



CELLULOSE DEGRADING ENZYMES AND THEIR POTENTIAL INDUSTRIAL APPLICATIONS

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ABSTRACT

Bioconversion of cellulose to soluble sugars and glucose is catalyzed by a group of enzymes called cellulases. Microorganisms including fungi, bacteria and actinomycetes produce mainly three types of cellulase components—endo-1,4- β -D-glucanase, exo-1,4- β -D-glucanase and β -glucosidase—either separately or in the form of a complex. Over the last several decades, cellulases have become better understood at a fundamental level; nevertheless, much remains to be learnt. The tremendous commercial potential of cellulases in a variety of applications remains the driving force for research in this area. This review summarizes the present state of knowledge on microbial cellulases and their applications.

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KEY WORDS

Cellulases, cellulose, enzymes, biopolymers, cellulolytic microorganisms.

INTRODUCTION

Cellulose is the most abundant and renewable biopolymer on Earth. Cellulose has been used by man for centuries; however, its enormous potential as a renewable source of energy was recognized only after the cellulose degrading enzymes or “cellulases” had been identified. During World War II, the U.S. Army was alarmed at the rate of deterioration of cellulosic materials including clothing, tents and sand bags, in the South Pacific. Several organizations within the Army, set up laboratories to find an immediate solution to this problem [134]. As a result, a parent strain QM6a was isolated from a shelter remains and identified as *Trichoderma viride* and later recognized as *Trichoderma reesei* [146]. The immediate benefits of the Army’s program led to further research on selection and characterization of hyper-cellulolytic *T. reesei* strains. These projects not only improved the production of cellulase by *T. reesei*, but also aroused worldwide research activity. The work at the Natick Army laboratory and in other places quickly led to the recognition that cellulosic wastes may be converted to glucose, soluble sugars, alcohols, single cell protein and other industrially useful chemicals through the agency of cellulases [33,34,105,137].

The past fifty years have witnessed remarkable progress in (a) isolation of microorganisms producing cellulases [76,81,128,136]; (b) improving the yield of cellulases by mutation, and protoplast fusion [23,43]; (c) purifying and characterizing the cellulase components [15,16,18,27-29,79,142,143,182-189]; (d) understanding the mechanism of cellulose degradation [48,49,188,190]; (e) cloning and expression of cellulase genes [11-14,52]; (f) determining the 3-D structures of cellulase components [4,37,38,40-42,85,139,152]; (g) understanding structure-function relationships in cellulases [30,38,60,159,162]; and (h) demonstrating the industrial potential of cellulases [11,33,34,105]. This review summarizes the present state-of-the-art on cellulases both from fundamental and applied viewpoints. Further background information can be found in other recent reviews [11,33,34,50,61,81,91,92,95, 100,105,178,179,190,196].

OCCURRENCE OF CELLULASE

Cellulolytic enzymes are produced by a wide variety of bacteria and fungi, aerobes and anaerobes, mesophiles and thermophiles [35,100]. However, relatively few fungi and bacteria produce high levels of extracellular cellulase capable of solubilizing crystalline cellulose extensively [84,175,176]. So far, most of the studies have been on the cellulase system of aerobic fungi *Trichoderma viride*, *Trichoderma reesei*, *Penicillium pinophilum*, *Sporotrichum pulverulentum*, *Fusarium solani*, *Talaromyces emersonii* and *Trichoderma koningii* [48,49,63,109,125,173,186]. Only recently, it has been recognized that the other microorganisms such as thermophilic aerobic fungi (*Sporotrichum thermophile*, *Thermoascus aurantiacus*, *Chaetomium thermophile*, *Humicola insolens*), mesophilic anaerobic fungi (*Neocallimastix frontalis*, *Piromonas communis*, *Sphaeromonas communis*) mesophilic and thermophilic aerobic bacteria (*Cellulomonas* sp., *Cellvibrio* sp., *Microbispora bispora*, and *Thermomonospora* sp.), mesophilic and thermophilic anaerobic bacteria (*Acetivibrio cellulolyticus*, *Bacteroides cellulosolvens*, *Bacteroides succinogenes*, *Ruminococcus albus*, *Ruminococcus flavefaciens*, and *Clostridium thermocellum*) as well as actinomycetes (*Thermomonospora fusca*) also produce active cellulases [1-15,17,53,54,64,66,67]. Among the above noted microorganisms, the cellulolytic thermophilic microorganisms are of particular interest, because of their ability to produce thermostable cellulases which are generally stable under a variety of severe conditions including highly acidic and alkaline pHs as well as temperatures up to 90°C [95]. Notable thermophilic cellulolytic microorganisms are *Clostridium thermocellum*, *Thermomonospora fusca*, *Thermoascus aurantiacus*, *Sporotrichum thermophile*, *Humicola insolens* and *Chaetomium thermophile* [14,15,17,86,95,157]. These microorganisms are also capable of fermenting a wide range of substrates with minimal risk of contamination by other pathogens [95]. Because of those advantages, the thermophilic cellulolytic microorganisms have attracted considerable research interest in recent years.

MEASUREMENT OF CELLULASE

The physical heterogeneity of cellulosic substrates together with the complexity of cellulase system produced by different microorganisms have led to the development of several assay procedures for the measurement of cellulase activities. The considerable difference in the nature of substrates used, variation in assay procedures adopted for measuring different cellulase components, and the synergistic action of cellulase components have made the comparison of results among laboratories difficult. Therefore, in 1984, the IUPAC Commission on Biotechnology published standard assay procedures for measuring cellulase activities [58]. Some of these recommendations have been readily accepted by biotechnologists, but many enzymologists feel that these procedures are quite restricted and not satisfactory for understanding the mechanism of action and substrate specificities of cellulases in detail. Consequently, Wood and Bhat [180] reviewed the cellulase assays used by laboratories working on fungal cellulases and highlighted the advantages and disadvantages of the various procedures. Although, the assay procedures described [180] were for *Trichoderma* cellulases, those methods can be adapted to other cellulases. The substrates and the estimation methods used in measuring cellulase activities are summarized in Table 1.

INDUCTION AND REGULATION OF CELLULASE PRODUCTION

Cellulase is an inducible enzyme system [91,92,140]. All microorganisms studied so far have produced the highest level of cellulase when grown on cellulose [140,156,175]. Cellobiose, lactose and sophorose are also known to facilitate the production of either complete or incomplete cellulase system by few microorganisms [107,108,121,140]. Synthetic compounds such as palmitate and acetate esters of disaccharides and thiocellobiose have also been shown to function as inducers of cellulases [135,140]. However, cellulose was found to be the best carbon source for the production of high levels of cellulase by many microorganisms [140, 156,175]. This observation raised a fundamental question with respect to induction and regulation of cellulase production, that is how an insoluble substrate which cannot enter the microbial cell, induces and regulates the production of cellulase.

The most generally accepted view of induction process is that the low levels of cellulase constitutively produced by the microorganism, first hydrolyses cellulose to soluble sugars [11,91,92]. These sugars are presumably converted into true inducers, which enter the cell and either directly or indirectly influence DNA binding protein and promote cellulase gene expression [92]. It has also been suggested that in case of *Trichoderma*, the conidial bound cellobiohydrolase hydrolyses the cellulose and releases cellobiose and CBL (cellobiono- δ -1,5-lactone). The cellobiose and CBL are taken up by the mycelia and promote cellulase synthesis [92,93].

Table 1. Substrates and assay procedures used for measuring cellulase activities. Reproduced from Wood and Bhat [180], with permission.

Enzyme	Substrate	Assay
Total cellulase	Cotton	Solubilization: 1. Estimation of cellulose residue [173] 2. Reducing sugar released [114, 151] 3. Weight loss [181] 4. Loss in tensile strength [181]
	Filter paper, Hydrocellulose, Avicel and Solka Floc	Solubilization: 1. Release of reducing sugars [106,114,151]
	Dyed Avicel [177]	1. Release of dyed soluble fragments [123,180]
Exo-1,4- β -D-glucanase (cellobiohydrolase, exocellulase or Avicelase)	Avicel Hydrocellulose Dyed Avicel [177]	Solubilization: 1. Release of reducing sugars [151] 2. Release of dyed cellobiose [123,180]
	Amorphous cellulose [177]	Solubilization: 1. Release of reducing sugar [151] 2. Decrease in turbidity [46,123]
	Substituted and unsubstituted cello- oligosaccharides	1. Increase in reducing power [39,151] 2. Analysis by HPLC [16]
Endo-1,4- β -D-glucanase (endoglucanase, CM- cellulase, endocellulase)	Carboxymethyl cellulose Hydroxyethylcellulose	1. Release of reducing sugars [151] 2. Decrease in viscosity [180]
	Substituted and unsubstituted cello- oligosaccharides	1. Increase in reducing power [151] 2. Analysis by HPLC [16]
	Cotton Amorphous cellulose [177]	1. Swelling in alkali [110] 2. Solubilization: release of reducing sugars [151] 3. Decrease in turbidity [123]
β -Glucosidase or Cellobiase	Cellobiose Cello-oligosaccharides	1. Release of glucose [180] 2. Increase in reducing power [151]
	o- or p-Nitrophenyl- β -D- glucosides	1. Release of o- or p-nitrophenol [180]

Mandels and Reese [108] predicted that cellobiose, the repeating unit of cellulose, could be the natural inducer of cellulase in *T. reesei*. However, it has been shown with *T. viride* that cellobiose could induce cellulase production only at a high concentration (1% or more) or in presence of a surfactant such as Tween 80 [135,136]. In contrast, the cellobiose induced the production of endoglucanase by *S. pulverulentum* at a concentration of $1 \text{ mg} \cdot \text{L}^{-1}$ [140]. Also, Garcia-Martinez *et al.* [56] reported that cellobiose induced CM-cellulase production at a concentration as low as 0.2%, and supported the production of the highest level of cellulase in the presence of 1% cellobiose in *C. thermocellum*. Recently, Bhat *et al.* [20] demonstrated that cellobiose at a concentration as low as 0.2% induced the production of both CM-cellulase and true cellulase activities by *C. thermocellum*. Therefore, it can be argued that cellobiose is the natural inducer of cellulase system at least in some microorganisms.

It is generally accepted that the transglycosylation product of cellobiose could be the true inducer of cellulase in *T. reesei* [140]. Also, it has been shown that sophorose (β -1,2-glucobiose), a contaminant in glucose preparation, is an effective inducer of cellulase in *T. reesei* and in other species of *Trichoderma*, even at a concentration of $0.3 \text{ } \mu\text{g} \cdot \text{mL}^{-1}$ [121,155,196]. However, sophorose did not induce the production of cellulase in other fungi and in a mutant of *T. reesei*, QM 9414 [116]. Thus, sophorose is not a universal inducer of cellulase. In contrast, Fritscher *et al.* [55] reported that cellobiose induced the production of cellulase system in *T. reesei*, either in the presence of β -glucosidase inhibitor or when a β -glucosidase defective mutant was used. These results strongly support the view that cellobiose could be the true inducer of the cellulase system in *T. reesei*.

Studies using inhibitors of protein synthesis have suggested that cellulase formation is regulated at the translational level [122]. Evidence based on the measurement of mRNA levels documented that the formation of cellulase occurs at the pre-translational level [87,113] and the cellulase gene transcription occurs within 20 min, after the addition of inducer [44]. With the exception of conidiation, all cellulase inducers promoted the synthesis of a constant ratio of three major cellulases: CBH I, CBH II and endoglucanase I in *T. reesei* [44,92]. This indicates that the expression of all three cellulase components is regulated coordinately. In contrast, investigation on the transcription of cellulase genes from other microorganisms revealed that transcription is not strictly coordinated [11]. However, Yagüe *et al.* [195] demonstrated that the nuclei of *Agaricus bisporus* isolated from cellulose-grown mycelium synthesized six times more Cell mRNA than nuclei from glucose-grown mycelium. Transcription of the *cex* and *cenc* genes of *C. fimi* was detected only in the cells grown on carboxymethyl cellulose (CMC) whereas *cenA* and *cenB* transcripts were present in cells grown in the presence of glycerol [11]. Also, *cenB* transcripts were present even in cells grown on glucose. However, high level transcription of *cenB* was observed in the presence of CMC. In *C. thermocellum*, transcription of several *cel* genes were induced sequentially when cellobiose concentration in the medium became

limiting [11]. Thus, there is considerable variation in the level of transcription as well as the type of cellulase genes transcribed, depending on the carbon source used for growth.

Carbon catabolite repression is another regulatory mechanism known to control cellulase production in bacteria and fungi. In this case, the end product of cellulose hydrolysis interacts with a cellular protein to form a complex which interacts with a particular gene at the transcription level and represses cellulase synthesis [99]. The carbon catabolite repression occurs in *Escherichia coli* [131], *Saccharomyces cerevisiae* [47] and *C. thermocellum* [83]. In the case of *C. fimi* and *T. reesei*, growth on non-repressing substrates, either slow feeding of glucose or mutation which affect catabolite type repression did not induce cellulase synthesis to the same extent as in control experiments [92,156]. However, Canevascini *et al.* [24] reported that the cellulase synthesis is regulated by both induction and catabolite repression in *S. thermophile*.

The proof for carbon catabolite repression is based on the fact that no cellulase is formed during the growth of a microorganism on glucose, glycerol and other carbon sources related to glycolytic metabolism. However, Messner *et al.* [113] demonstrated that the addition of glucose to *T. reesei* cultures producing cellulases decreases the rate of formation of only CBH I to about 40%, while production of other cellulases continues up to 40 h. Because there is no clear evidence that either glucose or a catabolite in fact controls the transcription of cellulase genes, Kubicek recommended not to use the term "catabolite repression" [91]. The only alteration observed with these *T. reesei* mutants was at the level of the endoplasmic reticulum, suggesting that glucose may effect either the development of the secretory pathway or some of its components. O-Glycosylation of cellulases, which takes place at the endoplasmic reticulum has been shown to control the secretion of cellulases in *Trichoderma* [91]. Because a decreased activity of dolichol-phosphate-mannose synthase, a key enzyme in the O-glycosylation pathway of *T. reesei*, has been reported during growth on glucose, it is questionable whether the glucose regulates the synthesis or the secretion of cellulases.

In the case of a wilt organism, *Verticillium albo-atrum*, the extracellular enzyme secretion was stimulated by using restricted supply of carbon source [32]. However, the cellulase production was repressed by conditions favoring rapid growth [80]. In *C. thermocellum*, the rapid growth on cellobiose repressed cellulase production, which was relieved during the slow growth on crystalline cellulose [83]. However, Bhat *et al.* [20] did not report any such repression in growth, endoglucanase and true cellulase production by *C. thermocellum* on cellobiose.

The involvement of end product inhibition during crystalline cellulose hydrolysis by a rumen fungus *Neocallimastix frontalis* RK21 was demonstrated [191]. An increase of 82% cotton solubilizing capacity of *N. frontalis* RK21 was shown in the presence of a methanogen, *Methanobrevibacter smithi*. The methanogen used the end products, formate and H₂ formed during cotton solubilization, as substrates and relieved the end product inhibition [191]. Although, induction, catabolite repression,

product inhibition and restricted carbon source supply are identified to be involved in the regulation mechanism of cellulase synthesis in fungi and bacteria [112], very little is known at the molecular level regarding the mechanism regulating the synthesis of cellulases. Recently, the presence of a putative regulatory protein in induced cells of *T. fusca*, and its binding to a 14-bp palindromic sequence located upstream from at least three endoglucanase genes has been reported [11]. Also, a motif similar to the sequence mediating cAMP-dependent regulation of rat tyrosyl amino transferase is identified upstream from the *cbhII* gene of *T. reesei* [11]. Further research at the molecular level on cellulase genes from different microorganisms is necessary for a better understanding of the regulation of cellulase synthesis.

FUNGAL CELLULASE SYSTEMS

The best characterized cellulase systems are those of the aerobic fungi, *Phanerochaete chrysosporium* [S. *pulverulentum*; 48,49], *F. solani* [182], *P. funiculosum/pinophilum* [16,18,185-189], *Talaromyces emersonii* [111], *Trichoderma koningii* [9,70,181,184] and *T. reesei* [35, 91-94]. The cellulase systems of these fungi consist of endo-1,4- β -D-glucanase [1,4- β -D-glucan glucanohydrolase; EC 3.2.1.4], exo-1,4- β -D-glucanase [1,4- β -D-glucan cellobiohydrolase (CBH); EC 3.2.1.91] and β -glucosidase [cellobiase or β -D-glucoside glucohydrolase; EC.3.2.1.21] [175,179, Table 2]. Generally, the endoglucanases randomly attack H_3PO_4 -swollen cellulose, CM-cellulose and the amorphous regions of the cellulose and release cello-oligosaccharides [180]. The CBHs hydrolyze H_3PO_4 -swollen cellulose and Avicel sequentially, by removing the cellobiose units from the non-reducing end of the cellulose chain [180]. Using radio-labeled cello-oligosaccharides, CBH capable of releasing cellobiose from the reducing end has also been reported [164]. The endoglucanase and CBH act synergistically to effect the extensive hydrolysis of crystalline cellulose, while the β -glucosidase completes the hydrolysis by converting the resultant cellobiose to glucose [34].

The exo-1,4- β -D-glucanases (1,4- β -D-glucan glucohydrolase; EC 3.2.1.74) from *P. funiculosum* [185] and *T. emersonii* [111] catalyze the removal of glucose residues from the non-reducing end of celloextrins, and do not interact cooperatively with endoglucanase in the hydrolysis of crystalline cellulose. The brown rot fungi (e.g., *Poria placenta*, *P. carbonica*) differ from the white (e.g., *S. pulverulentum*) and the soft rot fungi (e.g., *F. solani*, *P. pinophilum*, *T. reesei*) in producing only endoglucanases [49].

Most of the fungal cellulases are glycoproteins and exist in multiple forms [35]. Four immunologically related CBH from *T. viride* appear to differ from one another, in the composition of covalently attached neutral carbohydrates [68]. Similarly the four endoglucanases from *T. emersonii*, differ only in the extent of glycosylation [117]. In contrast, *T. reesei* [124] and *P. funiculosum* [189] produce immunologically distinct CBH I and II. The two CBHs differing in amino acid composition have also been identified from *Fusarium lini* [115].

Table 2. Components of aerobic fungal cellulases and their mode of action on the cellulose chain [140,175,180,183].

Enzyme Type	EC Code	Synonym	Mode of Action
Endo-(1-4)- β -D-glucanase	EC 3.2.1.4	Endoglucanase or endocellulase	$\begin{array}{c} \text{--G--G--G--G--} \\ \uparrow \quad \uparrow \\ \text{Cleaves linkages at random} \end{array}$
Exo-(1-4)- β -D-glucanase	EC 3.2.1.91	Cellobiohydrolase or exocellulase	$\begin{array}{c} \text{G--G--G--G--G-} \\ \uparrow \\ \text{Releases cellobiose either from} \\ \text{reducing or non-reducing end} \end{array}$
Exo-(1-4)- β -D-glucanase	EC 3.2.1.74	Exoglucanase or glucohydrolase	$\begin{array}{c} \text{G--G--G--G--G-} \\ \uparrow \\ \text{Releases glucose from non-} \\ \text{reducing end} \end{array}$
β -Glucosidase	EC 3.2.1.21	Cellobiase	$\begin{array}{c} \text{G-G, G-G-G-G} \\ \uparrow \quad \uparrow \\ \text{Releases glucose from cellobiose} \\ \text{and short chain cello-} \\ \text{oligosaccharides} \end{array}$

The extracellular cellulase components of most fungi are generally found to exist as individual entities [33]. However, the presence of aggregates of extracellular enzyme in *T. reesei* culture filtrate has been reported [153]. This complex consists of six proteins and exhibits cellulase, β -glucosidase and xylanase activities. Also, the proteins appear to be aggregated with the help of the remnants of the fungal cell wall, where, Ca^{2+} may be involved [153]. Although many fungi secrete separate cellulase components into culture medium, it is not yet clearly known how these components interact on the surface of crystalline cellulose and effect the extensive hydrolysis of cellulose.

Studies on rumen anaerobic fungi suggest that these microorganisms have a definite role in the initial colonization and degradation of lignocellulose in the rumen [9,10]. Anaerobic fungi have been shown to produce extracellular cellulases and xylanases which are important in the breakdown of lignocellulose [102,120]. Several studies have been carried out on the production and characterization

of cellulolytic enzymes from these microorganisms and indicated their importance in the degradation of cellulosic materials and subsequent fermentation in the rumen [169,170,191].

Anaerobic cellulolytic fungi belonging to genera *Neocallimastix*, *Cacomyces*, *Orpinomyces*, *Piromyces*, and *Ruminomyces*, have been described and classified [178]. At present, it is a well established fact that obligately anaerobic fungi are found in the saliva, alimentary tracts and feces of number of animals such as ruminants and herbivorous non-ruminant mammals [9,10,103,119,128]. Among these species, *Neocallimastix frontalis* has been studied in most detail [119,120,169,170]. Unlike that of aerobic fungi, the cellulase system of *N. frontalis* is a multicomponent enzyme complex termed crystalline cellulose solubilizing factor [CCSF; 190]. It has a molecular mass of 700 kDa and consists of a number of subunits ranging in molecular mass from 68-135 kDa [169,170]. So far, it has not been possible to dissociate the CCSF without losing much of its original activity towards crystalline cellulose. The presence of the multicomponent enzyme complex of this fungus suggests that it may hydrolyze the crystalline cellulose using a mechanism which is similar to that of the anaerobic cellulolytic bacterium *C. thermocellum*. The *N. frontalis* (RK 21) is the most efficient degrader of crystalline cellulose so far known and plays an important role in the digestion of cellulose in the rumen [191]. Therefore, it will be interesting to investigate the mechanism of cellulose degradation by the cellulase system of this fungus.

BACTERIAL CELLULASE SYSTEMS

Cellulolytic bacteria may be aerobes or anaerobes. Most of the bacteria produce mainly endoglucanases [175]. Only in case of actinomycete, *Microbispora bispora*, the presence of cellobiohydrolase in culture fluid has been convincingly demonstrated [8]. However, the β -glucosidase in this actinomycete is cell bound [8]. Also, the cellulase system of *M. bispora* was similar to those of aerobic cellulolytic fungi in effecting the hydrolysis of crystalline cellulose [8]. In contrast, the anaerobic bacteria, *C. thermocellum* [96], *Clostridium cellulovorans* [145], *A. cellulolyticus* [104] which degrade the highly crystalline cellulose such as cotton produce a high molecular-mass enzyme complex called cellulosome. In case of *C. thermocellum*, the cellulosome consists of several endoglucanases and at least three exoglucanases [14,21,148]. It has been suggested that these anaerobic bacteria must attach themselves to cellulose in order to effect cellulolysis [53,168].

The activity of some of the anaerobic bacteria, *C. thermocellum* [78,84], *A. cellulolyticus*, [104] and *Ruminococcus flavefaciens* [98], towards crystalline cellulose is dependent on either Ca^{2+} or Mg^{2+} and either DTT or other thiol reagents. The activities of *Ruminococcus albus* [150] and *Fibrobacter* (*Bacteroides*) *succinogenes* [54] against crystalline cellulose were diminished in the presence of air, but maintained under reducing conditions.

Although, these anaerobic bacteria hydrolyze crystalline cellulose, there is no definite proof for the production of exoglucanase activity [54]. Anaerobic bacterium, *A. cellulolyticus* produced a cell bound β -glucosidase [141]. The cells of *Bacteroides cellulosolvens* were believed to comprise cellobiose phosphorylase [EC 2.4.1.49; 67], whereas, the cells of *C. thermocellum* [3,130] and *R. flavefaciens* [98] produced both cellobiose phosphorylase and β -glucosidase. However, the β -glucosidase of *C. thermocellum* had a high K_m towards cellobiose and hence its role in the metabolism of cellobiose is difficult to understand [2]. In contrast, cellobiose phosphorylase from *C. thermocellum* showed a high affinity for cellobiose [3]. Also, *C. thermocellum* produced an intracellular cellodextrin phosphorylase (EC 2.4.1.49), but its role is not well understood [6].

Among the bacterial cellulase systems, the most extensively studied system is that of *C. thermocellum* [95,96]. This bacterium produces a very active cellulase in the form a complex termed cellulosome, which degrades the crystalline cellulose extensively in presence of Ca^{2+} and a reducing agent, preferably DTT [84]. The yellow affinity substance (YAS) which is produced by *C. thermocellum* during its growth on crystalline cellulose further facilitates the degradation of cellulose by binding the cellulosome to the substrate [101]. The morphological and biochemical properties of *C. thermocellum* cellulosome have been extensively reviewed [95]. Also, the genes coding for several endoglucanases of *C. thermocellum* have been cloned, expressed and the corresponding proteins purified and characterized [13,14,132]. Recently, recombinant EGA, EGC and EGD from *C. thermocellum* have been crystallized and their 3-D structures determined [4,40,42,85,97]. Despite substantial progress, the mechanism of cellulose degradation by the cellulosome of *C. thermocellum* is not entirely clear.

Attempts to dissociate and isolate the individual subunits of the *C. thermocellum* cellulosome in active form were unsuccessful for many years [95]. In 1988, Wu *et al.* [192] reported the isolation of S_L (S_L) and S_s (S_s) subunits of *C. thermocellum* cellulosome in active form and their interaction in solubilizing Avicel, but the extent of Avicel solubilization was low. Recently, Bhat and Wood [19] established conditions for the successful dissociation and reassociation of the cellulosome without losing much of its original activity. Using this novel method, four subunits of *C. thermocellum* cellulosome have been isolated and their interaction in the solubilization of crystalline cellulose has been studied [21]. One major endoglucanase and two major exoglucanases corresponding to three different subunits of the cellulosome have been purified and characterized [21,118]. It should now be possible to isolate all the subunits of *C. thermocellum* in active form and demonstrate the mechanism of cellulose degradation by this highly active cellulase system.

SYNERGISTIC INTERACTIONS AMONG CELLULASE COMPONENTS

Synergism between cellulase components during the hydrolysis of cellulose was first demonstrated by Giligan and Reese [59]. Subsequently several researchers showed the cooperative action between endo- and exo-glucanases (CBHs) during the solubilization of crystalline cellulose and the release of various higher cello-oligosaccharides and cellobiose [109,144,181]. The β -glucosidase completes the hydrolysis by cleaving the cello-oligosaccharides and cellobiose to glucose [34]. However, the degree of synergism varied with the type of cellulose used. In the presence of highly ordered crystalline cellulose (cotton and Avicel), the synergism was high, and was low in amorphous cellulose (H_3PO_4 -swollen cellulose) and was absent in the presence of soluble derivatives such as CMC [34].

Four types of synergism have been reported between fungal cellulase components. These include, between (a) endoglucanase and CBH [188,190]; (b) two immunologically related and distinct CBHs [51,75,94,186]; and (c) between β -glucosidase and either endoglucanase or CBH [49]. In the fourth type of synergism, a non-hydrolytic protein called C_1 is believed to be involved in the relaxation of inter-molecular H-bonding between adjacent cellulose chains [138]. Although, this hypothesis was never substantiated, isolation of CBH which acted synergistically with endoglucanase to degrade crystalline cellulose resolved this confusion [181,183]. In addition, synergisms between bacterial and fungal cellulases as well as between the subunits of *C. thermocellum* cellulosome have been reported [21,64].

The exo-exo type synergism between CBH I and II of *T. reesei* has been demonstrated during solubilization of cotton [51,75,94]. It has also been claimed that CBHs I and II, together, can effect the extensive hydrolysis of crystalline cellulose and that the endoglucanase plays only a minor role [45]. However, CBHs I and II from *P. pinophilum* acted synergistically during the hydrolysis of Avicel, but not cotton [186]. It has also been shown that CBH II tends to form complexes with endoglucanases, which act synergistically with the CBH I to degrade the cellulose [178,188,190]. Furthermore, CBHs I and II of *P. pinophilum* prepared free of endoglucanase [160] act synergistically only to a limited extent during hydrolysis of crystalline cellulose [188,190]. These results suggest that the exo/exo type synergism observed between CBHs I and II of *T. reesei* could be due to the presence of trace amounts of endoglucanase.

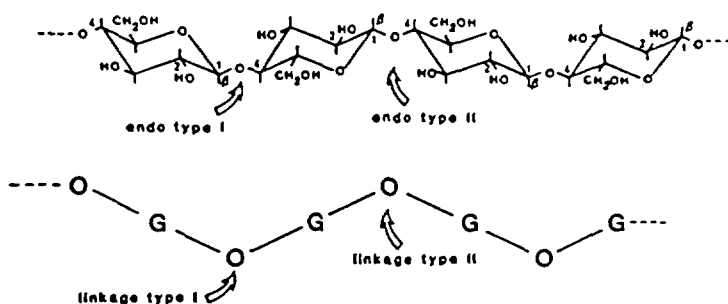
Using highly purified endoglucanases and CBHs from *P. pinophilum*, it has been demonstrated that specific types of endoglucanase must be added to the mixture of CBHs for the extensive hydrolysis of crystalline cellulose [188,190]. Thus, in the case of *P. pinophilum* cellulase, only two endoglucanases (EG III and EG V) which were strongly adsorbed onto cellulose showed a significant degree of synergism in the presence of CBHs I and II [18,188,190]. Maximum synergistic activity occurred when the CBHs I and II were in 1:1 ratio, with a trace amount of either endoglucanase III or V [188,190]. These results demonstrated, for the first time, that at least three enzymes (two CBHs + one

endoglucanase) are required for extensive hydrolysis of crystalline cellulose. Although, the number and type of enzymes required to degrade crystalline cellulose have been established, it is still not well understood how the cellulase components interact on the face of the cellulose crystallite.

An early model [181] envisaged that the cellulose chains cleaved by endoglucanases become the substrate for CBH. This model argued that the action of CBH was essential to prevent the reformation of the glycosidic bonds between glucose residues held in place in the crystal lattice [174]. However, this model does not explain either the synergism between CBHs I and II or the apparent inability of the CBHs to synergise with all endoglucanases from other microorganisms [176]. Therefore, with a view to refining the above model, it has been suggested that the competitive adsorption of enzymes and the formation of binary complexes are involved in CBH/CBH and CBH-endoglucanase interactions [75]. Moreover, it has been argued that the structural asymmetry in the cellulose crystallite and stereospecificity of the enzymes must be taken into consideration [187,188,190].

The rationale for the above suggestion was based on the following observations: firstly the cellulase enzymes appear to attack a particular face of the cellulose crystallite, and secondly the glycosidic linkages between glucose residues held rigidly in position by H-bonds will be stereochemically quite different [178]. Considering the above issues, the synergism between different endoglucanases and CBHs was best explained by Wood *et al.* [188,190] as follows (Figure 1). The attack of cellulose chains by the stereospecific endoglucanase will generate only one of two possible glycosidic linkages. As a result only one of two possible non-reducing end groups would be generated. These non-reducing end groups would be hydrolyzed by a stereospecific CBH which will result in successive removal of cellobiose units and expose another chain-end of a different configuration. This chain-end would be attacked by the other stereospecific CBH [175]. Hydrolysis of the two chain-ends in random would explain the synergism between the CBHs. The attack by another stereospecific endoglucanase, which may generate a non-reducing end of a different configuration would make the process more efficient [188,190].

Synergism between small (S_8) and large (S_L) subunits of *C. thermocellum* cellulosome during the hydrolysis of Avicel has been reported, but the overall extent of hydrolysis was low [192]. Recently, Bhat *et al.* [21] demonstrated the synergism between major CMCase (S_{11} subunit) and two major pNPCases (S_5 and S_8 subunits) of *C. thermocellum* cellulosome, during the hydrolysis of Avicel, when acting together either with or without S_1 subunit. The synergism was maximum when S_1 subunit was combined with pNPCases (S_5 , S_8) and CMCase (S_{11}). Thus, in case of *C. thermocellum* cellulase system, exoglucanase and endoglucanase-type activities together with S_1 are essential for exhibiting maximum synergism during the hydrolysis of crystalline cellulose. Furthermore, the S_1 which formed a complex with S_5 , S_8 and S_{11} subunits of cellulosome, supported the view that this subunit is responsible for scaffolding various subunits of cellulosome [21]. Formation of such enzyme complex may facilitate



Cleavage of cellulose chain can be effected by either endoglucanase type I or endoglucanase type II attacking type I or type II glucosidic linkages

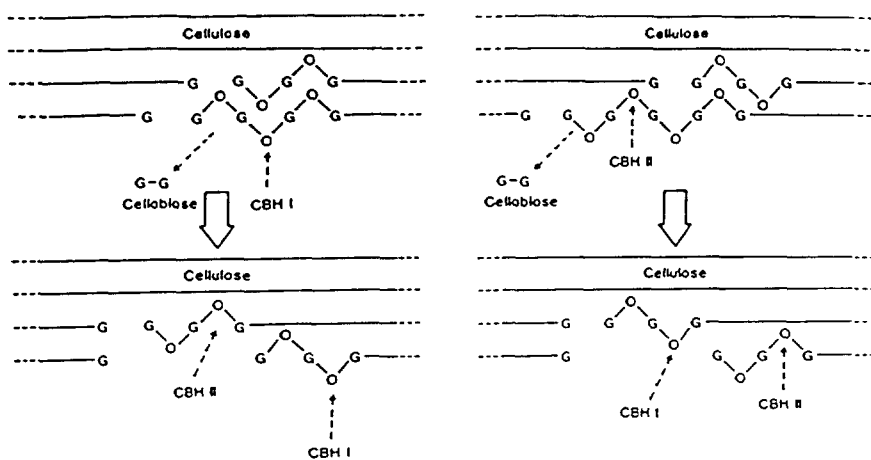
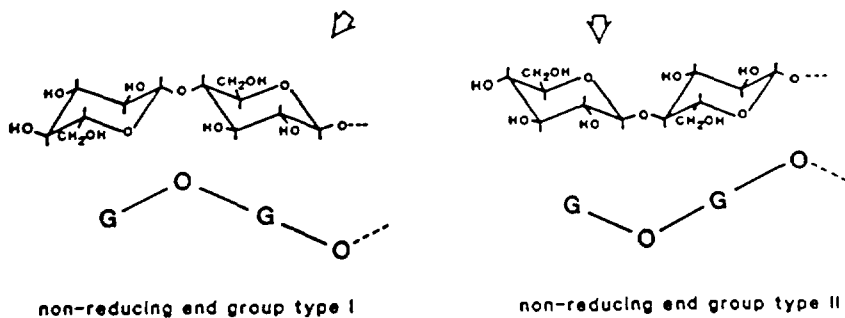


Figure 1. Possible mechanistic explanation of synergism among CBHs I, II and the various endoglucanases of *P. pinophilum* in solubilizing crystalline cellulose. Reproduced from Wood *et al.* [190] with permission.

the correct orientation of subunits during the hydrolysis of cellulose and improve hydrolytic efficiency. Therefore, to understand the mechanism of cellulose degradation by a multienzyme complex such as cellulosome, a knowledge of the synergistic interactions among various subunits of the cellulosome is crucial.

ORGANIZATION AND CLASSIFICATION OF CELLULASES

The cellulase systems of many fungi and bacteria contain multiple enzyme components which are required to cope with the physical heterogeneity of the substrate. Proteolytic cleavage of cellulases to separate the cellulose-binding and catalytic fragments demonstrated the presence of a two-domain organization in these enzymes [62,161,163]. These two domains are linked by a short amino acid sequence of varying length (6–59 residues), rich in proline and hydroxyamino acids which are often highly glycosylated [61]. The cellulose binding domain of the cellulase often helps to target the catalytic domain to cellulose, in order to effect efficient hydrolysis [127].

The CBDs from CBH I, CBH II, endoglucanase II and endoglucanase III of *T. reesei* and from CeX, CenA and CenB of *C. fimi* have been well characterized [62,161,163]. The CBDs from all three enzymes of *Cellulomonas fimi* contain around 100 amino acids with >50% sequence homology [126,172]. The most striking features of these bacterial CBD sequences are: (1) low content of charged amino acids; (2) high content of hydroxyamino acids; and (3) conserved tryptophan, asparagine and glycine residues. In contrast, the CBDs of *T. reesei* CBHs and endoglucanases contain only 33 amino acids and present, either at N or C terminal, with only 10 identical amino acids [61]. A 3-D solution structure determination of CBD of CBH I from *T. reesei* by NMR revealed a wedge-like shape with two flat surfaces, one of which was hydrophilic, while the other was hydrophobic [90]. Homologous CBDs have also been reported in cellulases of other fungi [7,26,30,147,171]. However, most endoglucanases of *C. thermocellum* characterized so far, except endoglucanase E, lack CBD, but the major CBD of the S₁ subunit of the cellulosome of *C. thermocellum* has been isolated, crystallized [95,97] and the 3-D structure determined [Lamed and Bayer, personal communication]. Also, the CBDs which are present either at N or C terminal, and attached to catalytic domains of different specificities, do not appear to be the determinants of CBH and endoglucanase specificities [61].

The catalytic domains of all cellulases are reasonably large and represent more than 70% of the total protein. Analysis of catalytic domains based on their sequence showed considerable variability between different cellulases. However, using hydrophobic cluster analysis of the catalytic domains, cellulases and hemicellulases have been classified into 11 structurally related families [73,74]. According to the initial classification, families A, B, F, H and K contain fungal and bacterial enzymes, family E contains bacterial and plant enzymes, family C contains only fungal enzymes and families D, G, I and J contain only bacterial enzymes. Members of each family possess the same protein fold and

display the same stereoselectivity, which suggests that they share a general mechanism of hydrolysis [57]. However, by comparing the amino acid sequences of 301 glycosyl hydrolases, Henrissat [72] grouped these enzymes into 35 families. Recently, Henrissat and Bairoch [73], using the amino acid sequences of 482 glycosyl hydrolases, classified these enzymes into 45 families. According to the recent grouping, the 11 cellulase families (A–K) correspond to glycosyl hydrolase families 5–12, 26, 44 and 45, respectively. Therefore, it can be speculated that the classification of cellulases into different families may help in predicting the 3-D structure of enzymes from the same family, if the structure of one family member is known.

STRUCTURE OF CELLULASES

Structures of CBH I and II from *T. reesei* were first determined by small-angle X-ray scattering analysis [1,50]. Both enzymes were found to have a tadpole like shape with an isotropic head and a long flexible tail (Figure 2).

Proteolytic cleavage of CBD led to determination of the 3-D structures of the catalytic domains of CBH I and II of *T. reesei* by X-ray crystallography [41,139]. In addition, the 3-D structures of an endoglucanase from *T. fusca* [152], CelA, CelC and CelD from *C. thermocellum* [4,40,85], endoglucanase A from *C. cellulolyticum* [42], β -1,4-glycanase Cex from *C. fimi* [166], and endoglucanase V from *H. insolens* [37] have been determined.

The catalytic domain of *T. reesei* CBH II is a large α/β protein with a fold similar to, but different from the widely occurring barrel topology first observed in triose phosphate isomerase [Figure 3b; 139]. The active site of CBH II is located at the carboxyl-terminal end of a parallel β -barrel, in an enclosed tunnel through which the cellulose threads [139]. The two aspartic acids located in the center of the tunnel are the most likely catalytic residues [139]. Also, it has been suggested that four glucose residues bind to subsites A, B, C and D [139]. The catalytic domain of CBH I is also a large single domain protein with two large antiparallel β sheets that stack face to face to form a β -sandwich [Figure 3d; 41]. Except for four short α -helices, the rest of the protein consists entirely of loops connecting the β -strands [41]. The active site tunnel of CBH I is about twice as long as that observed with CBH II and is estimated to possess seven glycosidic binding sites (A–G) [41]. The catalytic residues are believed to be the two glutamic acids [41]. However, it has been suggested that CBH I cleaves cellulose chains from the reducing end and releases cellobiose units, whereas CBH II hydrolyses the same substrate from the non-reducing end [41,139]. The CBH I and II belong to glycosyl hydrolase families 7 and 6, respectively [Figures 3d and b; 38]. The 3-D structure of endoglucanase from the fungus *T. fusca* (family 6, Figure 3c) is very similar to the catalytic core of CBH II of *T. reesei* [139,152]. However, a close examination of the above two structures suggests a large difference in

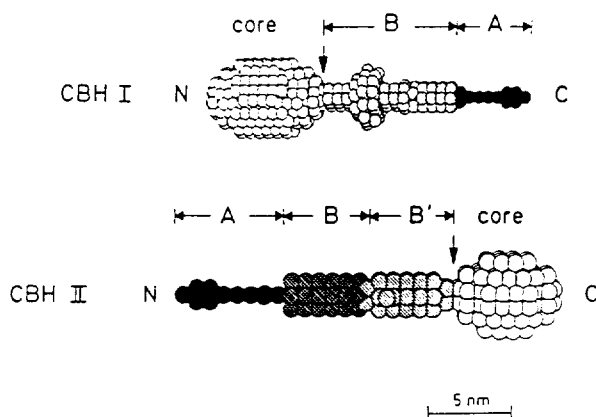


Figure 2. Small-angle X-ray scattering based model of CBHs I and II from *T. reesei*. A = CBD; B = linker region. Reproduced from Abuja *et al.* [1] with permission.

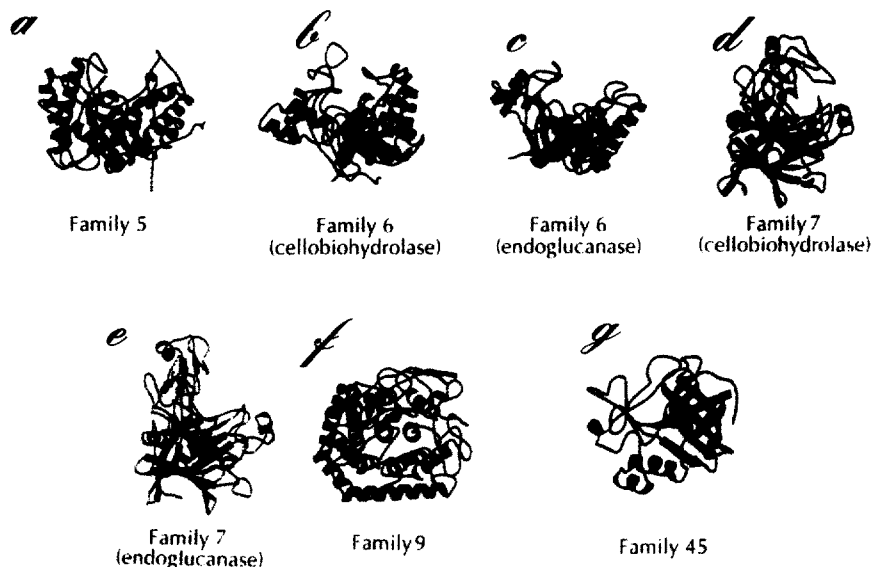


Figure 3. Ribbon representation of the main fold of the catalytic domain of cellulase from different families: (a) Endoglucanase from *C. cellulolyticum* (family 5); (b) CBH II from *T. reesei* (family 6); (c) endoglucanase from *T. fusca* (family 6); (d) CBH I from *T. reesei* (family 7); (e) endoglucanase I from *H. insolens* (family 7); (f) CelD from *C. thermocellum* (family 9); and (g) endoglucanase V from *H. insolens* (family 45). Reproduced from Davies and Henrissat [38], with permission.

their active site accessibilities and supports the hypothesis that the main difference between endo- and exo-glucanases is the degree of accessibility of the active site to the substrate [Figures 3b and c; 38].

The 3-D structure of CelD of *C. thermocellum* reveals that it has a globular and slightly elongated shape with two distinct structural domains; a small N-terminal β -barrel closely packed against a larger, mostly α -helical domain [Figure 3f; 85]. Also, an extended active site has been suggested since it is possible to fit cellobiose in the active site [85]. The CelD of *C. thermocellum* belongs to family 9 of glycosyl hydrolases (Figure 3f). The 3-D structure of CelA of *C. thermocellum* has been determined by multiple isomorphous replacement and refined to 1.65 Å resolution [4]. It has been shown that the protein folds into a regular $(\alpha/\alpha)_6$ barrel formed by six inner and six outer α helices (Figure 4a). Studies using cello-oligosaccharides revealed that these sugars bind to an acidic cleft containing at least five D-glucose-binding subsites (A–E) in such a way that the scissile glycosidic linkage lies between subsites C and D. It has also been shown that the strictly conserved Glu 95, which occupies the center of the substrate-binding cleft, is hydrogen bonded to the glycosidic oxygen and has been assigned as a proton donor. The present structure which belongs to family 8 cellulases also provides a basis for modeling homologous cellulases of this family. In addition, the 3-D structures of CelC from *C. thermocellum* [40,162] and CelCCA from *C. cellulolyticum* [Figure 3a; 42] belong to family 5 have been determined. Both enzymes show a regular $(\alpha/\beta)_8$ protein structures, commonly known as TIM barrels [38].

The 3-D structure of the catalytic core of endoglucanase V from *H. insolens* is quite distinct from that of CBH II of *T. reesei* [37,139] and endoglucanase D of *C. thermocellum* [85] and displays an unusual barrel topology [37]. It has been shown that this enzyme has a flattened sphere-shape with dimensions approximately $42 \times 42 \times 22$ Å and the major structural feature having a 6 stranded β -barrel domain which belongs to glycosyl hydrolase family 45 [Figure 3g; 38]. A large, deep groove runs across the complete 42 Å surface of the molecule, and the active site aspartates (10 and 121) are situated in a groove which shows a remarkable similarity to hen egg-white lysozyme [82]. It has also been suggested that the structure of this enzyme represents the third topology for cellulases [38]. The 3-D structure of endoglucanase I from *H. insolens* has also been determined [38]. This enzyme belongs to glycosyl hydrolase family 7 [Figure 3e; 38].

Based on small-angle X-ray scattering analysis, it has been suggested that the structure of β -1,4-glycanase (Cex) from *C. fimi* resembles a tadpole, in which the catalytic domain corresponds to the globular head and the cellulose-binding domain to the extended tail [167]. The 3-D structure of the catalytic core of Cex from *C. fimi* has now been determined with an R-factor of 0.217 for data extending to 1.8 Å resolution [167]. The protein forms an eight-parallel-stranded α/β -barrel, which is a novel folding pattern for a microbial β -glycanase of family 10 [Figure 4b; 167]. The active site is shown to have an open cleft on the carboxy-terminal end of the α/β barrel. An extensive hydrogen-bonding

network stabilizes the ionization states of the key residues, in particular, the Asp235-His205-Glu233 hydrogen-bonding network. Also, it is believed that this network may play a role in modulating the ionization state of Glu233 and in controlling the local charge balance during catalysis [167].

Determinations of 3-D structures of cellulases from different families and comparison of their structure-function relationships should facilitate precise protein engineering and improvement of catalytic efficiency for specific purposes.

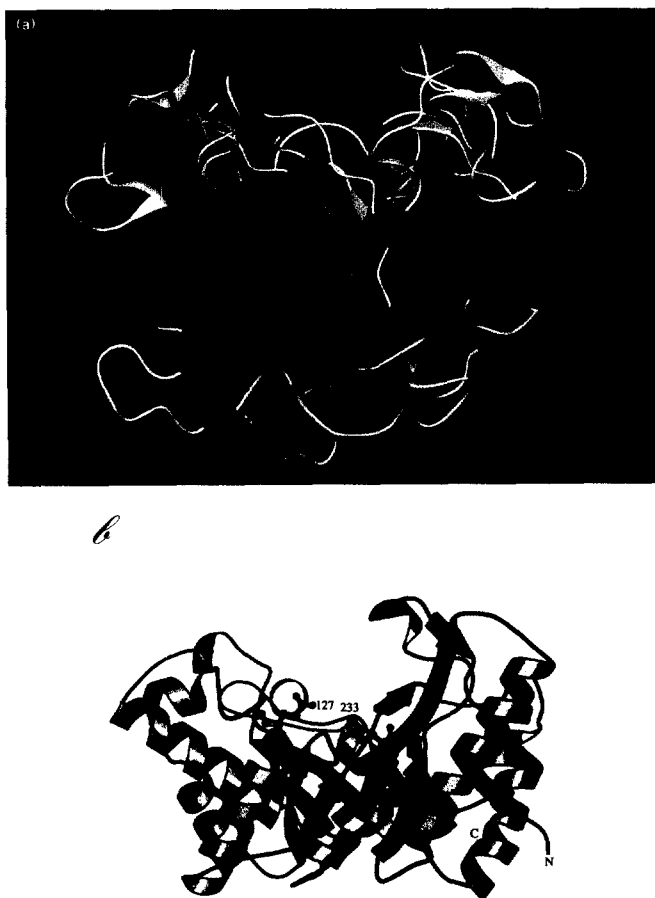


Figure 4. The 3-D structures of two bacterial endoglucanases: (a) Side view of endoglucanase CelA of *C. thermocellum* showing the active-site cleft at the N-terminal end of the inner helices; (b) ribbon representation of β -1,4-glycanase Cex from *C. fimi* showing the catalytic residues Glu127 and Glu233. The N and C represent the amino and carboxy termini, respectively. Reproduced from Alzari *et al.* [4] and White *et al.* [167], with permission.

CATALYTIC MECHANISM OF CELLULASES

Most cellulases follow either retention or inversion mechanism. The hydrolysis mechanism for retaining enzymes was based initially on work with hen egg-white lysozyme, the first enzyme for which a 3-D structure was determined [22,82]. However, additional data on hen egg-white lysozyme suggested that the proposed mechanism was inadequate [149]. An alternative mechanism was put forward by Sinnott [149], which elaborated on Koshland's [89] proposal and could describe the mechanistic data of all retaining glycosidases [Figure 5a, 38]. The mechanism for the inverting glycosidases is similar to that of retaining enzymes except that the hydroxyl group is in the inverted position in the anomeric carbon [Figure 5b; 38]. The mechanism of catalysis of CBH I and II of *T. reesei* has been elegantly demonstrated by NMR spectroscopy using methyl β -D-glycosides of cellotriose and cellotetraose [31]. Further studies revealed that the representatives of a given family follow the same catalytic mechanism [57].

GENETIC ENGINEERING OF CELLULASES

The low levels and the multiple forms of cellulase produced by various microorganisms together with the wide range of cellulase applications, drove the isolation, cloning and the expression of cellulase genes. So far, the cellulase genes from fungi, bacteria and actinomyces have been cloned, expressed and the recombinant enzymes purified and characterized [11,14]. Detailed information on cloning and expression of cellulases from different microorganisms has been published [7,11-14,26,43,47,52,66,99,154,165,172,193-196]. Using genetic engineering techniques, attempts have been made to change the proportion of different cellulases produced by *T. reesei* [71]. In addition, an expression vector, pAMH110, containing the promotor sequences of the strongly expressed *cbh 1* gene has been used to over-express a cDNA coding for EGI of *T. reesei* [71]. Similarly, *in vitro* modified *cbh 1* cDNA, incapable of coding for the active enzyme has also been produced [71]. Using the above approach, novel strains with either mixtures of CBHs, endoglucanases and xylanases or mixtures completely free of CBHs and strains over-expressing the endoglucanase have been produced [71]. Such genetic engineering is likely to produce hybrid strains capable of producing either one or all cellulase components at high levels and could solve some of the current industrial problems.

PROTEIN ENGINEERING OF CELLULASES

At present, the protein engineering of cellulases is in its early stages. So far, cellulases have been engineered mainly at three levels. Firstly, the catalytically important amino acids have been identified and/or mutated by site directed mutagenesis in order to understand the active site architecture and the role of active site amino acids in catalysis [25,36,129]. Secondly, the domains of cellulases are either being engineered or exchanged in order to purify a specific enzyme and also to produce hybrid

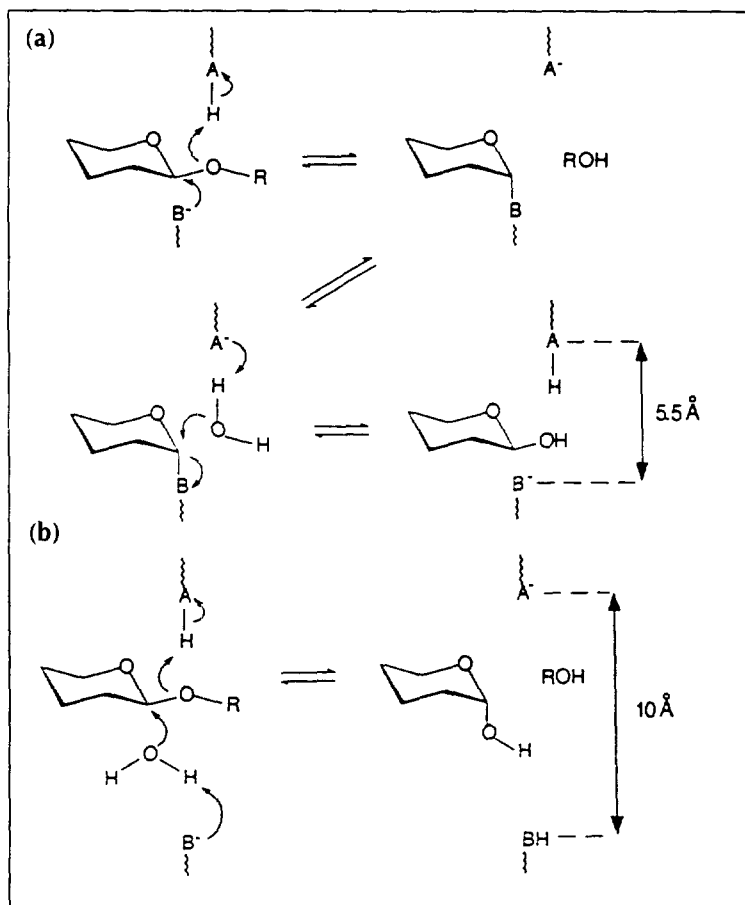


Figure 5. The two major mechanisms of enzymatic glycosidic bond hydrolysis as first proposed by Koshland [89]: (a) The retaining mechanism, in which the glycosidic oxygen is protonated by the acid catalyst (AH) and nucleophilic assistance to aglycon departure is provided by the base B⁻. The resulting glycosyl enzyme is hydrolyzed by a water molecule and this second nucleophilic substitution at the anomeric carbon generates a product with the same stereochemistry as the substrate. (b) The inverting mechanism, in which protonation of the glycosidic oxygen and aglycon departure are accompanied by a concomitant attack of a water molecule that is activated by the base residue (B⁻). This single nucleophilic substitution yields a product with opposite stereochemistry relative to the substrate. Reproduced from Davies and Henrissat [38], with permission.

enzymes. For example, cellulose binding domain (CBD) has been used as an affinity tag for the purification of heterologous polypeptides and for the immobilization of enzymes [5,62]. Thirdly, cellulases have been engineered to understand the contribution of disulfide bridges in the stability of cellulases [133]. The protein engineering studies to date have been confined to endoglucanase, exoglucanase and cellulose binding domain of exoglucanase of *Trichoderma*, *Cellulomonas* or *Bacillus* [5,25,36,62,129,133].

Site directed mutagenesis studies of endoglucanase from *C. fimi* (family B) indicated that Asp-252 and Asp-392 are the essential acid and base catalysts, whereas in endoglucanase from *Bacillus* sp. KSM-330 (family D), the most likely catalytic residues are Glu-30 and Asp-191 [36,129]. Twelve carboxyl residues were mutated to Ala in CelD of *C. thermocellum* by site directed mutagenesis and Glu-555 was identified as the most likely amino acid participating in catalysis [25]. These studies together with the 3-D structures of cellulases demonstrated that these enzymes generally follow acid-base catalysis and possess either two Asps or one Asp and one Glu in their active site, and participate in catalysis.

Many cellulases possess a large catalytic core domain linked by a relatively long O-glycosylated peptide to a small cellulose binding domain (CBD) [161]. CBDs are also found in xylanase from *P. fluorescens* [88] and mannanase from *T. reesei* [154], but their precise function is not yet known. Also, it has been shown that CBDs when fused to heterologous polypeptides can be used as affinity tags either for the purification or immobilization of fusion proteins [62,65,127]. Recently, the CBD gene of either endoglucanase (CenA) or exoglucanase (Cex) from *C. fimi* has been fused either to alkaline phosphatase gene of *E. coli* or β -glucosidase gene of *Agrobacterium* sp. and used for easing purification of the recombinant proteins [62]. Similarly, the CBD gene of exoglucanase from *C. fimi* has been fused to Factor-X gene and the resultant fusion protein was purified using cellulose affinity chromatography [5]. In addition, a hybrid CelC-Cel'D of *C. thermocellum* capable of binding to CipA—a protein that acts as a scaffolding component of *C. thermocellum* cellulosome—was successfully produced by fusing a duplicated 22 amino acid segment from CelD gene to CelC gene of *C. thermocellum* at the 3'-end [162]. Binding of CelC, a non-cellulosomal endoglucanase to Cel'D, which has identical catalytic properties of CelC, confirms that foreign proteins can be incorporated into the cellulosome of *C. thermocellum* to modify its catalytic properties [162]. Thus, CBD appears to be ideal for the production of a variety of affinity tags, immobilization of proteins and enzymes as well as for the incorporation of desired proteins and enzymes into protein complexes. All these possibilities are expected to have tremendous impact in biotechnology and industry.

Disulfide bonds are commonly found in extracellular proteins, and it is widely accepted that they contribute to stabilizing the native conformation of proteins [133]. It has been shown that 1,3-1,4- β -glucanase from *Bacillus* possesses a highly conserved disulfide bridge connecting a β -strand with a

solvent-exposed loop lying on the top of the extended binding site cleft [133]. The contribution of the disulfide bond and individual cysteins (Cys-61 and Cys-90) in the *Bacillus licheniformis* 1,3-1,4- β -glucanase towards stability and activity was investigated by protein engineering [133]. The reduction in the number of disulfide bonds has no effect on kinetic parameters, but has a small effect on the activity-temperature profile at high temperatures, and destabilizes the protein by less than 0.7 kcal-mol⁻¹ at 37°C. However, replacing either of the Cys residues with Ala destabilizes the protein and lowers the specific activity. The Cys90Ala retains 70% of the wild type activity, whereas Cys61Ala and the double mutant (Cys61Ala/Cys90Ala) retain only 10% of the wild type activity. A large change in free energy of unfolding is seen by equilibrium urea denaturation for the C61A mutation (3.2 kcal-mol⁻¹ reduced relative to wild type) as compared with the C90A mutation (1.8 kcal-mol⁻¹ reduced relative to wild type), while the double mutant is 0.8 kcal-mol⁻¹ less stable than the single C61A mutant. The effects on stability are believed to be due to the change in hydrophobic packing which occurs upon removal of the sulfur atoms in Cys to Ala mutations [133].

Other protein engineering studies include the conformational modeling of substrate binding to endocellulase from *T. fusca* [158] and molecular dynamics simulation of fungal cellulose binding domains [77]. Future protein engineering on cellulases should aim to improve their biochemical and catalytic properties, so that cellulases with desired properties and potential applications can be produced in large quantities.

APPLICATIONS OF CELLULASES

Cellulases have a wide range of applications. The main potential applications are in food, animal feed, textile, fuel and chemical industries [11,33,34,105]. Other areas of application include the paper and pulp industry, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment [11,33,34,105].

In food industry, cellulases are being used (a) in the extraction of fruit juices and oil from seeds, (b) in the clarification of fruit juices, (c) to increase the soaking efficiency and homogeneous water absorption of cereals, (d) in the removal of external soybean coat in production of fermented soybean foods such as soysauce and miso, (e) in the isolation of proteins from soybean and coconut, (f) for the efficient isolation of starch from corn and sweet potato, (g) for the gelatinization of seaweeds to increase digestibility, (h) for the extraction of agar from seaweeds, and (i) to digest ball-milled lignocellulose which can be used as food additive [11,33,34,105]. Cellulases can also be used for (a) improving the nutritive quality of fermented foods, (b) improving the rehydrability of dried vegetables and soup mixtures, (c) the production of cello-oligosaccharides, glucose and other soluble sugars from cellulosic wastes, and (d) for the removal of cell wall which will facilitate the release of flavors, enzymes, polysaccharides and proteins [105].

In brewery and wine industries, cellulases are used to (a) hydrolyze β -1,3 and β -1,4 glucan which is present in low grade barley and help in the filtration of beer, and (b) to increase the aroma in wines. The recombinant yeasts producing β -1,3 and β -1,4-glucanases have already been used in brewery industry [11].

In animal feed industry, the cellulases are used (a) as a supplement in feed for ruminants and monogastric animals, (b) in pretreating lignocellulosic material, dehulling of cereal grains, treating silage to improve the digestibility of ruminants and monogastric animals [105]. Another interesting application is that the cellulase genes can be cloned to produce transgenic animals which would secrete the required cellulases into the gastrointestinal tract of the animal and help in the digestion of roughage efficiently [11].

In textile industry, cellulases are being used for (a) removing excess dye from the denim fabric in pre-faded blue jeans (biostoning), (b) removing the microfibrils which stick out from cotton fabrics after several washing cycles, and (c) for restoring the softness and color brightness of cotton fabrics [11,105].

In addition, either cellulases or mixture of glucanases have been used for the production of plant and fungal protoplasts, in producing hybrid strains as well as in other genetic engineering experiments [10]. Thus, cellulases have a wide range of potential applications, but considerable future research effort is necessary to exploit the commercial potential of cellulases to the fullest extent.

CONCLUDING REMARKS

Research on cellulase has progressed very rapidly for the last five decades. Remarkable progress has been made in understanding the production of cellulase, biochemistry of cellulose degradation, cloning and expression of cellulase genes as well as the determination of 3-D structure of a few cellulases. In addition, potential industrial applications of cellulases have been identified. Major impediments to exploiting the commercial potential of cellulases are the yield, stability, specificity and the cost of cellulase production. Therefore, future research should focus on producing high yields of thermostable, alkaline and acidic cellulases with broad substrate specificity and improved catalytic efficiency, using low cost media and fermentation techniques. Research should also aim at exploiting the commercial potential of existing and new cellulases.

Acknowledgments

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