} + \alpha\text{-D-glucose-1-P}$
3.1.6.7	Cellulose sulphate sulphohydrolase	Cellulose polysulphatase	Hydrolysis of the 2- and 3-sulphate groups of the polysulphates of cellulose and charonin
3.2.1.4	1,4(1,3;1,4)-β-D-glucan 4 glucanohydrolase	Cellulase	Hydrolysis of internal 1,4-β-D-glycosidic linkages in cellulose, lichenin and cereal β-D-glucans
3.2.1.21	β-D-glucoside glucohydrolase	β-Glucosidase	Hydrolysis of cellobiose and removal of glucose from non-reducing ends of cello-oligosaccharides and glycosyl transfer to cellobiose
3.2.1.74	1,4-β-D-glucan glucohydrolase	Glucan 1,4-β-glucosidase	Hydrolysis of 1,4-linkages in 1,4-β-D-glucans so as to remove successive glucose units
3.2.1.91	1,4-β-D-glucan cellobiohydrolase	Cellulose 1,4-β-cellobiosidase	Removal of cellobiose units from non-reducing ends of cellulose chains
5.1.3.11	Cellobiose 2-epimerase	Cellobiose epimerase	Cellobiose ⇌ D-glucosyl-D-mannose
—	—	Yellow affinity substance	Facilitates binding of the cellulase complex to cellulose
—	—	Cellulosome	A cell surface organelle that binds cellulose, promotes adherence of the bacterium to cellulose and exhibits cellulase activity
—	—	Microfibril generating factor	Generates short fibres or microfibrils on interaction with cellulose and acts synergistically with cellulases to effect digestion of cellulose
—	—	H_2O_2 + iron	Effects loss of strength and weight and rapid depolymerization of cellulose and increases swelling, alkali solubility and enzyme susceptibility of residual substrate

The recommended and systematic names given in this table are those listed in the most recent issue of *Enzyme Nomenclature* (1984) published for IUB by Academic Press, Inc. Elsewhere in the text the names used are those that obtained prior to the publication of this issue.

However, whether their roles are in synthesis or in degradation, or in the regulation of cellulase synthesis, is not yet understood.

PHOSPHOROLYTIC ENZYMES

Some bacteria, both aerobic and anaerobic, including *Ruminococcus flavefaciens, Cellvibrio gilvus, Cellulomonas fimi* (Ayers, 1959), *Clostridium thermocellum* (Alexander, 1968) and possibly *Bacteroides cellulosolvens* (Giuliano and Khan, 1984) synthesize a relatively non-specific intracellular enzyme, cellobiose phosphorylase (EC 2.4.1.20), that catalyses the reversible phosphorylation of cellobiose (*Table 2*). *C. thermocellum* also produces an intracellular cellodextrin phosphorylase (EC 2.4.1.49) that effects phosphorolysis of cello-oligosaccharides from cellotriose up (Sheth and Alexander, 1969). As some of these organisms do not produce a β-glucosidase it seems reasonable to assume that the phosphorylases provide a means of metabolizing cellulose degradation products and of diminishing the inhibitory effect of cellobiose on cellulase activity (*see later*).

OXIDATIVE ENZYMES

In 1974 Eriksson and co-workers reported that culture filtrates of *Polyporus versicolor* and *Phanerochaete chrysosporium* (then called *Sporotrichum pulverulentum*) contained an FAD-enzyme, cellobiose dehydrogenase (quinone) (EC 1.1.5.1) (Westermark and Eriksson, 1974a,b, 1975; Eriksson, 1978). This enzyme catalyses the oxidation of cellobiose and oligosaccharides up to cellopentaose, to their corresponding lactones. The electron acceptors used are lignin or its degradation products such as quinone or semiquinone radical. As such the enzyme was seen to act as a link between the degradation of lignin and cellulose by this white-rot fungus (Eriksson, 1978).

Subsequently, Dekker (1980) found that *Monilia* sp. also produced an enzyme, cellobiose dehydrogenase (acceptor), EC 1.1.99.18. This inducible cell-bound and extracellular enzyme also catalysed the oxidation of cello-oligosaccharides, including cellobiose, to their corresponding lactones but did not use quinones as electron acceptors. Rather, the electron acceptor specificity was restricted to those with a redox potential of +220 mV, e.g. 2,6-dichlorophenol indophenol (Dekker, 1980). An extracellular cellobiose dehydrogenase is also found in culture filtrates of the non-lignolytic *Sporotrichum thermophile* after growth on cellulose and cellobiose (Coudray, Canevascini and Meier, 1982). This enzyme oxidizes cellobiose and cellodextrins to the corresponding lactones using a variety of electron acceptors.

An obvious role for cellobiose dehydrogenases would be in the oxidative, rather than hydrolytic, catabolism of cellobiose within the cell. A second function would be to alleviate inhibition of cellulase action by cellobiose. *Sporotrichum thermophile* does not produce a β-glucosidase (Coudray, Canevascini and Meier, 1982), an enzyme that in other cellulolytic fungi would fulfil these functions. The fact that, in each of these organisms,

cellobiose dehydrogenase activity is induced in parallel with cellulase activity, underlines its importance in cellulose utilization.

In 1974 Eriksson and his colleagues also found that culture filtrates of *Phanerochaete chrysosporium* (then called *Sporotrichum pulverulentum*), *Polyporus adustus*, *Myrothecium verrucaria* and *Trichoderma reesei* catalysed considerable degradation of cotton in the presence of oxygen but only half that amount if the oxygen (air) was replaced by nitrogen (Eriksson, Pettersson and Westermark, 1974). Since cellobiose dehydrogenase does not utilize oxygen they concluded that a separate oxidative enzyme must be involved in cellulose digestion. The enzyme in question, cellobiose oxidase, has since been purified (Ayers, Ayers and Eriksson, 1978; Eriksson, 1978). It contains one mole of haem and one mole of flavin per mole of enzyme and catalyses the oxidation by O_2 of cellodextrins to the corresponding lactones. Substrates include cellobiose up to celloheptaose but not cellulose. The oxidation of cellobiose may effectively overcome its inhibition of cellulase action (Sternberg, Vijakumar and Reese, 1977) and so stimulate cellulose saccharification. Moreover, 'terminal reducing glucosyl residues formed as a result of endocellulase action may be oxidized by this enzyme thereby rendering the hydrolytic reaction irreversible' (Ayers, Ayers and Eriksson, 1978). Still to be resolved is the fate of the second atom of molecular oxygen in the reaction catalysed by cellobiose oxidase (*Table 2*). The end result is not, apparently, H_2O_2 (Ayers, Ayers and Eriksson, 1978). The ability of culture filtrates of *Trichoderma koningii*, *Penicillium funiculosum* or *Fusarium solani* to degrade cotton or of *Talaromyces emersonii* to hydrolyse Avicel is the same whether the reaction is carried out in an air or a nitrogen atmosphere (Wood and McCrae, 1979; McHale and Coughlan, 1980). Clearly, cellulose hydrolysis by these filtrates does not involve an oxygen-dependent enzyme unless it has been inactivated by the growth conditions before harvesting. However, oxidative enzymes that utilize acceptors other than O_2 may be engaged in the utilization of cellulose by these organisms.

OTHER FACTORS

In 1950 Reese, Siu and Levinson put forward their now classic C_1C_x hypothesis outlined below to explain the enzymic digestion of crystalline cellulose:

$$\text{Crystalline cellulose} \xrightarrow{C_1} \text{Reactive cellulose} \xrightarrow{C_x} \text{Soluble sugars}$$

They proposed that crystalline substrate is first rendered susceptible to hydrolysis by C_1 component, a non-hydrolytic chain-separating or hydrogen-bond-splitting enzyme; the modified cellulose is then hydrolysed by the multicomponent C_x fraction. This theory and minor variations on the theme

(*see* e.g. Leatherwood, 1969) held sway for some considerable time. However, the C_1 components from culture filtrates of *Trichoderma viride* and *Penicillium funiculosum* (Selby, 1969), *Chrysosporium lignorum* (Eriksson, 1969a), *Trichoderma koningii* (Halliwell and Riaz, 1971; Wood and McCrae, 1972, 1975; Halliwell and Griffin, 1973), *Fusarium solani* (Wood, 1969) and *Trichoderma reesei* (Pettersson, 1975) were isolated and shown to be exocellobiohydrolases. Indeed, more recent theories (discussed later in the section on mechanism of action) suggest that enzymic degradation of the crystalline substrate involves the synergistic action of endo- and exocellulases (Mandels and Reese, 1964; Selby, 1969). Nevertheless, the existence of an essential non-hydrolytic component continues to be mooted by some investigators. Thus, Halliwell and Riaz (1970) and Halliwell (1975) claimed that a component of the *Trichoderma koningii* cellulase system was required for the formation of short fibres from cotton. This component appeared to be independent of carboxymethyl cellulase (i.e. endocellulase) or exocellulase activities. On its own it effected little solubilization. However, Halliwell (1965) had earlier suggested that short-fibre formation was the initial stage in the attack on recalcitrant substrates. Griffin *et al.* (1984) have now isolated, from commercial preparations of *Trichoderma reesei* cellulase, a factor that generates microfibrils or short fibres from filter paper without causing the release of soluble sugars. The factor contains iron (additional ferric iron is needed for maximal activity), does not appear to be an enzyme, has a very low molecular weight, is relatively stable to boiling, lyophilization and extraction with methanol, and acts synergistically with endo- and exocellulases to effect saccharification of cellulose. The involvement of iron in this short-fibre-generating factor recalls the observation of Koenigs (1975), that H_2O_2 plus Fe^{2+} catalysed extensive depolymerization of cellulose and a rapid increase in alkali solubility at low weight losses, and that the residue was more susceptible than the untreated substrate to cellulase action. Depending on the ratio of H_2O_2 to Fe^{2+}, the C_2, C_3 or C_6 carbons were oxidized with consequent disruption of the ordered packing of the cellulose chains. Because the H_2O_2 plus Fe^{2+} system was effective under mild conditions of temperature and pH, Koenigs (1975) has suggested that brown rot fungi, such as *Lenzites trabea*, may use a similar mechanism for cellulose digestion, but the enzyme(s) generating the H_2O_2, the source of the iron (the substrate or the fungus) and state of the iron (whether free or protein-bound) have not been described. Whereas H_2O_2 plus Fe^{2+} generated significant amounts of short fibres from filter paper only after overnight incubation, the factor isolated from culture filtrates of *Trichoderma reesei* did so in one hour or less (Griffin *et al.*, 1984).

Clostridium thermocellum, an anaerobic thermophile and a powerful cellulolytic bacterium, excretes large quantities of endocellulases (Ait, Creuzet and Forget, 1979; Ng and Zeikus, 1981; Petre, Longin and Millet, 1981), as well as producing cellobiose phosphorylase (Alexander, 1968), cellodextrin phosphorylase (Sheth and Alexander, 1969) and β-glucosidase (Ait, Creuzet and Cattaneo, 1982). Products of action of the endocellulase presumably are taken into the cell and metabolized further by the hydrolytic and phosphorolytic enzymes. In addition to the extracellular enzymes,

Ljungdahl *et al.* (1983) have recently isolated a low-molecular-weight water-insoluble 'yellow affinity substance' from culture filtrates of *C. thermocellum*. This substance binds to the cellulose fibres in the growth medium and promotes the binding of the endocellulases to the substrates. It is possible that the affinity substance may so orientate the endocellulase on the surface of the crystalline cellulose that the lack (apparently) of an exocellulase does not restrict substrate degradation.

Other workers (Ait, Creuzet and Forget, 1979; Petre, Longin and Millet, 1981) have considered the extracellular cellulolytic enzyme system of *C. thermocellum* to be part of a larger protein complex. More recently, Lamed *et al.* (1983) compared the properties of the extracellular and cell-associated cellulase systems of this organism and decided that they were essentially the same. These workers coined the term 'cellulosome' for a discrete cell-surface organelle of this bacterium. This cellulosome is thought to be a multisubunit complex with a molecular weight of 2·1 million daltons, responsible for adherence of the cell to the cellulosic substrate, and said to contain both endo- and exocellulase enzymes (Lamed *et al.*, 1983). The cellulosome may facilitate delivery of the products of action of these enzymes to other components of the cellulose-utilizing system of the bacterium. What part, if any, the yellow affinity substance isolated by Ljungdahl *et al.* (1983) plays in the cellulosome has yet to be determined.

Detection, measurement and purification of cellulases

SCREENING

Enzyme-production costs represent 40–60% of overall costs of processes designed to saccharify cellulosic substrates and ferment the products (e.g. Wilke, Yang and Von Stockar, 1976; Ryu and Mandels, 1980). Accordingly, the isolation of new organisms, or mutants of existing species, that synthesize more efficient cellulases or exhibit enhanced enzyme productivity is of the utmost importance. Rautela and Cowling (1966) and subsequently Tansey (1971) showed that organisms producing extracellular cellulases could effect clarification of agar gels containing cellulose: the depth or extent of clarification correlated reasonably well with the ability to utilize cellulosic substrates, to effect loss of weight in cotton and to hydrolyse carboxymethyl cellulose. Thus, clarification of cellulose-containing agar could be used to screen for cellulase-producing organisms. Montenecourt and Eveleigh (1977) adapted this technique so that many mutants could be screened simultaneously on a single plate. In addition to phosphoric acid-swollen cellulose their agar gels contained oxgall, to limit the size of the colonies and Phosphon D, apparently to facilitate secretion of the cellulases. The inclusion of dextrose in the gels allowed distinction to be made between constitutive and repressed mutants and to determine the concentration of dextrose at which repression occurred. Other workers including Nevalainen, Palva and Bailey (1980) and Saddler (1982) have also reported on the utility of the cellulose–agar clearing

technique for screening of cellulolytic organisms. Saddler (1982) has, moreover, examined the various physical, chemical and microbiological factors that affect the ability of the growing colony to clarify the agar.

In an extension of this technique, Considine and Coughlan (1982) showed clarification of cellulose-containing agar by culture filtrates to be a linear function of the logarithm of cellulase (filter-paper-degrading) activity (*Figure 4*). Moreover, such activity was not affected by the presence of inhibitors such as cellobiose in filtrates but was seriously impaired by the inclusion of such inhibitors in the agar. These findings permit simple and quantitative estimation of cellulase activities to be made, usually without any need for tedious preliminary sample preparation (*Figure 4*). The inclusion of cellobiose in the agar facilitates identification of cellulases not susceptible to product inhibition. Removal of β-glucosidase from culture filtrates or addition of this component to filtrate samples in the wells did not affect the clarification process (Considine and Coughlan, 1982). Thus, depending on the cellulosic substrate incorporated into the agar gel, this technique would be suitable for selecting mutants producing increased quantities of endo- or exoglucanases or both.

DETECTION

As stated earlier, 'complete' cellulases are composed minimally of endoglucanases, exoglucanases and β-glucosidases, each often existing in multiple forms, thus making location and identification of the individual species on electrophoretic or isofocusing gels a tedious business. The insolubility of many of the otherwise most suitable substrates and the large molecular weight and low diffusibility of the soluble substrates are added difficulties. Cellulases separated by electrophoresis or isofocusing can be located by slicing the unstained gels, eluting the enzyme(s) therein and assaying the eluate for activity. Such procedures, while they may provide definitive confirmation of enzyme identity, are laborious and unsuited to routine investigation.

Cellulases

Fägerstam and Pettersson (1979) used an immunochemical procedure to detect cellulases after electrophoretic separation. This method involved protein estimation after immunoreaction with antisera prepared specifically against purified preparations of the endo- or exoglucanase components. A similar approach using monospecific and polyspecific antisera was adopted by Schülein, Schiff and Dambmann (1981). Nummi *et al.* (1980) improved on this immunoelectrophoretic approach by incubating sliced immunoplates in contact with plates containing amorphous cellulose in agarose. Cellulolytic activity was located as 'clear zones in the turbid gels'. Among the drawbacks of the above techniques are the need to prepare antisera and the time, up to 24 h, needed to produce clear zones.

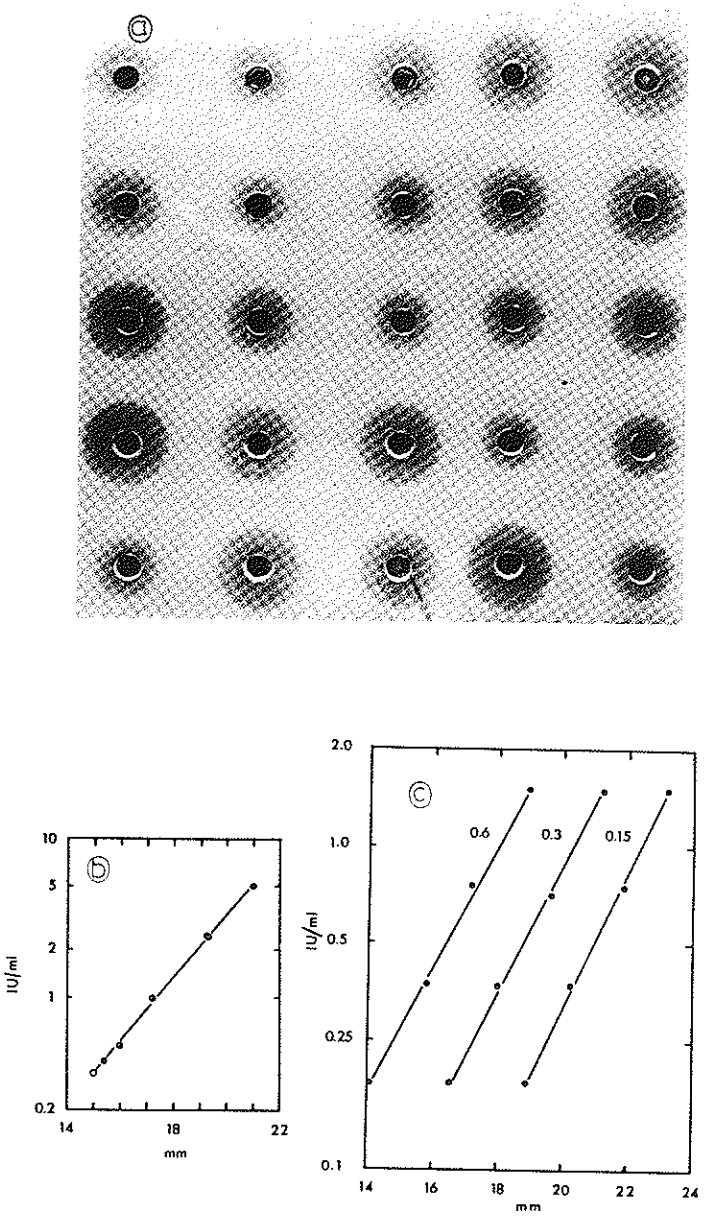

Figure 4. Quantitative determination of total cellulase activity. (a) Clarification of acid-swollen cellulose-containing agar by culture filtrates of *Talaromyces emersonii* or *Trichoderma viride*. To minimize inconsistencies in the agar, replicate samples were randomly placed in wells. (b) Plot of $\log_{10}$ enzyme activity (IU/ml) versus diameter (mm) of clear zone for the *T. emersonii* (○) and *T. viride* (●) enzymes. (c) Effect of cellulose concentration (% of w/v) on the extent of clarification (from Considine and Coughlan, 1982, courtesy of *Bioscience Reports*).

Endocellulases

Eriksson and Pettersson (1973, 1975a) sprayed isofocusing gels with viscous solutions of carboxymethyl cellulose (CMC). The reducing-sugar products soaked into an overlaid paper and the areas corresponding to the location of the enzymes were detected with a reagent for reducing sugars. Saddler and Khan (1981) located the position of endoglucanases by incorporating CMC in gels. After electrophoresis the gels were stained with dinitrosalicylate. McHale and Coughlan (1981a) immersed gels in agar containing phosphoric-acid-swollen cellulose azure. During incubation the dye released from the substrate diffused into the disc gel at the locations corresponding to the endo- and exoglucanases. Beguin (1983) made use of the fact that Congo red binds strongly to polysaccharides containing adjacent β-1,4-linked glucopyranosyl residues to visualize bands of cellulolytic activity. Gels, after electrophoresis, were overlaid by thin sheets of agar containing carboxymethyl cellulose. After incubation and staining of the agar replica with Congo red, the locations corresponding to the endocellulases were revealed by their lack of colour.

Exocellulases

Bartley, Murphy-Holland and Eveleigh (1984) made use of Beguin's findings to differentiate between endo- and exocellulases. The former activities were located as outlined by Beguin (1983). General β-1,4-glucanase activity was located by soaking post-electrophoretic gels in solutions containing sodium borohydride-reduced cellulo-oligosaccharides and detecting the release of reducing sugars by reaction with triphenyl tetrazolium chloride. The endo-glucanases are active in both assays whereas the exoglucanases are active only in the latter.

β-Glucosidases

The location of β-glucosidase activity on gels may be determined by immersing the gels in solutions containing *p*-nitrophenyl-β-D-glucoside or cellobiose. After a light wash the gels are immersed in alkaline buffer or in a solution containing the glucose oxidase reagent. The yellow colour of the *p*-nitrophenolate ion, or of the colour of the oxidized chromogen in the oxidase reagent, diffuse into the gel at the regions corresponding to the location of the enzyme. Fluorescence detection using 4-methylumbelliferyl-β-D-glucoside as substrate is another possibility (Sprey and Lambert, 1983).

ASSAY OF CELLULOLYTIC ACTIVITY

The methods whereby the activities of complete cellulase systems and of the individual components thereof may be assayed are summarized in *Table 3*. Substrates may be crystalline or amorphous or both, soluble or insoluble and pure or (as in the case of native materials) admixed with other substances

Table 3. Assay of cellulolytic activity

Enzyme or system assayed	Substrate used	Product or property measured
Complete cellulase	Cotton	Release of reducing sugars
	Avicel	Loss of weight
	Filter paper	Decrease in turbidity of solutions
	Solka floc	Decrease in tensile strength of fibres
	Dyed cellulose	Release of dye
	Cellulose-agar	Clarification of agar
Endoglucanase	Carboxymethyl cellulose	Release of reducing sugars
	Hydroxyethyl cellulose	Decrease in viscosity
	Cello-oligosaccharides	Clarification of agar
	CMC-agar	
Exocellobiohydrolase	Amorphous cellulose	Reducing sugar
	Cello-oligosaccharides	Cellobiose
	p-Nitrophenyl-β-D-cellobioside	*p*-Nitrophenol
Exoglucohydrolase	Amorphous cellulose	Reducing sugar
	Cello-oligosaccharides	Dextrose
β-Glucosidase	Cello-oligosaccharides	Reducing sugar
	Cellobiose	Dextrose
	p-Nitrophenyl-β-D-glucoside	*p*-Nitrophenol

such as lignin. The multicomponent nature of cellulase systems, the variety of substrates that may be, and are, used to measure actvity, the different experimental conditions and units used to express activity have bedevilled comparative evaluation of reports from different laboratories. The amorphous regions of celluloses are readily broken down by endo- or exocellulases whereas neither type of enzyme alone can effect extensive hydrolysis of crystalline material. For this both types of enzyme and, in the long run, β-glucosidase as well, would be required. Typical examples of cellulose hydrolysis by two culture filtrates are shown in *Figure 5*. In each case there is an initial fast rate of release of reducing sugar (line 1). This represents the attack on the amorphous part of the substrate. This phase is followed in those systems lacking exoglucanases by cessation of activity (line 3) whereas hydrolysis by complete cellulase systems continues, although at reduced rates (line 2). In this phase the crystalline substrate is attacked. It is obvious, therefore, that initial rates of hydrolysis may be meaningless. The extent of hydrolysis of 'real' substrates is also an important criterion. Failure to appreciate these niceties may, as pointed out by Mandels (1975), lead to exaggerated claims being made about the effectiveness of cellulolytic systems. For all of the above reasons the International Union of Biochemistry, perhaps with some prompting, invited a number of experts in this field to draw up recommendations for assaying each of the cellulolytic activities (Ghose *et al.*, 1983). Another useful source of methods for determining cellulolytic activities is the short review written by Rosenberg and Oberkotter (1977).

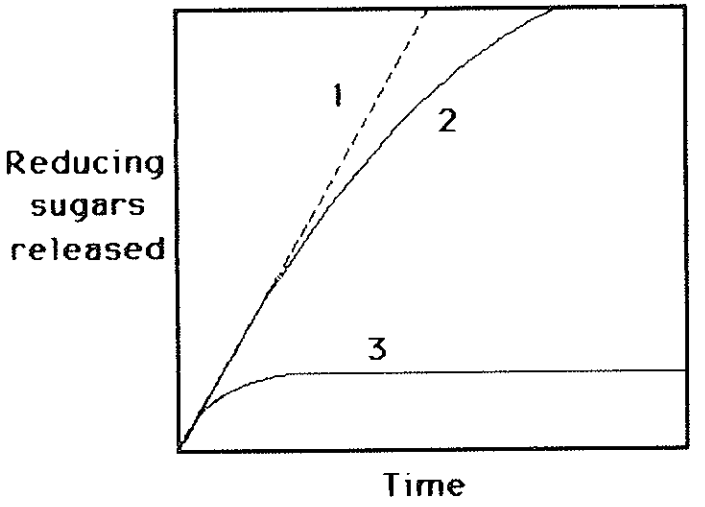

Figure 5. Release of reducing sugars on hydrolysis of cellulose by complete and incomplete cellulolytic systems. Details in the text.

Total cellulase activity

The most popular assay for complete cellulase activity is that developed by Mandels, Andreotti and Roche (1976) in which the quantity of filtrate (enzyme) required to effect 4% hydrolysis of filter paper in one hour at optimum pH and temperature is determined. This assay strikes a reasonable compromise between practical convenience (including a relatively short incubation time) and an extent of hydrolysis of this substrate that requires the synergistic action of both endo- and exoglucanases. Montenecourt *et al.* (1978) suggest that the use of special filter-paper antibiotic-assay discs instead of ordinary filter paper would require less preparation; blank values would be lower and, being more resistant, would reduce the number of enzyme dilutions to be assayed. Bailey (1981), and Joglekar, Karanth and Srinivasan (1983) demonstrated that the β-glucosidase levels in filtrates had a marked effect on filter-paper hydrolysing activity, even during incubation times of one hour or less. It should be mentioned here that many investigators will wish to estimate the effectiveness of their preparation under saccharification process conditions. For this there is no substitute for using as substrate the waste, agricultural residue or other cellulosic material that is to be hydrolysed, and long incubation times.

Endocellulase activity

Endocellulase activity is usually determined by measuring the release of reducing sugar from soluble cellulose derivatives of low degrees of substitution. The effect of dilution on such assays is apparently quite critical (Cauchon and Le Duy, 1984). Alternatively, the decrease in viscosity, or

increase in fluidity, accompanying hydrolysis of such derivatives may be assayed viscosimetrically. Eriksson (1969b) and Almin, Eriksson and Pettersson (1975) report such a procedure whereby enzyme activity may be determined in absolute terms. Plots of increase in relative fluidity versus reducing equivalents released may be used to compare individual endoglucanases and serve as an indicator of the 'randomness' of attack on the substrate (e.g. Suzuki, Yamane and Nisizawa, 1969; Wood and McCrae, 1978).

Exoglucanase activity

The activity of isolated exoglucanases may be determined by measuring the release of reducing sugars or more specifically of cellobiose or dextrose, as appropriate, from amorphous cellulose or cello-oligosaccharides. Measurement of exoglucanase activity in mixtures of enzymes or in filtrates is a different matter. It is true that exoglucanases will degrade acid-swollen cellulose but not carboxymethyl cellulose whereas endoglucanases are active against both substrates. Moreover, exoglucanase as well as endoglucanase activities are needed for hydrolysis of crystalline substrates such as Avicel. Indeed, some workers take the activity of a culture filtrate against Avicel or similar substrate to be a measure of exoglucanase activity. At best, however, such measurements can be regarded as only approximations. Reese (1969) attempted to estimate exoglucanase activity in crude solutions by measuring the relative rates of hydrolysis of cellotetraose and cellobiose. Unfortunately, the relative rates of hydrolysis of each substrate by purified preparations of exoglucanase and β-glucosidase must first be ascertained. These ratios will vary from the filtrate of one organism to the next, and cellobiose phosphorylase, if present, will also interfere. No mention was made of the possible complex interference due to the presence of multiple forms of exoglucanases and β-glucosidases but the fact that the latter might be inhibited by gluconolactone was alluded to. Deshpande, Eriksson and Pettersson (1984) also reported an assay for the selective determination of exoglucanases in systems also containing endoglucanases and β-glucosidases. The basis of the assay is that exoglucanases specifically hydrolyse only the agluconic bond of *p*-nitrophenol-β-D-cellobioside to yield cellobiose and *p*-nitrophenol. The interference by β-glucosidase which acts on both the agluconic and holosidic bonds is prevented by inhibition with gluconolactone. Interference by endoglucanases, which also acts on both bonds, must be compensated for by prior standardization of the assay procedure with a purified endoglucanase from the cellulase system under investigation. Once again, the possible effects of multiple forms of endoglucanases with different relative specificities for the bonds in question was not considered.

β-Glucosidase activity

β-Glucosidase activity is determined by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucoside or by the release of dextrose from cellobiose or cello-oligosaccharides. The dextrose is measured using the

glucose oxidase/peroxidase/chromogen reagent (Werner, Rey and Wielinger, 1970). Note that some commercial preparations of this reagent are contaminated with cellobiase activity so that suitable precautions must be taken if solutions are also known or suspected to contain cellobiose. Dextrose concentration may also be determined by measuring the amount of NADPH + H^+ formed using the hexokinase, $NADP^+$, glucose-6-phosphate dehydrogenase reagent.

The dinitrosalicylate (DNS) (Miller, 1959) and the Nelson–Somogyi (Nelson, 1952) methods are by far the most popular procedures used for the estimation of reducing sugars released on hydrolysis of purified cellulases or lignocellulosic substrates such as municipal or agricultural wastes. Neither method is specific for sugars, with the result that other reducing substances present can cause significant interference. Moreover, the response to either reagent varies considerably from sugar to sugar. These drawbacks do not matter too much when the saccharification of purified cellulosic substrates is under examination. However, as pointed out by Rivers *et al.* (1984), the DNS method can seriously overestimate the extent of hydrolysis (i.e. reducing sugars released) when impure lignocellulosic materials are used. The release of coloured inks and tannins, for which adequate blanks cannot be included, on hydrolysis of these substrates seriously interferes with the assay (Rivers *et al.*, 1984). Breuil and Saddler (1985) have also commented on the inadequacies of the reducing-sugar assay procedures. Holtzapple and Humphrey (1983) developed a method whereby hexoses can be measured using chromotropic acid without interference by pentoses. More recently Mullings and Parish (1984) reported a simple assay for reducing sugars based on the production of a coloured formazan and solvent extraction. Ultimately, high-pressure liquid chromatography must be the most reliable procedure for analysis of the sugar content and composition of hydrolysates. Honda (1984) has written a most extensive and thorough review of this subject. However, our personal experience of attempts to analyse enzymic hydrolysates of sugar-beet pulp indicates that even HPLC cannot easily solve all of the problems. Thus, mixtures contain both neutral and acidic sugars, thereby necessitating individual separation techniques for each group. Moreover, the presence of proteins, amino acids and other compounds affects refractive index or ultraviolet-absorption monitoring techniques. One procedure that, although time-consuming, appears to work is that described by Puls and co-workers (Sinner and Puls, 1978; Koerner *et al.*, 1985). Neutral reducing sugars are identified as copper I bicinchoninate complex by postcolumn reaction following HPLC separation. Acidic sugars are then selectively eluted by alteration of buffer conditions and analysed as before.

CELLO-OLIGOSACCHARIDE PREPARATION

Short-chain cello-oligosaccharides have proved to be very useful for investigation of the substrate specificities of cellulolytic enzymes. They may be prepared by acetylation of cellulose, neutralization, precipitation and deacetylation (Miller, Dean and Blum, 1960) and then separated by gel

filtration on Biogel P-2. An alternative procedure involves hydrolysis of cellulose by HCl/CO_2, neutralization and separation on charcoal or Biogel P-2 (Halliwell and Vincent, 1981; Bartley, Murphy-Holland and Eveleigh, 1984). The neutralization steps above generate considerable quantities of salts that interfere with subsequent separation of the oligosaccharides. To obviate this, Voloch *et al.* (1984) hydrolysed cellulose with sulphuric acid, precipitated the products with ethanol and effected their separation by ion-exchange on Aminex resin in the Ca^{2+} form.

ENZYME PURIFICATION

Fractionation and isolation of the individual components of cellulase systems is a necessary prerequisite to a fuller understanding of the relative importance and mechanism of action of each enzyme in the hydrolysis of cellulose. However, the fact that in complete systems there are at least three groups of enzymes involved and that each type may exist in numerous forms does not make the task an easy one. All of the usual protein purification techniques can be, and are, used for the purification of cellulases. These would include ammonium sulphate fractionation, gel filtration, ion-exchange chromatography, electrophoresis and isoelectrofocusing. However, it is a sobering experience for a novice in the field to see dialysis tubing and some cellulose-based resins disappear before one's eyes unless suitable precautions are taken. A number of more specific isolation procedures are worthy of mention. Gong, Chen and Tsao (1979) exploited the differential affinity of β-glucosidase and endoglucanase for concanavalin A–agarose to isolate the latter enzyme from partially purified preparations of culture filtrate of *Trichoderma viride*. The β-glucosidase did not bind to concanavalin A whereas the endoglucanase did, indicating that it is a glycoprotein and that its associated carbohydrate contains α-D-manno- and gluco-pyranosides. Fähnrich and Irrgang (1984) purified an endoglucanase from culture filtrates of *Chaetomium cellulolyticum* by affinity chromatography on concanavalin A–Sepharose. They also found that linking the triazine dye, Procion Red HE3B, to agarose proved to be useful in separating β-glucosidase from cellobiose dehydrogenase.

Cellulolytic enzymes bind with different degrees of tenacity to cellulosic substrates. Various investigators have made use of this fact to effect fractionation of culture filtrates of *Trichoderma* spp. (Halliwell and Griffin, 1978; Weber, Foglietti and Percheron, 1980; Nummi *et al.*, 1981). Vaidya *et al.* (1984) found that the glycoprotein nature of an endoglucanase from culture filtrates of *Fusarium lini* markedly retarded its elution from Biogel P-150. This provided a rapid and simple method for its purification. Van Tilbeurgh *et al.* (1984) used a biospecific adsorbent, *p*-aminobenzyl 1-thio-β-D-cellobioside, attached to a BioRad Affigel-10 to isolate the cellulases in filtrates of *Trichoderma reesei* QM9414. The endoglucanases did not bind to the ligand and so could be separated from the exocellobiohydrolases (CBHs) which did. The CBHs were then separated from one another by selective elution, CBH I with lactose and CBH II with cellobiose (Van Tilbeurgh *et al.*, 1984).

Properties of complete cellulase systems

ADSORPTION/DESORPTION

By contrast with most enzyme–substrate interactions, that between native lignocellulose, a structurally heterogeneous material, and the equally heterogeneous cellulase system involves the interaction of soluble enzymes with an insoluble substrate. More than twenty years ago Halliwell (1961) showed that the enzymes in question were adsorbed by the substrate. The factors influencing the adsorption and desorption processes have since been subjected to considerable scrutiny by many investigators. The cost of cellulases can, as stated earlier, account for as much as 60% of the total cost of saccharification (Wilke, Yang and Von Stockar, 1976). Thus, an understanding of adsorption/desorption behaviour is essential for economic enzyme utilization.

Factors affecting the adsorption of cellulases to cellulose may include the nature of the substrate, its purity, pretreatment and the extent to which it is crystalline or amorphous, as well as the enzyme/substrate ratio, the affinity of the multicomponent enzyme system used for the substrate used, the fact that the topography of the substrate changes as digestion proceeds, together with factors such as inactivation of the bound or free enzyme, and the effects of products of action or other substances that might promote or inhibit adsorption.

When relatively pure celluloses are used, the cellulases are rapidly adsorbed. This initial phase is followed by a gradual release of enzyme to the solution as hydrolysis proceeds (Mandels, Kostick and Parizek, 1971; Huang, 1975; Wilke and Yang, 1975; Bisaria and Ghose, 1977; Kolankaya and Kaytan, 1980; Moreira, Phillips and Humphrey, 1981a; Moloney and Coughlan, 1983). By contrast, using milled newspaper Brandt, Hontz and Mandels (1973) found that although protein was desorbed as digestion proceeded, the adsorbed enzyme activity was not returned to solution .They concluded that the enzyme was being inactivated—a phenomenon that did not occur when pure cellulose was used as adsorbent. Subsequently, however, Castanon and Wilke (1980) on the basis of enzyme assays and electrophoresis of reaction mixtures were of the opinion 'that enzyme once bound to newspaper remains immobilized within the supramolecular organization of the substrate even after extensive degradation has taken place'. Clearly, as has been shown by several research groups (e.g. Peitersen, Medeiros and Mandels, 1977; Deshpande and Eriksson, 1984) the nature of the substrate greatly influences adsorption. Indeed, Lee, Shin and Ryu (1982) state that the adsorption of cellulase usually parallels the rate of hydrolysis of cellulose. This may be true for purified substrates but does not hold for impure lignocellulosic materials (*see* e.g. Moloney and Coughlan, 1983), almost certainly because the undigested lignin fraction of such materials may adsorb up to 50% of the endo- and exoglucanase activities (Deshpande and Eriksson, 1984).

Most investigators find that the extent of adsorption increases as the cellulose concentration or enzyme concentration is increased, the adsorption behaviour obeying Michaelis–Menten kinetics or the Langmuir adsorption

isotherm (Mandels, Kostick and Parizek, 1971; Peitersen, Medeiros and Mandels, 1977; Lee, Shin and Ryu, 1982; Goel and Ramachandran, 1983; Moloney and Coughlan, 1983). Generally speaking adsorption, or specific adsorption, in the initial rapid phase (*see above*) is greatest under the conditions of pH and temperature that are optimal for hydrolysis (Mandels, Kostick and Parizek, 1971; Bisaria and Ghose, 1977; Rabinovich, Van Viet and Klesov, 1982; Moloney and Coughlan, 1983). However, desorption in the succeeding slow phase is also greatest under these conditions (Mandels, Kostick and Parizek, 1971; Bisaria and Ghose, 1977; Moloney and Coughlan, 1983). Thus, for example, at 4°C the extent of adsorption in the initial rapid phase is low but such adsorption continues until ultimately much more enzyme is bound than at higher temperatures (Moloney and Coughlan, 1983). Presumably, the greater rate of hydrolysis of substrate at the higher temperatures effects a more rapid rate of decrease in the number of sites to which the enzymes can bind, thereby giving rise to a greater rate of desorption (but *see below* for an alternative explanation).

Mandels, Kostick and Parizek (1971) and Bisaria and Ghose (1977) showed that the extent of cellulase adsorption to a given cellulose increased as the substrate particle size decreased, i.e. the surface area increased. However, methods for measuring available surface area are not as satisfactory as those for measuring crystallinity (Mandels, 1985). Indeed, the most direct measure of available surface area is the absorption of cellulase: the rate of hydrolysis is proportional to surface area measured thus (Berezin and Klesov, 1980). In more recent studies Lee, Shin and Ryu (1982) claimed that the extent of adsorption of enzyme in the initial phase correlated well with the specific surface area (SSA) and with the crystallinity index (C_rI) of cellulosic substances; the effects of the crystallinity of milled cotton on adsorption were similar to those on hydrolysis. In contrast, Peitersen, Medeiros and Mandels (1977) and Lee, Shin and Ryu (1982) showed that the extent of adsorption on Avicel was similar to that on Sweco-270, even though the respective SSA and C_rI values of Avicel (5·4 m^2/g and 80·8%) and Sweco-270 (24·2 m^2/g and 12·5%) were very different, while Goel and Ramachandran (1983) found that particle size and crystallinity had only a limited effect on the adsorption of the cellulases in *T. reesei* D1-6 culture filtrates. In consequence of their findings, Lee, Shin and Ryu (1982) concluded that the adsorption affinity as well as structural properties have a significant influence on adsorption.

The differential affinity of cellulolytic components for substrate has been exploited for the purposes of enzyme fractionation and purification (*see above*). Non-cellulolytic proteins such as albumin are not adsorbed on cellulose or are much less tightly bound (Bisaria and Ghose, 1977; Lee, Shin and Ryu, 1982), nor is the β-glucosidase component in filtrates of *T. reesei* bound by pure celluloses, whereas the endo- and exoglucanases are (Ghose and Bisaria, 1979; Mandels *et al.*, 1981; Reese, 1982; Ooshima, Sakata and Harano, 1983). Other investigators find that all extracellular cellulolytic components of *Trichoderma* spp. (Enari, 1983; Goel and Ramachandran, 1983; Deshpande and Eriksson, 1984) and *Talaromyces emersonii* (Moloney and Coughlan, 1983) are bound. Indeed, Goel and Ramachandran's findings

indicate that β-glucosidase is more tenaciously bound than the endo- or exoglucanases which were bound equally tightly. The endo- and exocellulase components of the *Trichoderma reesei* system are adsorbed to the same extent (Wilke and Yang, 1975; Bisaria and Ghose, 1977) as are those of *Talaromyces emersonii* (Moloney and Coughlan, 1983). However, Li, Flora and King (1965), Mandels, Kostick and Parizek (1971) and Wilke and Mitra (1975) claimed that the exoglucanase fraction of *Trichoderma viride* filtrates adsorbs more strongly than the endoglucanase to Solka floc. Ooshima, Sakata and Harano (1983) found the ratio of adsorbed components to be temperature-dependent, the endoglucanases being preferentially bound at low temperatures (5°C), whereas at the temperature at which activity was maximal (50°C), the exocellobiohydrolase was the more tightly adsorbed.

Lee, Shin and Ryu (1982) compared the adsorption/desorption behaviour of cellulases on a number of substrates. They concluded that enzymes were continuously adsorbed when the *initial* adsorption was hindered by the inaccessibility of the substrate or by the presence of non-hydrolysable materials. By contrast, enzyme proteins that adsorbed maximally at the initial stages of hydrolysis were gradually released with reaction time as the crystalline and inaccessible fractions of substrate increased. These conclusions may explain the effects of substrate type and of temperature discussed above.

More recent findings by Ryu, Kim and Mandels (1984) who examined the adsorption of the separated cellulolytic components of *Trichoderma reesei* MCG 77 on Avicel have mechanistic significance. They postulated that the endoglucanases and cellobiohydrolases adsorb at distinctly different sites on cellulose, these sites corresponding to the sites of hydrolysis. Moreover, the addition of cellobiohydrolase to substrate to which endoglucanase is bound 'affects' the latter in such a way as to speed up its action and bring about its desorption. Conversely, endoglucanase on binding to cellulose speeds up the rate of scission by cellobiohydrolase and brings about its desorption. This competitive adsorption which (the authors hypothesize) explains the synergistic interaction of these components in cellulose hydrolysis, was most evident when both components were present in that ratio found in the crude filtrate (*Figure 6*).

During the course of the above investigations, Ryu, Kim and Mandels (1984) found that the endoglucanase fraction consisted of adsorbable and non-adsorbable components. Similar findings have been reported by Manning and Wood (1983) with respect to the endoglucanases synthesized by *Agaricus bisporus* when grown on microcrystalline cellulose. One may wonder what is the function of endoglucanases that do not adsorb to cellulose. Is it to hydrolyse the long-chain cello-oligosaccharides released during saccharification? The reports of Klesov and colleagues (Rabinovich, Van Viet and Klesov, 1982; Klesov *et al.*, 1982) may be pertinent, in that they investigated the adsorption of the endoglucanases, produced by 12 different organisms, on a variety of cellulosic substrates differing in specific surface area, degree of crystallinity and of polymerization. They conclude that the only decisive factor in the effective hydrolysis of amorphous cellulose is the amount of

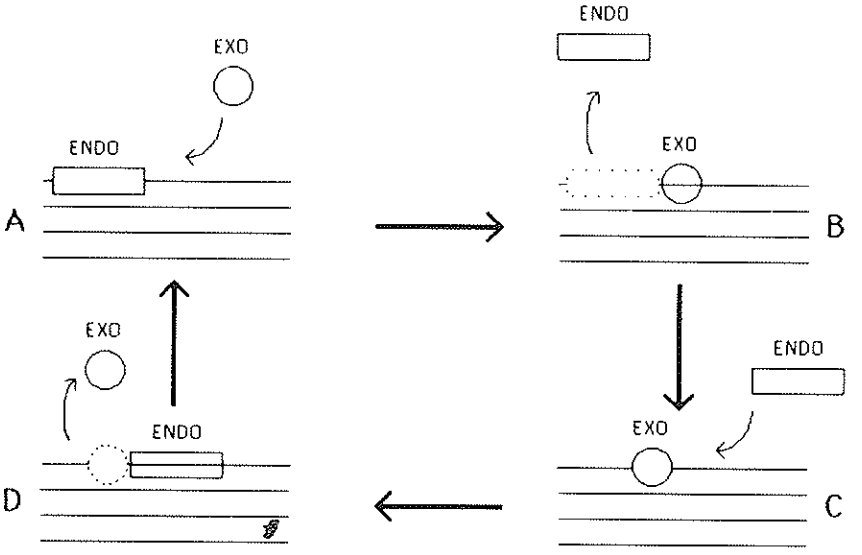

Figure 6. Competition between endo- and exoglucanases for adsorption to substrate (based on the findings of Ryu, Kim and Mandels, 1984). (A) Endoglucanase has bound to a cellulose chain and catalysed cleavage of a glycosidic linkage. (B) Exoglucanase then binds to a contiguous but non-overlapping site and promotes desorption of the endoglucanase. (C) The exo enzyme has catalysed cleavage of a glycosidic linkage and an endo enzyme is seen approaching a binding site that does not overlap the exo site and need not be contiguous with the latter. However, binding of the endo-acting enzyme will influence binding of the exo enzyme and promote desorption of the latter (as in D). In this scheme the exoglucanases do not act in a processive manner, i.e. they do not continue to remove cellobiose units as they diffuse along a chain. Rather, must they dissociate following bond cleavage and reassociate again contiguous to an endoglucanase. However, there is no reason to believe that several molecules of exoglucanase cannot simulate processivity with amorphous substrates.

enzyme bound to it. By contrast, hydrolysis of the crystalline substrate by complete systems depends not only on the extent but also on the 'quality' of binding of the endoglucanase, this being characterized by the adsorption equilibrium constant. In this context, endoglucanases fall into two classes, those that adsorb 'strongly' and those that adsorb 'weakly'. The former predominate in cellulase systems that are highly active against crystalline cellulase, whereas the 'weakly' binding forms predominate in filtrates that are relatively inactive against such substrates. Both forms of enzyme are equally active against soluble substrates.

Bisaria and Ghose (1977) and Rabinovich, Van Viet and Klesov (1982) claim that adsorbed cellulases can be removed by buffer, whereas Mandels, Kostick and Parizek (1971) and Enari (1983) find that these enzymes are desorbed only on hydrolysis of the substrate. Castanon and Wilke (1981) reported that the presence of the surfactant, Tween 80, increased the rate and extent of cellulose saccharification by filtrates of *Trichoderma reesei* QM9414, even though a larger proportion of the enzyme remained in solution. The increased saccharification could be explained by an effect of Tween 80 in

decreasing non-productive binding to the substrate: however, Mandels, Kostick and Parizek (1971) and Moloney and Coughlan (1983) also investigated the use of Tween 80 and found no such effect. Reese (1982) showed that substances such as urea, guanidine-HCl, and various solvents did promote desorption of the enzyme system from Avicel and that the enzymes were sufficiently stable to permit recovery; however, Deshpande and Eriksson (1984) reported recovery of only 50% of the cellulolytic enzymes in soluble form even after almost complete hydrolysis of impure lignocellulosic substrates. The remaining enzymes ultimately could be recovered from the lignin to which they were bound but the authors question whether such recovery is economically feasible. Mandels, Kostick and Parizek (1971) found that cellulose can adsorb sufficient enzyme for its digestion without replenishment of enzyme, even when the sugar-containing liquid phase was continuously removed. As cellulose was digested, the released enzyme was readsorbed on freshly added substrate with retention of activity. The substrate used in these studies was Solka floc, which is a relatively pure cellulose. Nevertheless, the implications of this approach for continuous saccharification processes are clear.

SYNERGISM

The first demonstration of synergism in cellulose digestion was reported 30 years ago by Gilligan and Reese (1954) who fractionated culture filtrates of *Trichoderma viride* and *Myrothecium verrucaria* by chromatography on calcium phosphate gel. They found that the extent of hydrolysis of Walseth cellulose, an acid-swollen partially degraded substrate, by the combined fractions was somewhat greater than the calculated sum of the extents of hydrolysis by the individual fractions. Almost a decade later, Mandels and Reese (1964) and Li, Flora and King (1965) showed that the endoglucanase and C_1 (now known to be exoglucanase) fractions of *Trichoderma viride* acted synergistically to effect hydrolysis of more recalcitrant substrates such as cotton, hydrocellulose and Avicel. Since then various investigators have shown that the individual exoglucanase (exocellobiohydrolase), endoglucanase and β-glucosidase fractions by themselves have little action on crystalline substrates, but mixtures of the endoglucanase and exoglucanase fractions do allow extensive hydrolysis. The addition of the β-glucosidase fraction to the above mixture usually gives a further small increase in activity up to that shown by the unfractionated filtrates (*see* e.g. *Figure 7*). Synergism between endo- and exoglucanases has been shown for the enzymes from *Trichoderma viride* and *Penicillium funiculosum* (Selby, 1969), *Trichoderma koningii* and *Fusarium solani* (Wood, 1969, 1975, 1980; Wood and McCrae, 1972, 1975, 1977a), *Sporotrichum pulverulentum* (Eriksson, 1975) and *Talaromyces emersonii* (McHale and Coughlan, 1980). Synergism is most marked when highly crystalline substrates are used, is low with amorphous cellulose and absent with soluble derivatives (Wood and McCrae, 1979). Synergy is at a maximum when the components are used in the ratios in which they occur in the original fungal fermentation filtrates (Wood, 1975). Cross-synergism between the

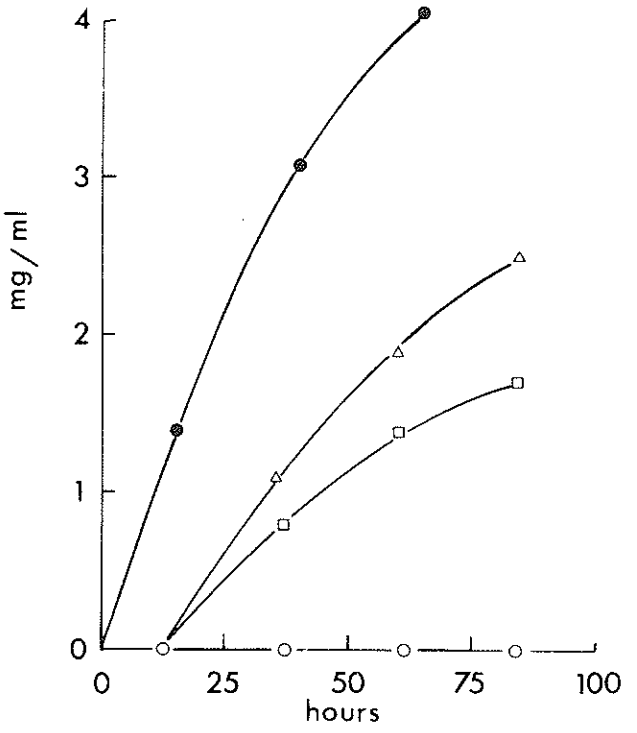

Figure 7. Synergistic hydrolysis of cellulose by components of *Talaromyces emersonii* filtrates. (●) Full complex added at zero time; (○) endocellulase alone added at zero time; (□) endocellulase added at zero time and exocellulase added at 12 h; (△) endocellulase added at zero time and exocellulase plus β-glucosidase added at 12 h (from McHale and Coughlan, 1980, courtesy of *FEBS Letters*).

exoglucanases produced by one organism and the endoglucanase fraction of another has also been demonstrated (Selby, 1969; Wood, 1969, 1975, 1980; Wood and McCrae, 1975, 1977a; Moloney *et al.*, 1985). This cross-synergistic interaction in the hydrolysis of cotton was strongest between the endo- and exoglucanase fractions produced by fungi that readily secrete active exocellobiohydrolase into the medium. Thus, cross-synergism between the appropriate components of *Trichoderma viride*, *Trichoderma koningii*, *Penicillium funiculosum* and *Fusarium solani* (Wood, 1975, 1980) is high. By contrast, that between the exoglucanase component from any of the above organisms and the endoglucanases synthesized by *Myrothecium verrucaria*, *Stachybotrys atra*, *Gliocladium roseum* or *Memnoniella echinata* is low (Wood, 1969, 1975, 1980). Although the last four organisms named above grow well on cotton and so may be presumed to produce exocellobiohydrase and endoglucanase, only the latter is found in culture filtrates (Wood, 1975).

In an extension of the above investigations, Wood (1980) grew *Trichoderma koningii* on a variety of cellulosic substrates and isolated the endo- and exoglucanase fractions. The cellobiohydrolase induced on one

cellulose source was mixed with the endoglucanase induced on another and the extent to which cotton was solubilized was measured. Regardless of the inducing substrate used, all of the cellobiohydrolases showed the same capacity for hydrolysing cotton when acting with the endoglucanase induced on this substrate. By contrast, considerable differences were observed between the extents to which cotton was solubilized when the cotton-induced cellobiohydrolase was mixed with the endoglucanases induced on different cellulosic sources; this suggests that synergism between endo- and exocellulases may be dictated primarily by the endocellulase. This supposition is strengthened by the observation that not all of the separated endoglucanases in a single filtrate can act synergistically or equally synergistically with the exoglucanase fraction from the same filtrate (Selby, 1969; Eriksson, 1975; Wood, 1975). In addition, the individual endoglucanases in these instances exhibited considerable differences in physicochemical and biological properties, perhaps because of differences in primary structures. By contrast, the four endocellulases isolated from filtrates of *Talaromyces emersonii* are similar, apart from their p*I* values and carbohydrate contents; each co-operates equally with the exoglucanase fraction in the hydrolysis of Avicel (Moloney *et al.*, 1985). These findings apparently indicate the primacy of the endocellulase components in 'endo–exo' synergism. The nature of the endocellulase component may determine whether or not complex formation with the exoglucanases can take place on the surface of the substrate (Wood, 1975, 1980). Similarly, it may be a deciding factor in the competitive adsorption between the endo- and exoglucanase fractions (Ryu, Kim and Mandels, 1984). Nevertheless, the immunologically unrelated cellobiohydrolases I and II of *Penicillium funiculosum* can both act synergistically with the endoglucanase produced by this organism to solubilize cotton, but only form I can co-operate with the endoglucanases of *Fusarium solani* and *Trichoderma koningii* (Wood, 1985a). All of the synergistic phenomena above are of the endo–exo type in which it is envisaged that endoglucanases cleave cellulose chains at random, thereby creating ends from which the exocellobiohydrolases remove cellobiose units. White and Brown (1981), using electron microscopy, have demonstrated the synergistic interaction between endo- and exo-acting components in the hydrolysis of crystalline cellulose. Both components must be present simultaneously (*Figure 8*). The β-glucosidase component completes the synergistic action by cleaving the cellobiose to dextrose, thus relieving inhibition of cellulase action.

Synergism between endocellulases and the exoglucohydrolase type of enzyme did not occur, at least in the case of the enzyme from *P. funiculosum* (Wood and McCrae, 1982). However, two unusual types of synergism have been documented. Kanda, Wakabayashi and Nisizawa (1976b) reported that considerable hydrolysis of crystalline cellulose could be brought about by the combined actions of *Irpex lacteus* endoglucanases differing only in the manner of their attack on soluble cellulose; we have no explanation of this endo–endo synergistic interaction. However, as noted earlier, hydrolysis of crystalline cellulose by *Clostridium thermocellum*, which may (Lamed *et al.*, 1983) or may not (Ljungdahl *et al.*, 1983) produce exocellulases in addition to

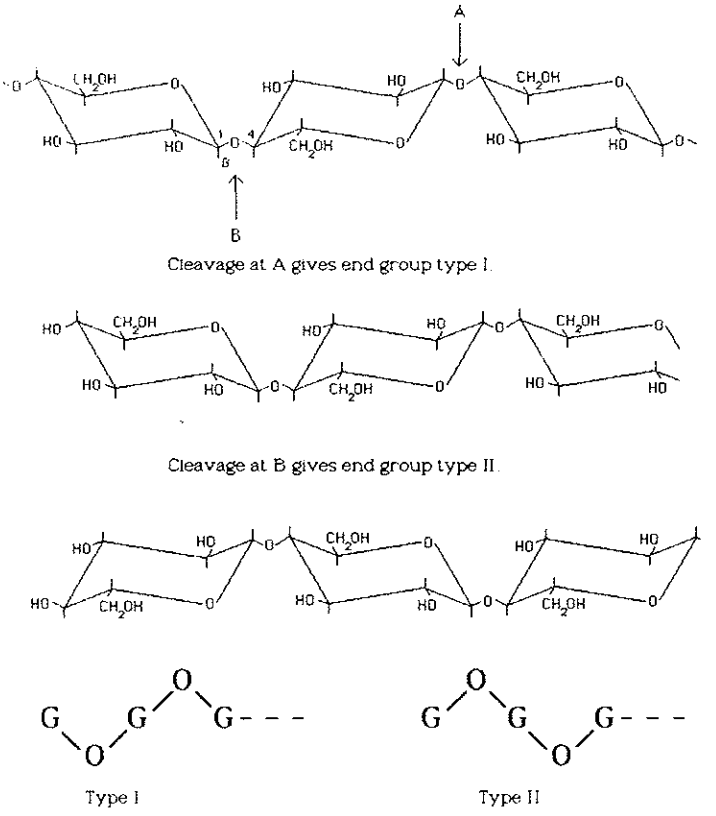

Figure 8. Proposed mechanism for the synergistic hydrolysis of cellulose by endo- and exoglucanases and of the need for multiple forms of each enzyme (after Wood, 1981, 1985b). The order in the structure of cellulose is such that glycosidic linkages cleaved by endoglucanases acting alone will rapidly re-form. However, if exocellobiohydrolase is also present it will remove cellobiose units from the chain ends provided by the endoglucanases. For stereochemical reasons the glycosidic linkages between successive residues, locked in position by hydrogen bonds (*see Figure 1*), will be in different planes. Moreover, the array of hydrogens and hydroxyls on successive residues differ. Thus, at least two endoglucanases differing in stereospecificity will be required for cleavage of glycosidic linkages in native cellulose (positions A and B). Furthermore, depending on the linkage cleaved, two different non-reducing end groups will be generated. This in turn means that at least two stereospecific types of exocellobiohydrolase will be required (Wood, 1981, 1985a,b).

endocellulases, is brought about by the latter acting in an ordered multi-subunit complex that may allow appropriately orientated binding of the enzymes and of the bacterium to the substrate (Enari, 1983; Lamed *et al.*, 1983). Another type of synergistic interaction, an exo–exo kind, has been reported by Fägerstam and Pettersson (1980), who found that the hydrolysis of crystalline cellulose by the combined action of cellobiohydrolases I and II of *Trichoderma reesei* was at least twice that calculated from the extent of hydrolysis by each enzyme separately. It is unfortunate that the actual extent of hydrolysis was not reported in this paper. However, Wood (1985b) has also

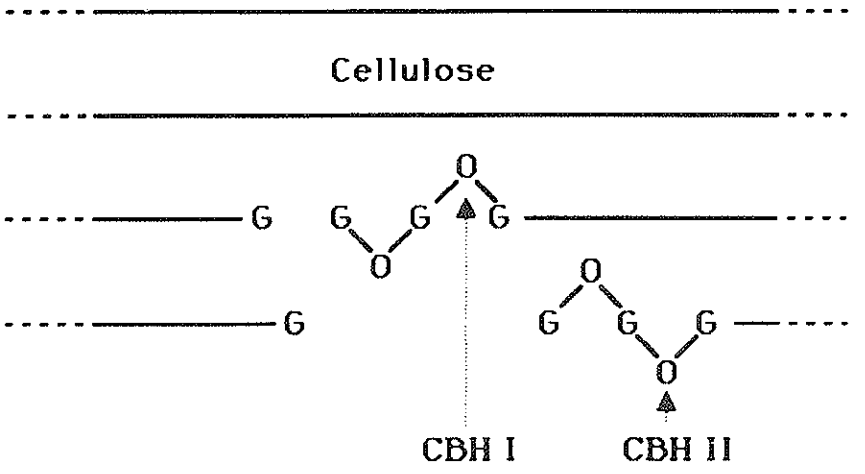

Figure 9. Proposed explanation for exo–exo synergism in the hydrolysis of cellulose (after Wood, 1985b). Details in the text.

recently claimed that the two cellobiohydrolases of *Trichoderma reesei* and *Penicillium funiculosum* interact synergistically in solubilizing Avicel and cotton fibre. Among the possible explanations for this exo–exo synergism considered by Fägerstam and Pettersson (1980) was that it reflected structural asymmetry in the substrate. Wood (1985b) has put forward a proposed explanation on just such lines (*Figure 9*): the two cellobiohydrolases I and II could exhibit different substrate stereospecificities, each attacking one of the two different non-reducing end groups that may be found in the substrate. Removal of cellobiose units from one type of non-reducing chain end could expose, on an adjacent chain, a non-reducing end of the other type from which the second stereospecific cellobiohydrolase would remove cellobiose. The successive operation of each enzyme on the adjacent chains could then explain the observed synergism.

KINETICS

Among the factors that might reasonably be expected to influence the hydrolysis of cellulosic substrates are the nature of the enzyme system itself, the physical attributes of the substrate and the interaction(s) between the enzyme and the substrate. At least three different enzymes, endoglucanase, exocellobiohydrolase and β-glucosidase are required for extensive hydrolysis of crystalline cellulose. Each enzyme is generally found to exist in several forms and these forms may differ in substrate specificity, ability to absorb to the substrate and in their capacity to interact synergistically with other enzymes in the system. Furthermore, non-enzymic factors may be involved in substrate conversion. The substrate may be a pure cellulose or, for example, an agricultural waste containing other polysaccharides, including hemicellulose and pectin, and also lignin which can bind a considerable quantity of

enzyme in a non-productive manner. Even with pure celluloses the degree to which it is amorphous or crystalline (the C_rI value), the specific or effective surface area and even the degree of polymerization will have an important bearing on reaction kinetics. Moreover, the topology of the substrate will change as the hydrolysis reaction proceeds. With respect to enzyme–substrate interaction, the adsorption-desorption behaviour of the individual components must be considered, as must the fact that adsorption may or may not be productive. The possibility of thermal or shear inactivation of enzymes during the saccharification process must be considered, and so must the effects of cellobiose, glucose and other products on enzyme activity. A useful summary of existing kinetic models for cellulose hydrolysis has been given in a paper by Wald, Wilke and Blanch (1984).

The model of Howell and Stuck (1975) did not, in the initial stages of reaction, differentiate between amorphous and crystalline regions of the cellulose used (Solka floc, BW 200) nor between the bulk and effective substrate concentrations. Account was taken of adsorption of the *Trichoderma viride* enzymes and did include non-competitive inhibition by cellobiose. The proposed model agreed with the experimental data up to 65% of substrate conversion at substrate concentrations between 10 and 15 g/ℓ. Deviation from predicted behaviour was observed at greater extents of hydrolysis and at lower substrate concentrations. Huang (1975) examined the hydrolysis of phosphoric-acid-swollen Solka floc (an amorphous substrate) by filtrates of *Trichoderma viride*. The proposed kinetic model included a fast adsorption phase, a slower reaction phase and inhibition by cellobiose and to a lesser extent by dextrose. Adsorption was considered to occur so quickly that equilibrium was established at all times. The dissociation of the enzyme–substrate (i.e. cellulase–cellulose) complex to free enzyme plus products (mainly cellobiose and dextrose) was said to be rate-limiting and the inhibition to be competitive. Correlation between model and data was good up to 70% saccharification. Maguire (1977) using cellobiohydrolase isolated from filtrates of *Trichoderma koningii* investigated the initial rates of cellobiose release from fibrous α-cellulose. Adsorption was observed to obey the Langmuir adsorption isotherm. Experimental observations on the time course of the reaction up to 40% hydrolysis of substrate fit a competitive inhibition model better than other types of inhibition. The K_i value for cellobiose was calculated to be 1·13 mmol and the E_a value to be 5·3 kcal/mol between 5° and 60°C at pH 5·2. Inspection of reaction velocity as a function of pH indicated enzyme pK values of 3·8 and 5·5. Brown and Waliuzzman (1977) considered that hydrolysis of milled newspaper by filtrates of *Trichoderma viride* QM9123 operated by Michaelis–Menten kinetics with competitive inhibition over the first 10 hours, after which, deviation from the proposed model was found.

The model proposed by Okazaki and Moo-Young (1978) was also based on Michaelis–Menten-type kinetics for simultaneous endo- and exo- attack on substrate. The model incorporated end-product inhibition and the operation of three separate enzymes. The experimental findings suggested that the hydrolysis kinetics are much affected by the degree of polymerization of the

substrate and the ratio of endo- and exo-acting enzymes in the reaction. Lee and Fan (1982) studied the kinetics of hydrolysis of Solka floc by filtrates of *Trichoderma reesei*. The initial hydrolysis rate was seen to depend mainly on the initial extent of enzyme adsorption and the ability of the adsorbed enzyme to effect hydrolysis. The initial extent of adsorption reflected the starting concentrations of enzyme and cellulose and the specific surface area of the latter. The ability of the bound enzyme to effect hydrolysis was governed by the initial crystallinity index. These workers emphasize that the Michaelis–Menten-type kinetics are inappropriate and that a mechanistic kinetic model should incorporate the effects of cellulose structure, enzyme adsorption and effectiveness. Lee and Fan (1983) extended these studies to longer reaction times. The marked decline in the hydrolysis rate during the early period of the reaction was consequent on product inhibition and conversion of the substrate to a less digestible form with an increased crystallinity index and a decreased specific surface area. They claimed that product inhibition was caused by deactivation of the adsorbed protein by the products. Deactivation was said to be linearly related to dextrose concentration but related to cellobiose concentration in a hyperbolic fashion.

Holtzapple, Caram and Humphrey (1984a) investigated the hydrolysis of Solka floc in a batch reactor using a commercial (Meicelase) *Trichoderma viride* enzyme preparation. Fifty different hydrolysis conditions were examined over a tenfold range of enzyme concentrations and a thirtyfold range of substrate concentrations. The abilities of various models to predict the experimental findings were compared. The model that most accurately correlated with the data was called, appropriately enough, the HCH-1 model, and incorporated product inhibition and adsorption of enzyme to the substrate, and distinguished between free and total binding sites on cellulose. Using this model the authors found that cellobiose and dextrose non-competitively inhibited the hydrolysis of cellulase by filtrates of *Thermomonospora* spp. and they determined the binding constants for both inhibitors (Holtzapple, Caram and Humphrey, 1984b): cellobiose was 14 times more effective in this regard. The authors include a convincing explanation for the general finding that inhibition of cellulose hydrolysis by cellobiose is non-competitive rather than competitive. Competitive inhibition will obtain for soluble substrates if the inhibitor binds to the active site and so prevents ES formation, whereas non-competitive inhibition will be observed when the inhibitor binds to a site (control site) other than the active site so as to bring about alteration of the three-dimensional conformation of the latter. Non-competitive inhibition may also be observed with insoluble substrates when the inhibitor binds to the control site of the enzyme. However, competitive and non-competitive patterns may be manifested if the inhibitor binds to the active site. Cellobiose, being of low molecular weight, can readily diffuse to the active site. If the substrate is also able to do so it could successfully compete with cellobiose. Such substrates would be largely amorphous, in which case a considerable proportion could be engaged in enzyme–substrate complex formation. Crystalline substrates, on the other hand, may not diffuse rapidly to the enzyme active site. Cellobiose would then be able to bind to

most of the enzyme molecules and non-competitive inhibition would be exhibited. In more recent papers Holtzapple, Caram and Humphrey (1984c) and Wald, Wilke and Blanch (1984) have presented kinetic models that successfully simulated the saccharification of 'real' substrates (poplar wood and rice straw, respectively), under conditions that might reasonably be expected to be operative in commercial processes. Both models allow for enzyme adsorption, product inhibition and multiplicity of enzyme and substrate forms.

Properties of the major components of cellulase systems

ENDOGLUCANASES

Among the outstanding characteristics of these enzymes is their ability to catalyse the hydrolysis of amorphous cellulose, soluble derivatives of cellulose, such as CMC, and cello-oligosaccharides, together with an inability to effect significant degradation of crystalline substrates. Endoglucanases may be classified on the basis of the 'randomness' of their attack on substrates such as CMC. The greater the decrease in substrate viscosity (increase in fluidity) for a given increase in reducing sugars, the more random the attack on the substrate. Thus, the action of an enzyme with a highly random action is accompanied by a marked increase in fluidity with little release of reducing sugar. Almin, Eriksson and Pettersson (1975), using a viscometric technique, compared the activities against CMC of the five endoglucanases produced by *Sporotrichum pulverulentum*. They found that enzyme–substrate interactions could not be interpreted on the basis of K_m values, but that the A_m values (i.e. molecular activity—bonds cleaved per second per enzyme molecule) were more informative.

The affinity of endoglucanases for cello-oligosaccharides and the rate at which they hydrolyse these substrates increase with increasing polysaccharide chain length. Indeed, the use of chemically or radioactively labelled cellodextrins has done much to distinguish between the mechanisms of action of different endoglucanases. Two examples of such use are shown below. In A, the glucosyl residue at the non-reducing end of the cellohexaose is ^{14}C-labelled, whereas in B the glucosyl residue at the other end has been reduced to sorbitol (King and Vessal, 1969).

(A)

$$^{14}C\text{—}G^1\text{—}G^2\text{—}G^3\text{—}G^4\text{—}G^5\text{—}G$$

(B)

$$G^1\text{—}G^2\text{—}G^3\text{—}G^4\text{—}G^5\text{—}S$$

If the endocellulase in question cleaves the glycosidic linkage between residues two and three (bond no. 2 as indicated) the products will be ^{14}C-cellobiose plus cellotetraose in A and cellobiose plus triglucosylsorbitol in B. Thus, analysis of the products will establish the site of attack and show whether the endoglucanase in question preferentially acts at internal linkages

or at the non-reducing ends of the cellodextrins. The enzymes isolated from culture filtrates of *Trichoderma koningii* (Halliwell and Vincent, 1981) and of *Aspergillus niger* (Hurst, Sullivan and Shepherd, 1978) act in the former fashion. Halliwell and Vincent (1981) consider that the cellodextrins 'associate with the enzyme in a symmetrical fashion so that all glucosyl units are available to the active site'. However, the relative amounts of product formed from cellodextrins indicate that internal linkages are the preferred points of cleavage. Endoglucanases II and IV of *Trichoderma viride* also display a preference for internal linkages of such substrates, whereas endoglucanase III from filtrates of this same organism preferentially cleaves cellobiose units from the non-reducing ends (Shoemaker and Brown, 1978a,b).

In the case of the endocellulases from *Aspergillus niger* (Hurst, Sullivan and Shepherd, 1978) and *Thermoascus aurantiacus* (Shepherd, Cole and Tong, 1981) the use of reduced cellodextrins has also shown that for optimal activity the substrate should be five to six glucosyl residues long. This is reminiscent of the situation that is operative in the case of lysozyme and, indeed, more than 20 years ago it had been postulated that all glycosidases might operate via a lysozyme-type mechanism (Vernon and Banks, 1963); however, it was not until recently that Canadian investigators showed that this is, in fact, the case. They determined the *N*-terminal sequence of an endoglucanase (EG-1) produced by *Schizophyllum commune* (Yaguchi *et al.*, 1983). The sequence from Glu-33 to Tyr-51 was homologous with the active-site sequence of hen egg-white lysozyme including the lysozyme catalytic residues (Glu-35, Asp-52) and the substrate-binding residue, Asn-44. This catalytic site homology has since been extended to a second endoglucanase (EG-2) from this organism by Paice *et al.* (1984), who also showed that the active site region, residues Glu-65 to Asp-74, in an exocellobiohydrolase produced by *Trichoderma reesei* is homologous with the catalytic sequence Glu-11 to Asp-20 in the lysozyme produced by phage T4. Demonstration of these similarities between the catalytic sites of an exocellobiohydrolase from *Trichoderma reesei* and endoglucanases of *Schizophyllum commune* justifies the hypothesis by these authors that cellulases and lysozymes utilize a common catalytic mechanism (*Figure 10*). It will be noted later that an aspartyl side chain is also present at the active centre of β-glucosidase. Goksoyr *et al.* (1975) compared the amino-acid composition of a large number of cellulases, and found that, with the exception of the *Pseudomonas* enzymes, all showed a high degree of similarity, thereby providing further evidence in favour of the Canadian group's hypothesis. A summary of the properties of well-documented endoglucanases is given in *Table 4*. The fungal enzymes are usually extracellular acidic glycoproteins and range in size from 12 500 to over 90 000 daltons. The bacterial enzymes are also mainly acidic glycoproteins but usually form part of larger molecular assemblies probably attached to the cell surface. The evident disparities between the reports on these enzymes and their properties, even when the same organism is being investigated, may be explained in part by differences in the strain in use and in medium and cultural conditions. Endoglucanases may differ from one another not only in bond preference (internal or external) but also in their

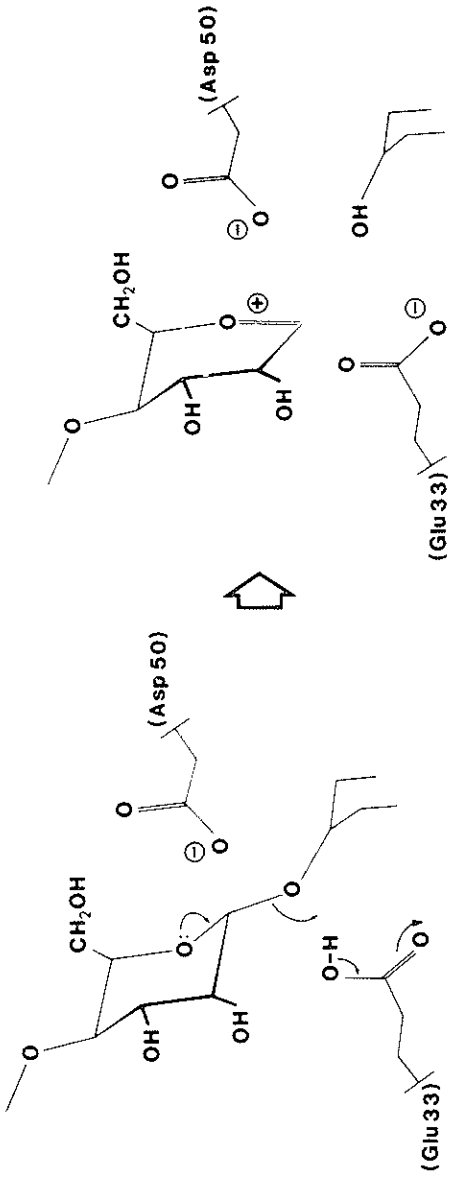

Figure 10. Proposed mechanism for the endoglucanase-catalysed cleavage of β-1,4 linkages in cellulose (from Yaguchi *et al.*, 1983, courtesy of *Biochemical and Biophysical Research Communications*). By analogy with the mechanism of action of lysozyme (Stryer, 1981) an H$^+$ is transferred from GLU33 to the oxygen of the glycosidic bond which is thereby cleaved. The resultant carbanion is stabilized by ASP50. Some of the products diffuse away at this stage. The hydrolysis reaction is completed by the addition of -OH$^-$ to the carbanion intermediate, an H$^+$ to the side chain of GLU33 and release of the remaining product.

Table 4. Properties of endoglucanases

Organism	Enzyme	$M_r \times 10^{-3}$	Carbohydrate content (% of dry wt)	p*I*	Optimum pH	Optimum temperature (°C)	T½	Reference
Aspergillus fumigatus		12·5	0	7·1	4·8	60	90 min at 50°C, pH 4.8	Parry, Stewart and Hepinstall (1983)
BW (a thermophilic bacterium)		91–99	2	3·85	6·4	60	1 h at 85°	Creuzet and Frixon (1983)
Clostridium thermocellum		83–94	11·2	6·72	5·2	62	—*	Ng and Zeikus (1981)
Clostridium thermocellum		56	—	6·2	6·0	65	Deviates from Arrhenius above 65°	Petre, Longin and Millet (1981)
Fusarium lini		28	2·9	8·3	—	—	—	Vaidya *et al.* (1984)
Fusarium solani	1	37	—	4·75				Wood (1971)
	2	37	—	4·80–4·85				
	3	37	—	5·15				
Humicola grisea		75	9·4		5·0	50	Rapidly degraded above 55°	Yoshioka, Anraku and Hayashida (1982)
Irpex lacteus	S1	56·0	12·2	—	4·0–5·0	50	20 min at 65°C, pH 4	Kanda, Wakabayashi and Nisizawa (1976a, 1980)
	E_n1	15·5	0·75	—	4·0	50	Killed in 10 min at 70°C	
Irpex lacteus	E_2A	—	—	—	4·0	60	Killed in 10 min at 70°C	Kubo and Nisizawa (1983)
	E_2B	—	—	—	4·0	60	30% loss in 10 min at 70°C	

Sporocytophaga myxococcoides	I	46	—	7·5	6·5–7·5	—	—	Osmundsvag and Goksoyr (1975)
	II	52	2	4·75	5·5–7·5	—	—	
Sporotrichum pulverulentum	T_1	32·3	10·5	5·32	—	—	—	Eriksson and Pettersson (1975a)
	T_{2a}	36·7	0	4·72	—	—	—	
	T_{2b}	28·3	7·8	4·40	—	—	—	
	T_{3a}	37·5	4·7	4·65	—	—	—	
	T_{3b}	37·0	2·2	4·20	—	—	—	
Talaromyces emersonii CBS 814.70	I	35·0	27·7	3·19	5·5–5·8	75–80	At 75°,pH 5,104min	Moloney *et al.* (1985)
	II	35·0	29·0	3·08			93 min	
	III	35·0	44·7	2·9–3·0			75 min	
	IV	35·0	50·8	2·86			66 min	
Thermoascus aurantiacus	III	34·5	1·8	—	—	—	—	Tong, Cole and Shepherd (1980)
Trichoderma koningii	E_1	13·0	—	4·73	5·5†	60†	Instability begins above 35°C†	Wood and McCrae (1978) Halliwell and Vincent (1981)
	E_{3a}	48·0	—	4·32				
	E_{3b}	48·0	—	4·34				
	E_4	31·0	—	5·09				
Trichoderma viride	IIA	30·0	12–14	—	4·5–5·0	60	100°C for 10 min, 27% retained	Okada (1975, 1976)
	IIB	43·0	12–14	—	4·5–5·0	50	41% retained	
	III	45·0	—	—			40% retained	
Trichoderma viride	I	12·5	21·0	4·60	—	—	—	Berghem, Pettersson and Axio-Fredriksson (1976)
	II	50·0	12·0	3·39	—	—	—	
Trichoderma viride	II	37·2	4·5	—			Labile above pH 8, 45°C	Shoemaker and Brown (1978b)
	III	52·0	15·0	—	4·0–4·5			
	IV	49·5	15·2	—				
Trichoderma viride	I	20·2	0	7·52	—	—	—	Hakansson *et al.* (1978, 1979)
	II	51·0	0	4·66	—	—	—	

* A dash indicates that there are no reported data. † Reported by Halliwell and Vincent (1981).

ability to adsorb to the substrate and interact synergistically with exoglucanases. Possible reasons for the observed multiplicity of enzyme forms are given elsewhere in this review.

EXOCELLOBIOHYDROLASES

Cellobiohydrolases bring about the hydrolysis of cellulose by cleaving cellobiose units from the non-reducing ends of cellulase chains. As noted in the previous section, this action may take place by way of a lysozyme-like mechanism. Acting alone, the cellobiohydrolases do not degrade cotton but can effect considerable saccharification of microcrystalline celluloses. They are also active against swollen partially degraded amorphous substrates and cellodextrins but do not hydrolyse soluble derivatives of cellulose, a fact that differentiates these enzymes from the endoglucanases. Cellobiose, and to a lesser extent dextrose, are inhibitory—hence the need for the β-glucosidases. Most exoglucanases are glycoproteins and exist as single polypeptides, the range of molecular weights being remarkably narrow (*Table 5*). As is the case with the other components of cellulolytic systems, these enzymes are acidic and are most active and most stable under these conditions.

There is as yet no firm proof of synthesis of exocellobiohydrolases by bacteria, even in those effecting extensive breakdown of crystalline cellulose. A common explanation of this phenomenon is that endoglucanases of different specificities (*see above*) when organized in a macromolecular assembly and appropriately orientated with respect to the substrate, can by themselves bring about large-scale hydrolysis of the crystalline substrate. Circumstantial evidence that such bacteria may produce cellobiohydrolases has recently been published (Johnson *et al.*, 1982; Lamed *et al.*, 1983), while Creuzet, Berenger and Frixon (1983), using a plate assay method, appear to have demonstrated the presence of an exocellobiohydrolase in filtrates of *Clostridium stercorarium*. The enzyme was inactive against filter paper but did hydrolyse phosphoric-acid-swollen cellulose, cellobiose being the main product. When recombined with endoglucanase it did allow extensive hydrolysis of crystalline cellulose, the degree of synergism between both components being quite marked. Further characterization of this enzyme is awaited with interest.

β-GLUCOSIDASES

These enzymes, which have been reviewed recently by Shewale (1982) and by Woodward and Wiseman (1982), have a vital role in the saccharification of cellulose because they hydrolyse the products of cellulase action and, because of their glycosyltransfer abilities (*see* e.g. Mahadevan and Eberhart, 1964), may be involved in the induction of cellulase synthesis (*see* page 45). They catalyse removal of dextrose from the non-reducing ends of oligosaccharides up to cellohexaose and effect the cleavage of cellobiose. The latter is important in that it relieves inhibition of cellulase action. β-Glucosidases act on a variety of β-linked glucopyranosides including cellobiose, trehalose,

Table 5. Properties of exocellobiohydrolases

Organism	Enzyme	$M_r \times 10^{-3}$	Carbohydrate content (%)	pI	Optimum pH	Optimum temperature (°C)	Stability	Reference
Fusarium lini	CBH I	57	5·4	6·4	4·5–5·5	55	—	Mishra *et al.* (1983)
	CBH II	50	6·5	6·2	4·5–5·5	55	—	
Fusarium solani	A	45	21·0	4·75	—	—	—	Wood and McCrae (1977b)
	B	45	10·0	4·90	—	—	—	
	C	45	12·0	4·82	—	—	—	
	D	45	1·0	4·95	—	—	—	
Penicillium funiculosum		46·3	9·0	4·36	2·5 & 4·0–5·5	60	90% loss, 10 min, 70°C	Wood, McCrae and Macfarlane (1980)
	CBH 1	51	9·0	4·7	2·5			Wood (1985a)
	CBH 2	51	19·0	5·0	4·2			
Sporotrichum pulverulentum		48·6	0	4·3	—	—	—	Eriksson and Pettersson (1975b)
Thermoascus aurantiacus	C_{11}	49	2·6	—	—	—	—	Tong, Cole and Shepherd (1980)
Trichoderma koningii	CBH_1	62	9·0	3·80	—	—	—	Wood and McCrae (1972)
	CBH_2	62	33·0	3·95	—	—	—	
Trichoderma reesei		65	10	3·6–4·2	—	—	—	Nummi *et al.* (1983)
	CBH I	—	—	3·95	—	—	—	Fägerstam and Pettersson (1980)
	CBH II	—	—	5·0–5·6		—	—	
Trichoderma viride		46	3·3	3·79	—	—	—	Berghem and Pettersson (1973)
		42	9·2	—	4·8	—	Denatured in 3 min at 78°C	Berghem, Pettersson and Axio-Frederiksson (1975)
	A	53	1·4	—	—	—	30 min at 60°C, 64% retained	Gum and Brown (1977)
	B	53	5·8	—	—	—	68% retained	
	C	53	10·4	—	—	—	65% retained	
	D	53	6·7	—	—	—	65% retained	

sophorose, laminaribiose and gentiobiose but at different rates. Generally speaking the rate of hydrolysis of oligosaccharides decreases as chain length increases, but affinity increases (or more accurately K_m decreases) with increase in chain length. The β-configuration is retained on hydrolysis, a fact that distinguishes these enzymes from exoglucohydrolases (*see Table 2*). Most β-glucosidases are strongly inhibited by gluconolactone, subject to excess substrate inhibition and to product inhibition by dextrose. Product inhibition has been variously reported to be competitive (e.g. Emert and Brown, 1977; McHale and Coughlan, 1981b; Shewale and Sadana, 1981; Parr, 1983; Evans, 1985) or non-competitive (e.g. Gong, Ladisch and Tsao, 1977), while Dekker (1981) reported that, of the three β-glucosidases synthesized by a *Monilia* sp., two were competitively inhibited and the other non-competitively inhibited by dextrose. These disparate findings may be reconciled by reports of investigations on the inhibition of the β-glucosidase of *Aspergillus foetidus* by dextrose (Gusakov *et al.*, 1984). These workers showed product inhibition to be of the mixed type, with competitive binding of dextrose to the enzyme and non-competitive binding to the enzyme–substrate complex. These authors also showed the upward deflection in Lineweaver–Burk plots of cellobiose hydrolysis at high substrate concentration to be due not only to excess substrate inhibition but also to the transglycosylation reaction that takes place under these conditions. From the literature one notes that V_{max} values for cellobiose cleavage range from 33 to 175 μmol/min/mg protein.

Considerable variation is shown in the physicochemical properties of these enzymes. However, they are usually acidic and are most active at acid pH values. Thermal stability is also markedly pH dependent, but the pH value that is optimum for activity is not necessarily that for stability. Examination of the effects of pH on the kinetic parameters of β-glucosidases from several organisms suggests the involvement of a carboxylate group and a protonated imidazole group at the active centre (Umezurike, 1971; Maguire, 1977; Hirayama, Nagayama and Matsuda, 1979; Shewale and Sadana, 1981). The involvement of a carboxyl group in catalysis was also indicated by affinity labelling with active site-directed inhibitors, ^{3}H-conduritol-β-epoxide (a structural analogue of dextrose), D-glucal, and ^{3}H-*p*-nitrophenyl-2-deoxy-D-glucose (Woodward and Wiseman, 1982). On denaturation of the enzyme the labelled substrate was found to be attached to an aspartyl side chain. The carboxylate may act as a general acid catalyst protonating the leaving group, as is the case with lysozyme (*see Figure 10*). Indeed De Bruyne, Aerts and De Gussem (1979) postulate that splitting of the aglycone moiety results in the formation of an enzyme–glucosyl complex; this is cleaved in turn to yield dextrose and free enzyme.

Many of the β-glucosidases are glycoproteins, the carbohydrate content varying from zero to 90% by weight as in the case of the enzyme in filtrates of *Coriolus versicolor* (Evans, 1985). Molecular weight values range from 35 000 to 440 000, the latter being the value reported for the enzyme from *Sporotrichum thermophile* (Meyer and Canevascini, 1981). The enzymes from *Talaromyces emersonii* exist as single polypeptides (McHale and Coughlan, 1982), that from *Aspergillus fumigatus* is a dimer (Rudrick and Elbein, 1975),

while Umezurike (1971) considers that the enzyme (M_r 332 000) from *Botryodiplodia theobromae* is composed of eight catalytic subunits, each of which is composed of four identical non-catalytic polypeptides. A summary of the properties of the β-glucosidases that have been most extensively studied is given in *Table 6*.

Trichoderma reesei is often regarded as being the most powerful producer of cellulases: hence, filtrates of this organism are frequently used in saccharification reactions. However, *T. reesei* produces relatively little extracellular β-glucosidase—in fact only 0·2% of the extracellular protein has such activity (Allen and Sternberg, 1980). This decreases the effectiveness of such filtrates in the saccharification of cellulosic substrates and necessitates supplementation of reaction mixtures with β-glucosidase from other sources (e.g. Sternberg, Vijakumar and Reese, 1977; Dekker and Wallis, 1983). Aspergilli are among the better sources (Enari, 1983); however, the search for alternative producers continues. Recently, Macris (1984) isolated a promising β-glycosidase from filtrates of *Alternaria alternata* and we note that this enzyme accounts for 20% of the extracellular protein content produced by *Coriolus versicolor* (Evans, 1985). It remains to be seen how useful these and other β-glucosidases will be under operating conditions.

Other means of supplementing mixtures with β-glucosidase have been examined. These include immobilization of the enzyme on various soluble and insoluble polymeric supports (Woodward and Wiseman, 1982; Rao and Mishra, 1984). Alternatively, the immobilized preparations may be incorporated into ultrafiltration devices or hollow fibre reactors (Klei *et al.*, 1981; Sarkar and Burns, 1984). Although increased stability does not necessarily follow on immobilization, it has sometimes been observed, and thus may allow a significant reduction in the amount of cellulase required for saccharification. An organism exhibiting cell-associated β-glucosidase activity may be encapsulated, rather than the free enzyme itself. In this way *Alcaligenes faecalis* has been immobilized in polyacrylamide (Wheatley and Phillips, 1984) while *Pichia etchelsii* (Jain and Ghose, 1984) and *Trichoderma* E58 (Matteau and Saddler, 1982a,b) have been encapsulated in calcium alginate. In the latter case the system had a half-life of more than 1000 hours when operating at 50°C, pH 4·8, and effected 47% conversion of substrate at a high dilution rate ($3h^{-1}$). Kierstan, McHale and Coughlan (1982) and Hahn-Hägerdal (1984) co-entrapped immbolized β-glucosidase and *Saccharomyces cerevisiae* in calcium alginate beads and demonstrated the ability of the system to produce alcohol from cellobiose. In fact the system prepared by Hahn-Hägerdal (1984) effected 80% conversion of substrate at a dilution rate of $0{\cdot}1\ h^{-1}$ and suffered no loss in productivity during continuous operation for 2 weeks.

Mechanism of cellulose saccharification

A proposal, based on the work of many groups (*see also Figures 6–10*), for the mechanism of saccharification of crystalline cellulose at the macromolecular level is given in *Figure 11*. In the first step, amorphogenesis, the crystalline

Table 6. Properties of β-glucosidases

Organism	Enzyme	$M_r \times 10^{-3}$	Carbohydrate content (%)	p*I*	Optimum pH	Optimum temperature (°C)	T½	K_m (m*M*) cellobiose	K_m (m*M*) 4 NPG**	K_i (m*M*) glucose	Reference
Aspergillus oryzae	E	218	14	4·3	4·5	—*	—	—	—	—	Mega and Matsushima (1979)
Aspergillus phoenicis	E	—	—	—	4·3	—	6 h at 60°C, pH 4.8	0·75	—	—	Sternberg, Vijakumar and Reese (1977)
Botryodiplodia theobromae	E	332	—	—	5·0	—	—	1·0	0·33	—	Umezurike (1971, 1975)
Chaetomium thermophile	BG1(1)†	—	—	—	5·5	—	50 min at 60°C, pH 5·5	1·1	1·1	91	Lusis and Becker (1973)
	BG2(1)	—	—	—	4·8	—		—	0·092	0·53	
	BG3(E)†	—	—	—	4·8	—		—	0·075	0·34	
Coriolus versicolor	E	300	90·0	—	4·3	45	20 h at 45°C, pH 4·5				
Macrophomina phaseolina	BG1(E)	323	—	—	—	—	—	1·66	1·25	—	Saha *et al.* (1981)
	BG2(E)	220	—	—	—	—	—	0·90	0·13	—	
Monilia sp.	G1(1)	480	—	4·40	4·2	60–65	—	20·0	1·67	1·63	Dekker (1981)
	G2(1)	37·5	—	8·30	4·5	50	—	5·7	0·08	0·67	
	G3(E)	37·5	—	8·30	4·5	50	—	5·7	0·08	0·67	
Penicillium funiculosum	E	—	—	—	3·0–5·0	50–60	16 min at 62°C, pH 5	2·1	0·4	1·7	Parr (1983); Rao and Mishra (1984)
Pyricularia oryzae	GB1(E)	240	—	4·15	5·5	55–60		0·91	—	1·4	Hirayama *et al.* (1978)
	GB2(E)	120	—	4·05	5·0	45		0·77–0·42	—	2·4	Hirayama, Nagayama and Matsuda (1979, 1980)

Sclerotium rolfsii	BG1(E)	95·5	+	4·10				3·65	1·07	—	Shewale and Sadana (1981)
	BG2(E)	95·5	+	4·55	4·5	65		3·07	1·38	—	
	BG3(E)	106·0	+	5·10				5·84	0·89	—	
	BG4(E)	95·5	+	5·55				4·15	0·79	—	
Sporotrichum pulverulentum	A_1(E)	165		4·80	4·25			4·5	0·15	0·00035	Deshpande, Eriksson and Pettersson (1978)
	A_2(E)	172		4·52							
	B_1(E)	165		5·15	4·25			3·7	0·21	0·0015	
	B_2(E)	172		4·87							
	B_3(E)	182		4·56							
	I	—	—	—	—	—	—	—	2·0	0·12	
Talaromyces emersonii	βGI(E)	138·0	51	3·4–4·17	4·1	70	410 min at 70°, pH 5	0·58	0·14	0·17	McHale and Coughlan (1981b, 1982)
	βGIII(E)	40·0	26	3·6	5·1	70	175 min	23·70	1·03	None	
	βGIV(I)	52·5	12	4·41–4·5	5·7	35	2 min	1·47	0·81	52·0	
Trichoderma koningii	BG1(E)	39·8	0	5·53							Wood (1980)
	BG2(E)	39·8	0								
Trichoderma viride	(E)	47·7	0	5·74	—	—	—	1·5	0·28	—	Berghem and Pettersson (1973)
Trichoderma viride	(E)	51·2	10·3	—	4·9	—	—	1·8	0·17	—	Emert and Brown (1977)
Trichoderma viride	1(E)	76·0	—	—	—	—	—	2·65	—	—	Gong, Ladisch and Tsao (1977)
	2(E)	76·0	—	—	—	—	—	2·50	—	1·22	
	3(E)	76·0	—	—	—	—	—	2·74	—	4·26	
Trichoderma reesei‡	(E)	35·0	0·01	—	—	—	—	—	—	—	Enari (1983)
	(E)	130·0	—	—	—	—	—	—	—	—	

* A dash indicates that there are no reported data.
** 4-Nitrophenyl-β-D-glucoside
† (E) = extracellular, (I) = cell-associated.
‡ *T. reesei* used to be known as *T. viride*.

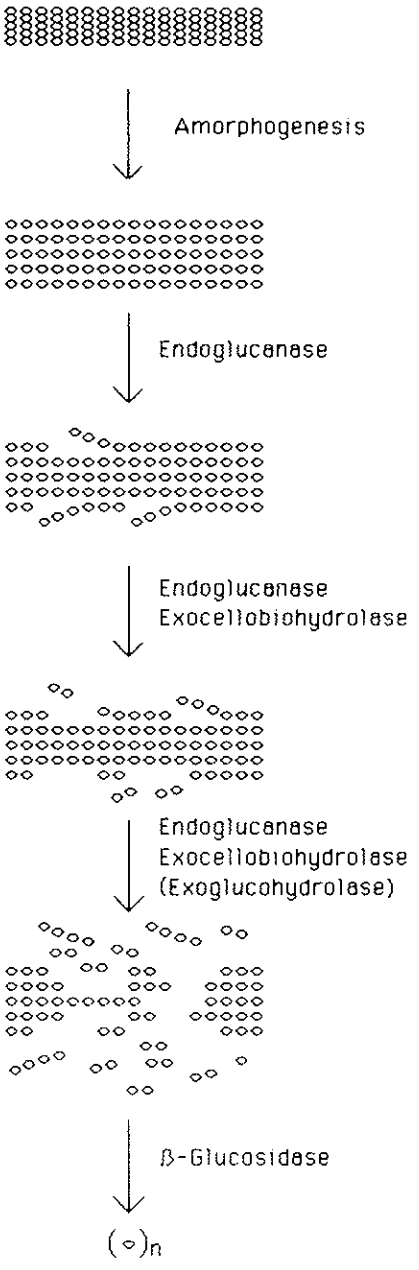

Figure 11. Proposed mechanism for hydrolysis of cellulose at the macromolecular level by complete fungal cellulolytic enzyme systems. Details in the text.

substrate is rendered more accessible to the hydrolytic enzymes. There is ample evidence in the literature that such a step is necessary but much disagreement as to whether factors other than the well-documented hydrolases may be involved. Amorphogenesis may be effected by enzymically produced H_2O_2 in the presence of iron (Koenigs, 1975), by the iron-containing microfibril-generating factor present in fungal filtrates (Griffin *et al.*, 1984) or by the short-fibre-forming C_2 factor in filtrates of *T. koningii* (Halliwell and Riaz, 1970; Halliwell, 1975). More recently Chanzy *et al.* (1983), using electron-microscopic techniques, claim to have demonstrated that cellobiohydrolase I from *T. reesei* acting alone effected the complete dissolution of highly crystalline cellulose from *Valonia macrophyta*. Disruption of the crystals was said to take place by erosion and fibrillation—splitting of lateral hydrogen bonds yielding narrower crystalline elements that retained their original length. During this disruption soluble sugars were released, but implicit in this paper was the idea that splitting of hydrogen bonds and glycosidic linkages were separate activities of CBHI. These findings are in contrast to those of White and Brown (1981), who used electron microscopy to study the action of the *T. reesei* cellulase system on cellulosic ribbons from *Acetobacter xylinum*. CBH alone effected no visible change in the substrate while endoglucanase alone brought about only slight splaying of the ribbon-like cellulose. However, binding of the combined cellulases produced visible splitting into bundles of microfibrils within 10 min and complete dissolution within 30 min.

Whatever the mechanism of amorphogenesis, further dissolution of cellulose is brought about by the combined actions of endoglucanases and exoglucanases utilizing a lysozyme-like mechanism (*Figure 10*), displaying inter- and intra-type synergism (*Figures 8, 9*) and perhaps competitive adsorption (*Figure 6*). Exoglucohydrolases, present in some fungal filtrates, may also be important in removing glucose residues from non-reducing chain ends. Lastly, β-glucosidases act on the cellobiose and short-chain oligosaccharides produced by the cellulases. The β-glucosidases may be extracellular, cell-bound or both, depending on the fungal species and growth conditions. The final result is the same, i.e. glucose is made available to the cell and inhibition of cellulase action is relieved. In some species cellobiose oxidase or cellobiose dehydrogenase (*see Table 2*) may replace or supplement this role of β-glucosidase.

The hydrolysis of cellulose by bacterial systems is illustrated in *Figure 12*. The cell-associated high-molecular-weight entity, the cellulosome, is thought to be responsible for adherence of the bacterium to the substrate, and the endoglucanases (exocellobiohydrolases may also be present) in the cellulosome are held in the appropriate juxtaposition with respect to the substrate to permit extensive hydrolysis (*see also* von Hofsten and Berg, 1972; Wood, Wilson and Stewart, 1982). Cellobiose and short-chain oligosaccharides produced by the cellulosome are metabolized by β-glucosidases or cellobiose oxidases in the periplasmic space.

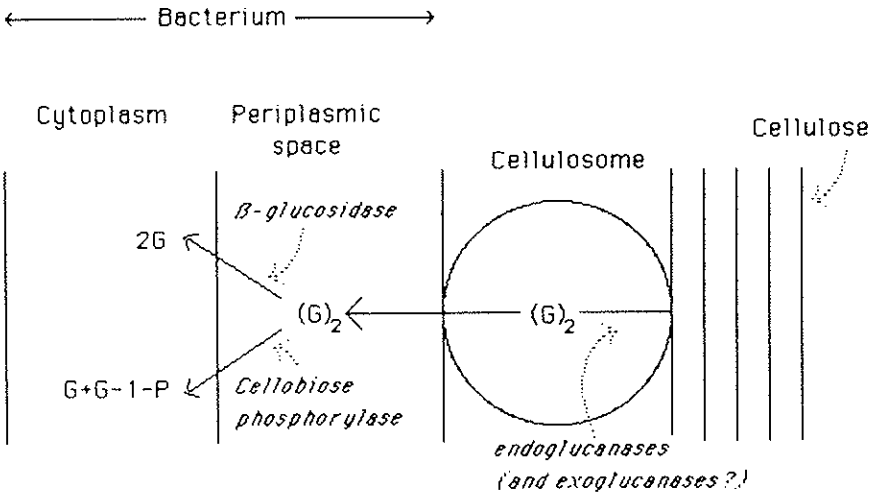

Figure 12. Hydrolysis of cellulose by bacterial cellulolytic enzyme systems (based on the findings of Lamed *et al.*, 1983). Details in the text.

Applications of cellulases

It is to be hoped that, in the future, cellulolytic enzyme systems may be utilized for the hydrolysis and fermentation of urban and agricultural wastes to yield desirable end-products and, indirectly, to expedite traditional fermentation processes, to improve fodder quality or to facilitate juice and flavour extraction. Indeed, almost ten years ago Yamada (1977) could state that 45 tonnes of cellulases then worth 170 million yen were produced in Japan alone for just such purposes. A summary of the current or potential applications of cellulases is given in *Table 7*.

For most of these present applications, the extensive hydrolysis of substrate is not required and enzyme use is thus small. No economically viable process yet exists for the direct conversion of cellulosic materials to desired end-products such as dextrose or ethanol. However, because lignocellulosic materials, whether primary sources or wastes of agricultural, domestic or industrial origin, are potentially huge storehouses of energy and chemical feedstocks (*see* e.g. Coughlan, 1985) the search for an economic process will continue. Mandels (1985) states that we must not be without hope of success.

Basically, the problems are twofold and interlinked. On the one hand, the crystallinity of the native cellulose and its coating with lignin render it inaccessible to the cellulolytic enzymes. A great variety of physical, chemical and biological pretreatments designed to render the substrate more amenable to hydrolysis have been and are being investigated. Such pretreatments add considerably to costs. Secondly, production of the necessary enzymes is expensive: large quantities are required; their synthesis is subject to catabolite repression; their action is bedevilled by end-product inhibition, while specific activity against crystalline substrates is low. A determination to overcome

Table 7. Application of cellulases and of cellulolytic organisms (from Mandels, 1985, courtesy of *Biochemical Society Transactions*)

I. Removal of cell walls, crude fibre
- A. Release cell contents
  - Flavours
  - Enzymes
  - Polysaccharides (Agar)
  - Protein (seeds, leaves)
- B Improve rehydratability of dried vegetables
  - Soup mixes
- C Oil-seed cakes
  - Straws
  - Barley
  - Mesquite
- D. Production of plant protoplasts
  - Genetic engineering, higher plants

II. Production of glucose, soluble sugars
- A. Animal feeds
  - Molasses direct or by-product
  - Increase nutritive value—add sugar to high-fibre feed
  - Single-cell protein—yeasts
- B. Industrial feedstock
  - Glues, adhesives
  - Chemicals—ethanol, butanol, etc.
- C. Raw material for fermentation industry
  - Antibiotics
  - Citric acid, etc.

III. Production of lignin
- Adhesives
- Resins
- Extenders
- Chemical raw material

IV. Miscellaneous food applications
- A. Cell-free protein
  - High productivity
  - High-quality protein
- B. Fermented foods
  - Addition of mycelial and extracellular protein
  - Removal of crude fibre
  - Conversion of fibre to sugar
  - Removal of other unwanted components
- C. Fungal proteases
  - Acid protease as a meat tenderizer

V. Decomposition of wastes and residues.

these twin problems of substrate recalcitrance and enzyme productivity is evident in the large number of relevant symposia held in the last few years. The reader may observe the progress, or lack of progress, that has been made by perusing the proceedings of symposia organized and/or edited by Gould (1969), Bailey, Enari and Linko (1975), Wilke (1975), Gaden *et al.* (1976),

Ghose (1977), Rijkens (1980), Bachofen (1982) and Ferrero, Ferranti and Naveau (1984). Review articles by Nystrom and Andren (1976), Mandels and Andreotti (1978), Dekker and Lindner (1979), Ryu and Mandels (1980), Flickinger (1980) and Linko (1985) are also particularly noteworthy as is the final report of the Natick group (Anonymous, 1981).

Ethanol, as a petrol replacer or as a chemical feedstock, is the end-product that might be saleable in the quantities utilization of the lignocellulosic substrates demands. There are essentially two ways whereby this conversion may be effected biologically. The first is the more traditional two-step process in which the lignocellulosic substrate is first hydrolysed to assimilable sugars by enzymes (filtrates) from organisms such as *Trichoderma* spp. The sugars are then fermented to ethanol by yeasts. The alternative procedure advocated by Wang and colleagues (Cooney *et al.*, 1978; Avgerinos and Wang, 1980; Wang *et al.*, 1983), Wiegel, Ljungdahl and co-workers (e.g. Wiegel, 1982) and Saddler's group in Canada (Saddler and Chan, 1984) is a one-step process. In this procedure the cellulose is simultaneously saccharified and fermented under anaerobic conditions by *Clostridium thermocellum* alone or in co-culture with *C. thermosaccharolyticum* and/or *C. thermohydrosulphuricum*. *C. thermocellum* effects hydrolysis of the lignocellulosic substrate and fer-

Table 8. Operational pilot and demonstration plants for cellulose hydrolysis and fermentation (based on Wilke *et al.*, 1983)

Process	A. Separate hydrolysis and fermentation in reactor (i) Stirred-tank (ii) Fluidized bed with entrapped enzyme (iii) Tapered fluidized bed (iv) Fixed bed (v) Solid-state cultivation B. Simultaneous hydrolysis and fermentation
Locations (USA unless stated otherwise)	Universities: Arkansas, Berkeley, Hahnemann Med. Sch., Michigan, Miyazaki (Japan), Pennsylvania, Purdue, Washington Technological Institutes: Indian Inst. Tech., Georgia Tech, M.I.T., Tokyo Inst. Tech. Others: Battelle (Switzerland), Dynatech, Forintek Corp. (Canada), Inst. du Petrole (France), Natick R & D., Oak Ridge Natl. Labs., Stake Technology (Canada)
Enzymes and organisms used in cellulolysis and/or fermentation	*Trichoderma reesei*, *Thermomonospora* spp., *Clostridium thermocellum*, *C. thermosaccharolyticum*, *C. thermohydrosulphuricum*, *Saccharomyces cerevisiae*, *Candida brassicae*
Substrates used	Municipal solid waste, corn stover, Solka floc, poplar
Pretreatments	Two-roll milling, steam explosion, solvent extraction, solubilization and reprecipitation
Products	Ethanol, acetone, butanol, single-cell protein, by-products.

ments the hexoses to ethanol while the other clostridia are added to ferment the pentoses released on hydrolysis of the hemicellulose fraction of the substrate. Despite the lack, as yet, of commercial success in the exploitation of cellulosic substrates, a number of pilot, demonstration and commercial plants are in operation or in the planning or design stages. Wilke *et al.* (1983) have described the more important of these, a summary of which is given in *Table 8*. Many institutions, especially in the USA, Canada, France and Japan, are engaged in such investigations. If success attends the efforts of these and many other institutions it cannot be very long before the potential of cellulose as a reservoir of food, fuel and chemical feedstock is realized.

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