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The cellulose paradox: pollutant *par excellence* and/or a reclaimable natural resource?

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Abstract

The various aspects of cellulose as a pollutant are considered in view of its lack of toxicity on the one hand and its recalcitrant durable nature on the other. The microbial degradation of cellulosics is discussed, and the contrast between its success in handling natural cellulosic wastes versus its failure to cope with man-made refuse is described. Research carried out in the past decade has demonstrated that cellulolytic organisms are provided with cell surface multifunctional multienzyme conglomerates, called cellulosomes, which are capable of solubilizing solid cellulosic substrates. The intriguing properties of such complexes include their cohesive nature, their many enzymatic components, and a characteristic glycosylated cellulose-binding, 'scaffolding' component. The latter appears to serve as a substrate-targeting carrier, which delivers the other (hydrolytic) components to the cellulose. Progress in establishing efficient model systems for *in vitro* solubilization of purified cellulose or natural cellulosic substrates has been achieved using purified cellulosome preparations, fortified with β-glucosidase and pectinase. The latter enzymes were required in order to alleviate the phenomenon of product inhibition which reduces the efficiency of the free cellulosome. Such combined enzyme systems are proposed as examples of future tailor-made cellulolytic systems for the degradation of natural cellulosics.

Introduction

Is cellulose, in fact, really a pollutant?

After all, it is common knowledge that paper is easy to degrade, or is it? There appears to be a common myth or delusion as to the biodegradability of cellulose. In any case, cellulose is not particularly toxic to the environment, but is it a serious problem? Figure 1 gives a schematic description of the fate of cellulose in the environment and the consequences of human activities.

Cellulose is the most abundant organic raw material in the world, comprising the major portion of plant matter. In its 'native' form, e.g., as the major (40–50%) component of wood in trees, cellulose is an ecological treasure. In other plant tissues, the cellulose content ranges from 20% in some grasses to over 90% in cotton fiber. But trees and other plants die, bringing the cellulose and other plant components (notably lignin and hemicellulose) to the soil, where, in time, natural forces (particularly microbes) are capable of accomplishing their degradation.

Fortunately, the process is reversible in that trees and other plants grow, thus replenishing the supply of cellulose and other organic polymers at

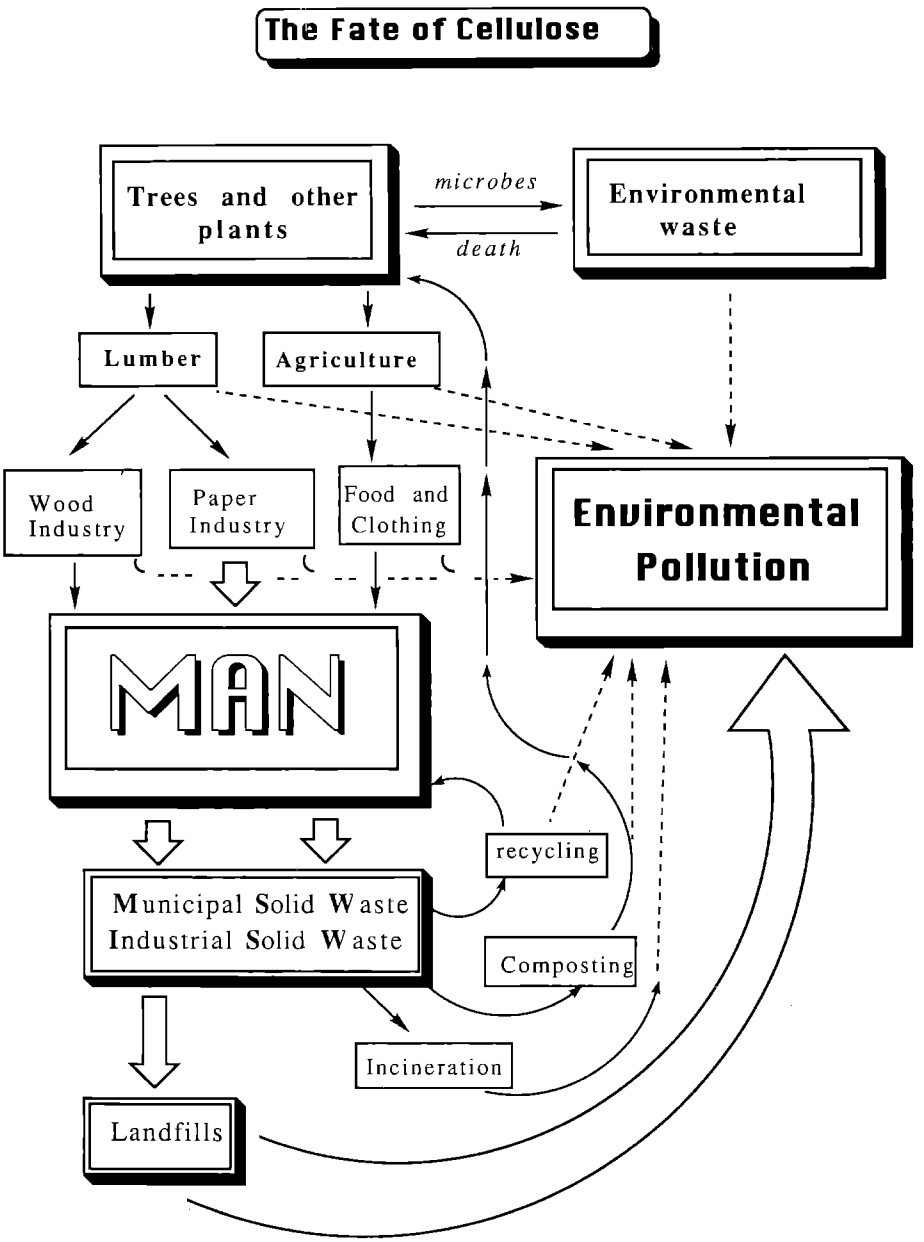

Fig. 1. Schematic view of the fate of cellulose in the environment resulting from both natural (microbial) degradation and human activities.

the expense of atmospheric carbon dioxide. In nature, the evolution of this cycle has been perfected over a period of hundreds of millions of years, and a variety of microbes exist in virtually every niche and clime, which are specifically suited for eliminating cellulosic waste. The facts speak for themselves: in the natural state there is no uncontrollable accumulation of cellulose-containing refuse. In other words, nature takes care of natural waste very nicely.

Human-generated trash is quite another story. Industrial wastes, agricultural wastes, commercial and residential wastes eventually become environmental waste. The industrial utilization of wood

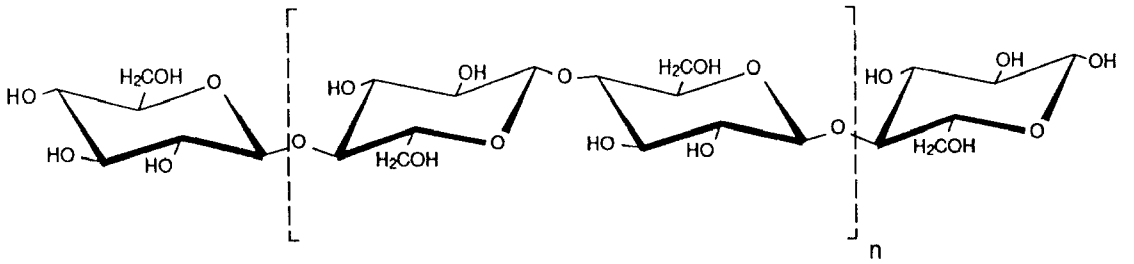

Fig. 2. Cellulose is composed of repeating units of cellobiose, 4-0-(β-D-glucopyranosyl)-D-glucopyranose where n is the degree of polymerization and is commonly about 10,000 units.

and other agricultural products is a serious source for depletion of viable plant matter which ultimately becomes cellulosic waste. Timber is prepared by logging and lumbering, and is used in construction, furniture and so on, before being returned in some way to the soil. In the sphere of agriculture, after crops are harvested, the residue is generally rich in cellulosics. Cotton waste is two-fold; the debris left in the fields are eventually joined by the derelict fabric. But, by far, the major source of cellulose waste in our environment is derived from paper.

On the one hand, the continued industrial processing of paper from wood is one of the important contributions to modern society. The dissemination of information in newspapers and books is dependent on the paper industry. The advent of computer technology has not alleviated this dependence; quite the contrary. Thus, wood is converted to paper, which is eventually converted to a lot of wastepaper.

The reason that paper has become such an important mainstay in our society is actually a function of the physical characteristics of cellulose. Cellulose is a remarkably stable polymer owing to its tertiary structure; the polymer consists of a simple undeviating syndiotactic linear arrangement of glucose residues, coupled exclusively by β [1, 4] glycosidic linkages. The simple disaccharide formed, called cellobiose [4-0-(β-D-glucopyranosyl)-D-glucopyranose], is the repeating unit of the cellulose polymer (Fig. 2). The resultant chains are organized in parallel fashion into fibrils, which are stabilized by interchain hydrogen bonding. Individually, the hydrogen bonding is quite weak, but collectively they result in a very strong associative force. The fibrils are considered to attain a 'paracrystalline' state, which accounts for about 75% of the cellulose fiber, the residual chains comprise 'amorphous' areas. The structural rigidity and stability of paper is thus a direct result of the molecular properties of cellulose.

For the same reasons, microbial attack of the cellulose is very slow. Although many microbes synthesize relevant enzymes, the cellulases, the types and number of individual microbes which can degrade efficiently, or solubilize, cellulose are indeed scarce. Under the best conditions in nature, cellulose degradation occurs at a sluggish pace. For certain uses, this is of course desirable; books are stable on the shelf for generations and sometimes centuries. In contrast, for other (even related uses) the stability of paper is perhaps exaggerated; the information recorded in newspapers is of only transient interest, and the newspapers are discarded on a daily basis. It should therefore come as no surprise that newspapers are the largest single item in landfills! And collectively, paper occupies almost 50% of the space in landfills. Thus, in terms of volume and mass, the accumulated cellulose waste can certainly be considered a problem.

Whether or not cellulose is indeed toxic, it has great potential as a source of biomass for conversion to soluble sugars that can serve as substrates for fermentation to fuels and chemicals. Low value cellulosic materials, such as wheat straw, sawdust, cornstover, sugar cane bagasse, and other agricultural residues are produced at a rate of about one billion tons (dry weight) annually in the United states (Tsao et al. 1987). It has been estimated that about 20% of this biomass can be

economically collected and fed into the production of chemicals. Many chemicals can be produced from cellulosic residue, including those useful in the production of energy [such as ethanol (Wright 1988; Fan et al. 1982), ethylene, propylene and related compounds (Hanselmann 1982)]. Other uses include production of high energy animal feeds (Gould 1985), polymer feedstocks and organic solvents (Hanselmann 1982).

The microbial degradation of cellulosics for combined waste management, by-product and energy resource has been a stated goal for over half a century. Cell-free enzymatic hydrolysis is also considered to be a promising approach for obtaining glucose from cellulosics, yet cellulase systems are neither sufficiently inexpensive nor efficient to achieve this objective. To date, the failure to design commercially viable technologies to achieve this goal can largely be attributed to the reliance on engineering finesse, often at the expense of understanding the basics of the cellulolytic phenomenon *per se*. This policy has resulted in decades of applied microbial and enzyme research, but, in retrospect, has led to poor potential with regard to the degradation of cellulosic materials.

In this chapter, we will concentrate on some of the intricacies of the microbial degradation of cellulose. In doing so, we will present some of the new principles discovered in our laboratory which deal with the bacterial degradation of cellulose. The primary role of cell adhesion to the substrate will be discussed. In this context, cellulolytic bacteria appear to produce a cell-associated, multifunctional, multienzyme complex, coined the cellulosome, which mediates the attachment of the bacteria to the cellulose surface, thus targeting the relevant cellulases to their sites of action. Knowledge gained from such studies has contributed to substantial progress in the efficient hydrolysis of pure and complex cellulosic substrates, using the purified cellulosome combined with other enzyme preparations.

The problem with cellulose pollution

As mentioned above, the real problem with cellulose waste is not necessarily one of direct toxicity or of wanton buildup in nature of immense quantities of cellulose which constitutes an environmental threat. Nevertheless, a variety of cellulosic wastes are often associated with the increasing problem of disposal and inherent negative environmental impact. These include residues from crop production, animal husbandry, forestry, paper pulp production, sawn wood and other forest product industries as well as food processing – wastes which range in moisture content from dry combustible straws to slurries and manures of very high water composition. These substances also offer the best potential short-term option as raw materials for energy generation as well as for composting and pulp production.

Significant amounts of non-hazardous industrial solid waste as well as the organic fraction of municipal solid waste are already disposed through energy- and resource-recovery routes, which include electricity generation, gas extraction from landfills, composting and combustion with district heating. Separation and utilization technologies, such as anaerobic digestion, pyrolysis and gasification offer opportunities for a massive increase in the utilization of such waste. Although some crops are specifically intended for the production of feedstock and energy, no emphasis will be placed in this chapter on trees and other crops grown for this purpose.

Ironically, most of the real pollution generated by the paper industry and the pulping process is not cellulosic in essence, but either relates to the chlorination of lignin aromatics or emanates directly from the bleached pulp which is then converted to paper. The chlorinated derivatives and sulfur emissions resulting from the processing of paper are not only toxic themselves, but they represent an added factor in secondarily inhibiting the biodegradation of the cellulose, thus exacerbating the overall problem. Agricultural residues, such as fiber from sweet sorghum, have also been found suitable for paper pulp production, but such straw-based factories have frequently been closed, due, emphatically, to the problems of pollution associated with the large amounts of water and chemicals used in the process (as well as the high costs involved), thus rendering

the product noncompetitive by conventional processes.

Processed paper waste not only constitutes the vast majority of cellulosic waste, but comprises a very large part of environmental trash as a whole. These include newspapers and magazines, telephone books, and packaging. By all accounts, present estimates of the paper waste (in terms of percentage bulk content of the total solid waste) currently hover around 40 to 50%. This value has more than doubled in the past 40 years or so and is expected to continue to rise in the future. A pointed example of this trend concerns the analysis of refuse from the city of Birmingham, England, where the content of paper and cardboard in trash rose from slightly less than 8% in 1934, to over 20% in 1960 and over 50% in 1970 (Bridgwater & Mumford 1979).

One of the great popular myths is that paper decomposes rapidly, but this perception is greatly erred; *legible* newspapers, buried up to 40 years in landfills, have been unearthed in archeologically oriented 'garbage digs' (Rathje 1991). Such studies have been designed to provide us with new insights into our society. They are extremely enlightening, and the accumulated data are currently serving both as a warning to the public concerning the nature of our recent and current trash and as guidelines towards future planning for its effective disposal. In fact, newspapers are used to 'date' other items, buried proximately. A combination of reasons may account for this astonishing stability, ranging from the anaerobic conditions of landfills, their deficiency in moisture content, the lack of necessary nutrients and proper agitation, and the presence of toxic wastes (e.g., solvents, preservatives, and the like).

Most municipal solid waste is disposed in landfills (McCarthy 1990). More than 70% of the trash in the United States is being buried in 5,500 active landfills. However, many are reaching capacity, and few new ones are being approved, mainly due to the increasing awareness of leachate-derived pollution of water, air pollution or other adverse effects on the environment. The knowledge gained through research into the mistakes of the past has contributed to more intelligent site selection, design, operation and reclamation of modern landfill sites (Crawford & Smith 1985). Modern alternatives to landfills include recycling and composting. But these too have their problems: recycling is hampered by market instability for certain materials and technical barriers for the recycling of mixed materials as well as pollution problems of its own. Composting can also cause environmental pollution, often compounded by the production of pathogenic organisms (Biddlestone & Gray 1987). Nevertheless, future use of these alternatives to landfill technology should continue to be explored.

In agriculture, perhaps one of the best examples of undue pollution is caused by the commonly employed practice of incineration for eliminating straw, leaves, and other low-moisture high-level cellulosic materials in the fields. Burning reduces waste volume by up to 90%, and the technology is currently available to establish incinerators which would convert the heat from burning garbage to steam or electricity. However, both air pollution as well as toxic ash would remain a problem. Notably, the pollution produced by smoke has instigated a recent law in the United Kingdom which bans this method, creating in turn great pressure for the development of alternative methodologies to digest surplus agricultural waste. Many other such wastes are high in cellulose content and often burned in the fields, some examples being banana husks, coffee peels, cotton straw, lemon grass and sugar cane begasse.

The potential for degradation of cellulose wastes

Three different approaches for the degradation of cellulosics are generally considered: the microbial approach, the use of purified enzymes, and combined chemical and/or physical approaches. In its natural lignocellulose state, cellulose is fairly well protected from microbial degradation, mainly due to the lignin and hemicellulose polymer components. These must be removed from the surface of the highly crystalline polymer. The degradability of lignocellulose under anaerobic conditions is indeed rather low, due to the near-absolute requirement of high levels of oxygen for microbial attack on

lignin. The susceptibility of lignocellulose to enzymatic hydrolysis depends on various factors, including particle size or surface area (Grethlein 1985), degree of crystallinity (Walseth 1952), and extent of lignification (Schwald et al. 1988). Different chemical and physical pretreatments, including steam explosion, ball-milling, pyrolysis or swelling in alkali, can markedly increase, in some cases, the extent of degradation (Marsden & Gray 1986). However, such procedures may considerably add to the overall operational costs. Moreover, degradation products of both chemical pretreatment processes and lignin-hemicellulose biodegradation are often toxic to strict cellulolytic microorganisms.

Microbial approach

Many microorganisms are known which can degrade cellulose in nature, including both fungi and bacteria, some of which exist under extreme environmental conditions, e.g., temperature, salts and pH. Microbial growth and decomposition of matter are generally divided into environmental categories (Haigler & Weimer 1991), usually based on temperature (thermophilic, mesophilic, cryophilic) and oxygen levels (aerobic and anaerobic). Other critical environmental factors are humidity, pH, and particularly in man-made environs, the presence of toxicants, such as heavy metal ions, solvents and other toxic chemicals, all of which may severely interfere with normal microbial growth. In any case, it appears that there are cellulolytic microorganisms which can exist in nearly every habitat (Table 1).

It should also be noted that, in nature, cellulolytic microbes do not occupy a given niche in pure culture. Rather, they exist in mixed culture with, perhaps, other cellulolytic organisms as well as those which degrade associated polymers (i.e., lignolytic and xylanolytic microbes). The polymer-degrading members are joined by others which utilize proficiently the polymer breakdown products (e.g., oligosaccharides and simple sugars), end-products (e.g., organic acids and molecular hydrogen), and toxic side-products (e.g., by-products of lignin and xylan hydrolysis).

Despite the anticipated complexity of natural systems, only one or a few strains may dominate the niche for a given time period, whereas other 'satellite' strains may provide assistance in facilitating the activity of the major strain(s). As the degradative process proceeds, the conditions of the microenvironment change, and the prosperity of a given strain would rise and fall according to demand.

The types of organisms necessary to achieve maximum results in a given niche would thus be dictated by the conditions of the given environment

Table 1. Some representative cellulolytic microorganisms.

BACTERIA
Aerobic Mesophiles
Cellulomonas fimi
Cellulomonas uda
Cellvibrio fulvus
Cellvibrio gilvus
Streptomyces falvogriseus
Aerobic Thermophiles
Thermomonospora curvata
Thermomonospora fusca
Anaerobic Mesophiles
Acetivibrio cellulolyticus
Bacteroides cellulosolvens
Clostridium cellobioparum
Clostridium cellulovorans
Fibrobacter succinogenes
Ruminococcus albus
Ruminococcus flavefaciens
Anaerobic Thermophiles
Caldocellum saccharolyticum
Clostridium stercorarium
Clostridium thermocellum
FUNGI
Aerobic Mesophiles
Aspergillus niger
Fusarium solani
Neurospora crassa
Penicillium pinophilum
Sporotrichum pulverulentum
Talaromyces emersonii
Trichoderma reesei
Aerobic Thermophiles
Chaetomium thermophile
Sporotrichum thermophile
Thermoascus aurantiacus
Anaerobic Mesophile
Neocallimastix frontalis

and the nature and complexity of the substrate. In this context, wood is much more difficult a substrate than paper, and, as mentioned above, pure cellulose itself is not so easy a substrate for microbial degradation. More than 100 species of bacteria and fungi have been considered for cellulose studies in the last century (Ljungdahl & Eriksson 1985), but to date none has been successfully applied (either in pure or in combined culture) in a commercially viable process for converting even pure cellulose to a desired product. The reasons for this failure range in character from low hydrolytic yields of the microbial cellulases, to the sensitivity of the microbial cells to the accumulation of end-products (such as ethanol). One recent study has registered some success in growing *C. thermocellum* in continuous culture on either microcrystalline cellulose or pretreated wood (Lynd et al. 1986). The pretreatment step was found to be essential, and substrate utilization was in the range of about 80%. The authors concluded that further efforts in identifying and overcoming the limiting factors would be necessary for greater conversion at higher rates. In any case, there is still a critical need to better understand the scientific aspects of cellulolytic microorganisms and their cellulases *before* allowing technology to proceed.

In view of the above considerations, the design of a successful applied system for the microbial elimination of cellulosics must take into account the composition of the substrate, the characteristics of the desired microbial strain (or strains) and component enzyme systems, the expected product pattern, and, of course, the costs involved. In the final analysis, it would be advantageous to couple the use of microbes to combat cellulose pollution with an economically feasible process. In this regard, certain products (such as soluble sugars, ethanol, acetone, and butanol) are preferred whereas others (acetic acid and lactic acid) are considered undesirable due to the difficulty in their separation and processing.

Application of cell-free cellulases

Individual microbial cellulases have been historically classified in several defined categories. The endoglucanases (endo-1, 4-β-D-glucan 4-glucanohydrolases, EC 3.2.1.4) sever the linear cellulose polymer presumably by random attack at sites within the chain. The exoglucanases (exo-1,4-β-D-glucan cellobiohydrolases, EC 3.2.1.91) remove cellobiose units from the nonreducing end of the chain. The β-glucosidases (β-D-glucoside glucohydrolases, EC 3.2.1.21) hydrolyze the free cellobiose molecules to two molecules of free glucose. Some cellulolytic systems, although rare, purportedly include glucohydrolases (1,4-β-D-glucan 4-glucohydrolases, EC 3.2.1.74), which cleave the terminal glucose unit from the nonreducing end of the cellulose chain. The neatness of this classification scheme is not generally matched by the actual characteristics of the purified, individual cellulases (especially those of bacterial origin), and, in due time, the establishment of a new scheme may prove beneficial.

Individual cellulases are incapable of hydrolyzing the paracrystalline cellulose polymer in an efficient manner. That is, a single cellulase enzyme cannot cause the total solubilization of the native cellulose polymer. One of the important doctrines, which has been almost sacred to the study of cellulolysis, is that the different enzymes are considered to act synergistically with each other. To explain this phenomenon, various hypotheses have been proposed in the past, including (1) the requirement for a noncatalytic hydrogen bond dissociating element (defibrillating enzyme), (2) the simple combination of endo- and exo-acting activities, (3) the requirement of two different cellobiohydrolases to account for the two possible stereochemical forms of the terminal chain glucose residue, and (4) the requirement for simultaneous multiple attack of the chains, which can be accomplished by higher order arrangement of the different enzymes. One thing is clear, there is a consensus that the end-product cellobiose is inhibitory to efficient cellulolysis, and the concerted action of β-glucosidase is essential for maximal synergistic effect. However, the mechanism(s) responsible for synergism is still under dispute, and the reader is referred to other sources for more detailed discourse of this issue.

Before the advent of molecular biology and the

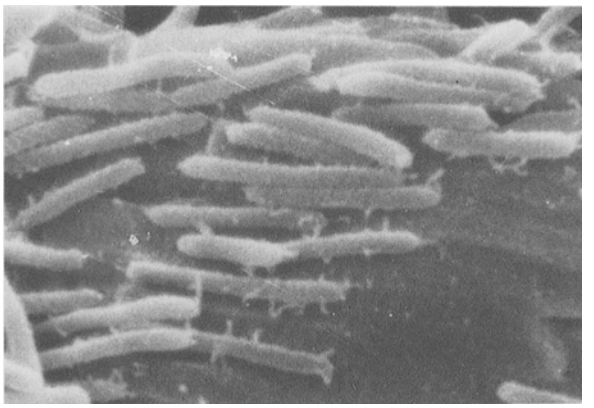

Fig. 3. Scanning electron micrograph of cationized ferritin-stained cells of *Clostridium thermocellum* growing on cellulose. Note the protracted protuberances which connect the cells to cellulose. Magnification: × 15,000.

recent strides in understanding the unique aspects of bacterial cellulase systems, fungal enzymes were the systems of choice. Unlike the bacterial enzymes, those from fungal sources are generally extracellular in nature, and their isolation and characterization have been relatively simple. Pure bacterial cellulases were sought for many years, but only in the last decade was it demonstrated that they are commonly cell-associated in the form of very large multienzyme complexes, called cellulosomes (Lamed et al. 1983a,b; Lamed & Bayer 1988a).

Despite the progress in this area, again, like in the studies using intact microbial systems, the use of cell-free cellulases for ambitious industrial application has been a frustrating endeavor. Why, then, has there been such marked success with amylases on the degradation of starches? The major reasons for this are manifold. First of all, the β-glucoside bond in cellulose is structurally very rigid and the cellulases are subject to strong product inhibition; in contrast, amylase systems generally lack of feedback inhibition, and glucose in the α configuration (which characterizes the starch polymer) is readily available to the enzymes. Incidentally, these properties reflect the natural roles of the respective materials in nature: i.e., cellulose relies on its natural stability for its role as a structural polymer, whereas starch is a storage material, which is subject to enzymatic breakdown when necessary. Thus, these vast differences in structural stability of these polymers and their consequences regarding their function in nature and propensity towards microbial and enzymatic degradation are all due to the simple difference in the configurational state of the anomeric carbon of the repeating glucose unit.

Chemical and physical approaches

Dilute acid hydrolysis combined with elevated temperatures is the only reasonable alternative to date as non-enzymatic methods of cellulose hydrolysis. Although this approach has been seriously considered in the past, especially for unprocessed wastes (e.g., municipal solid wastes), a maximum of only about 60% glucose can be expected after optimal hydrolysis conditions over a 24-h period (Green et al. 1988). The residue includes unwanted decomposition products of glucose and other sugars to furfural and derivatives, levulinic and formic acids and humic substances, in addition to the original acid and its by-products. Mechanical pretreatment, such as hammer-milling, trommeling and grinding are necessary to facilitate hydrolysis. These methods (inclusive) may also be appropriate for pretreatment of cellulosic wastes prior to microbial or cell-free enzymatic strategies.

The cellulosome of *Clostridium thermocellum*

Clostridium thermocellum is one of the few, and certainly the most prevalent, anaerobic thermophilic cellulolytic bacterium in nature (Lamed & Bayer 1991). Such bacteria are generally considered attractive for applicative research, mainly due to the fact that costly refrigeration and aeration procedures are unnecessary for maintaining their active culture. The processes are usually free from contaminating pathogens, and the thermophilic enzymes produced are generally of high activity and structural stability. *C. thermocellum* is considered to be the epitome of cellulolytic microorganisms, and its solubilizing potential of native crystalline cellulosics has been reported to be about 50 times higher than that of microbial systems (Johnson et

al. 1982). Indeed, the cellulolytic system of this bacterium has been a standard for decades of previous industrially oriented research and development.

In nature, the utilization of a polymeric substrate, like cellulose, by a microbial system would appear to be most efficient, were the hydrolytic enzymes not randomly secreted leading to free competition for the soluble products; rather, if these enzymes are positioned in the interface between the cell and the insoluble substrate, they would gain a diffusional advantage as well as possible synergistic action amongst the densely packed enzymes. Lastly, regulation by product inhibition would be very important to avoid waste of unused substrate, in cases where the cell is unable to absorb the soluble products generated by the hydrolytic action of the enzyme. It is therefore not surprising that one of our first observations, regarding the interaction of *C. thermocellum* with cellulose, was the very strong adhesion of the bacterium to its substrate prior to its degradation (Fig. 3).

To further study this phenomenon, we employed a combined genetic-immunochemical program which consisted of isolation of an adhesion-defective mutant combined with the preparation of a monospecific antiserum to analyze the cellulose-binding factor on the wild-type cells (Bayer et al. 1983). The factor was isolated and identified as a large 2-MDa multicomponent conglomerate, which not only exhibited the cellulose-binding function but also expressed very high levels of cellulolytic activity (Lamed et al. 1983a), estimated to be the major portion of the cellulolytic action in this organism. Electron microscopic and SDS-PAGE analysis supported the notion that the factor was organized into a cohesive multi-subunit structure. The subunits were examples of very large bacterial proteins, ranging in size from 210 kDa to 48 kDa (Fig. 4). Due to its proposed central and multifunctional role in the degradation of cellulose by *C. thermocellum*, the cellulose-binding factor was termed the cellulosome (Lamed et al. 1983b). The isolated cellulosome was shown to exhibit the same properties as the crude cellulase system from this bacterium; most notably, the purified cellulosome was alone capable of completely

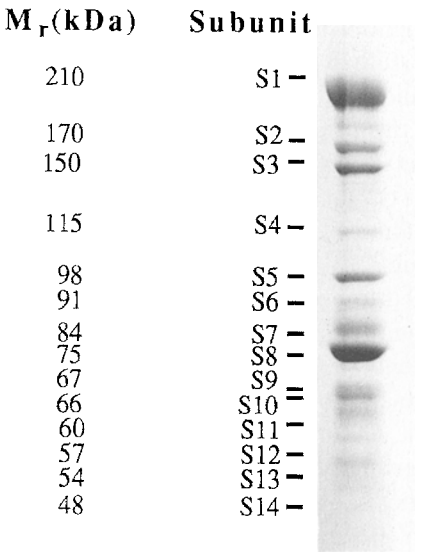

Fig. 4. Subunit composition of the cellulosome of *Clostridium thermocellum.*

solubilizing the paracrystalline structure of the insoluble substrate (Lamed et al. 1985). The presence of a discrete cellulosome complex was proposed to be a definitive property of the cellulase system in this organism, which promotes the synergistic action among its enzyme components.

Since these early reports, further study has been centered on characterizing the molecular and supramolecular nature of the cellulosome, with emphasis on functional and structural descriptions of the individual cellulosomal components. The major findings include the following: (1) descriptions of the disposition of the cellulosome on the cell surface, (2) the elucidation of a major nonenzymatic glycopeptide component (the S1 subunit) which is believed to play a decisive dual function, including both the crucial organizational role and the major cellulose binding role, (3) the analysis of the enzymatic components of the cellulosome, and (4) the isolation and characterization of a major exoglucanase, a cellobiohydrolase, which was found to exhibit many of the distinctive properties of the cellulosome. It was also discovered that many other cellulolytic bacteria, irrespective of their evolutionary or physiological profiles, appear to contain cellulosome-like entities which are similar in content and action to that of *C. thermocellum.*

Throughout the years, one of the major problems in studying the action of the components of the cellulosome is the exceptionally cohesive nature of the protein complex. Only treatment in detergent at high temperatures succeeded in dissociating the cellulosome into its component parts, and isolation of reasonable quantities of the individual subunits for conventional biochemical studies proved to be extremely difficult. Nevertheless, *in situ* zymogram assays of SDS-PAGE-separated components revealed that some subunits expressed very high activity levels on various substrates (Morag et al. 1990). In one case (Morag et al. 1991), limited proteolysis of the intact cellulosome led to the purification of a truncated form of a key subunit (S8), which was classified as a fairly typical cellobiohydrolase – the first and, as yet, the only confirmed example of this important enzyme type for this organism.

A second complementary direction in the study of the cellulase system of *C. thermocellum* has involved the cloning and expression of the relevant genes (Hazlewood et al. 1988; Beguin 1990). More than a dozen genes from this organism have been cloned and many of them have been expressed. By comparing the cellulase genes from *C. thermocellum,* both among themselves and with those from other species, it can be concluded that the cellulases are constructed in a modular fashion composed of multiple domains of defined function (Beguin et al. 1992). Interestingly, many cellulases contain a cellulose-binding domain separate from the catalytic domain. Many contain segments rich in proline, threonine and/or serine of as yet undetermined function.

Table 2. Some properties of the components of the cellulosome from *C. thermocellum* strain YS.

Subunit	M_r(kDa)	$\%_{app}$	Sugars	EGase	MUCase	CBHase	Xyl'ase
S1	210	24	+++	–	–		–
S2	170	5	–	++++	–		++++
S3	150	9	–	+	++		(++++)
S4	115	<2	–	±	±		–
S5	98	8	–	±	+++		±
S6	91	<2	–	–	?		–
S7	84	7	–	–	+		++
S8	75	29	–	–	–	+++++	(++++)
S9,10	66,67	7	–	–	+++		+++
S11	60	~2	–	(++++)	–	–	+
S12	57	<2	–	–	–		–
S13	54	4	–	+++++	–		+++++
S14	48	<2	–	?	–		?

$\%_{app.}$, the apparent percent of cellulosomal content, contributed by individual subunits, was determined by densitometric tracings of Coomassie Blue-stained SDS-PAGE gels.

Enzymatic activities are denoted as follows: EGase, endoglucanase; MUCase, methylumbelliferyl cellobiohydrolase; CBHase, cellobiohydrolase; Xyl'ase, xylanase.

Genuine CBHase activity was verified only for the purified (truncated) S8 subunit; the cloned endoglucanase D (termed EGD, and identified subsequently as cellulosomal subunit S11) definitely lacked this activity. Other activities were detected on zymograms after SDS-PAGE.

+++++, ++++, etc., the relative activity in each assay is denoted by the number of '+'s.

–, indicates that no activity could be detected in the given assay.

(++++), indicates that the designated activity was undetected in the normal zymogram, but could be verified in other assays or under altered conditions. Thus, EGase activity of the S11 subunit could not be detected in the zymogram, but was very high for the cloned and expressed enzyme (EGD). Likewise, xylanase activity was absent in S8 after SDS-PAGE, but was very high for the isolated subunit. The xylanase activity of S3 was heat sensitive (when samples were incubated above 70° C).

?, due to the low percentage of certain cellulosomal subunits (e.g., S6 and S14), their activities could have been masked by those of their neighboring subunits.

As time progresses, it becomes clear that certain cellulase gene products are equivalent to cellulosomal subunits whereas others are not. Thus, endoglucanase D (EGD), appears to be synonymous with the S11 subunit (Joliff et al. 1986; Lamed & Bayer 1988b), the xynZ gene product has been equated with the S5 subunit (Grepinet et al. 1988) and EGE corresponds to cellulosomal subunit S7 (Durrant et al. 1991). One of the intriguing findings that ensued from the cloning studies was the presence of a 24-amino acid reiterated domain in cellulase genes which seems to be associated with cellulosome-based enzymes (Navarro et al. 1991; Tokatlidis et al. 1991). The reiterated domain appears not to be involved in either binding to cellulose or catalysis thereof; one of the most plausible roles of this domain will be discussed below.

Some of the important enzymatic properties of the cellulosomal subunits are summarized in Table 2. With the exception of cellobiohydrolase (CBHase) activity, the other activities listed in the table were assayed on zymograms following SDS-PAGE of heated cellulosomal samples. Consequently, unless otherwise indicated for isolated components, the activities listed are those which were still recovered after treatment at either 70 °C or 100 °C in the presence of SDS (i.e., conditions which are required for separation of the cellulosome into its components). Endoglucanase activity (EGase) was measured using carboxymethyl cellulose as a substrate, 'mock' exoglucanase activity (MUCase) employed methyl umbelliferyl cellobiose as a substrate, and xylanase activity (Xyl'ase) was assessed using a soluble xylan fraction. The lack of detection of a given activity, under the described assay conditions, does not necessarily indicate the absolute lack of the said activity, since the relatively harsh conditions prior to enzymatic analysis would impede all but the stablest of enzymes. Indeed, in cases where enzymatic components were successfully isolated (i.e., S8 and S11), high levels of enzymatic activities were detected despite their absence in zymograms. Nevertheless, the information given in Table 2 provides some indication as to the character of the cellulosomal enzymes.

It is clear from the table that the different enzymatic subunits of the cellulosome express different patterns of cellulolytic activities. It is interesting to note that the subunits which express very high levels of endoglucanase (e.g., S2, S11 and S13) are all relatively minor components ($\leq 5\%$) of the cellulosomal protein in apparent quantitative terms. In contrast, the confirmed cellobiohydrolase (the S8 subunit) appears to account for almost a third of the cellulosomal protein. As stated above, this subunit is believed to play a central role in cellulose hydrolysis.

Another cellulosomal subunit which accounts for a major proportion of its protein is one of the most intriguing – i.e., the nonenzymatic, glycosylated, 210-kDa S1 subunit. The importance of this subunit to the cellulosome was recognized in the first publications, and, in view of its perplexing lack of enzymatic activity, various other roles have been proposed, including the crucial cellulose-binding activity and the central component carrier function. The reiterated domain, which has been associated with the other, mainly enzymatic, components of the cellulosome, may be responsible for the linkage of the respective component to the S1 subunit and, hence, to the cohesive cellulosomal complex. This would suggest a reciprocal subunit binding domain on the S1 subunit. Recent evidence, based on its anomalous dissociative behavior in SDS-PAGE gels (Morag et al. 1991b), has suggested that upon binding to cellulose, the S1 subunit undergoes dramatic conformational alterations in its structure (Morag et al. in press).

Thus, on the basis of this and other recent data, the general structure of the cellulosome and its initial interaction with cellulose can be envisioned as shown in Fig. 5. The cellulosome consists of a central S1 subunit which contains one or more cellulose binding domains (CBD) and a series of subunit binding domains (SBD). Bound to the latter in cohesive fashion are the other catalytic subunits via their reiterated sequences. The cellulosome first binds to the cellulosic substrate via the high-affinity CBD(s) on the S1 subunit, which undergoes conformational alterations on the surface of the cellulose. Upon binding, low-affinity CBDs on the other (catalytic) cellulosomal subunits may then bind the substrate, perhaps in a defined orienta-

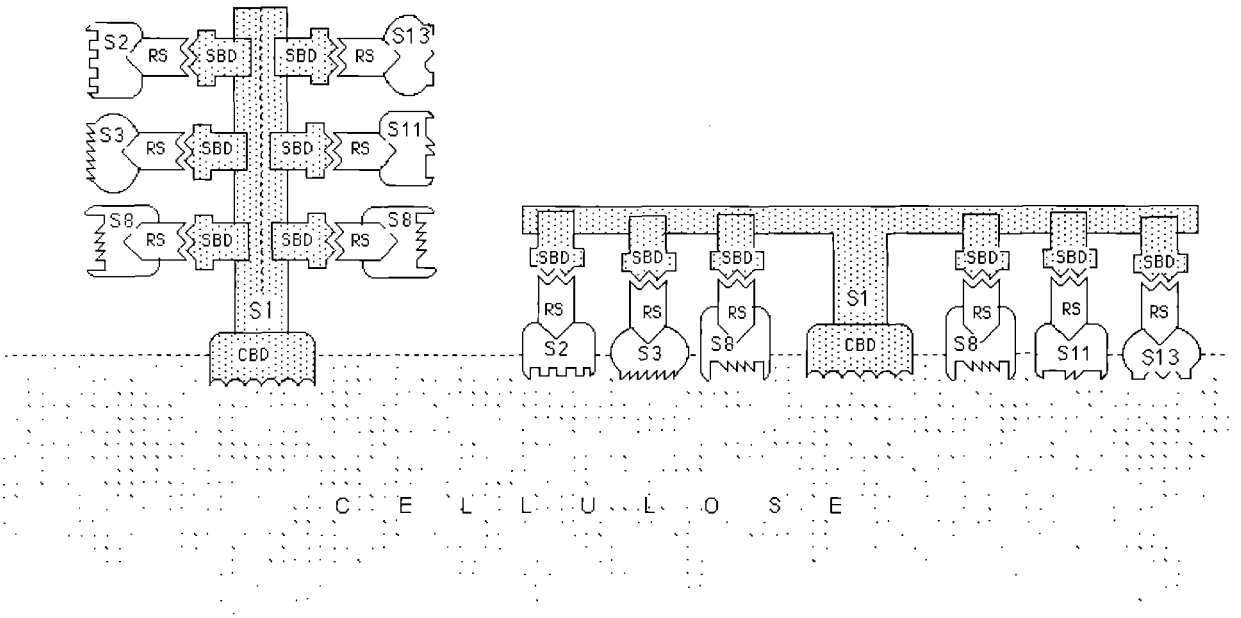

Fig. 5. Proposed model of cellulosome structure. The cellulosome comprises a central assembly or 'scaffolding' component, the S1 subunit, which exhibits a modular structure with multiple domains. The S1 subunit contains multiple subunit binding domains (SBD) which serve as attachment sites for the individual catalytic subunits (the cellulases). The S1 subunit also bears a very strong cellulose binding domain or domains (CBD) which targets the complex to the substrate. The cellulase subunits contain a 24-amino acid reiterated sequence (RS) which appears to be the site of attachment to the S1 (i.e., via the proposed SBDs, each of which may be selective for a specific RS). Upon binding to cellulose, the S1 subunit undergoes a conformational change which may be assisted by low-affinity CBDs known to occur on bacterial cellulases.

tion, and the concerted catalysis of the substrate ensues.

The binding of the cell-free cellulosome to cellulose actually reflects a more important function which the cellulosome performs, i.e., the binding of the cell itself to cellulose by virtue of the cell-

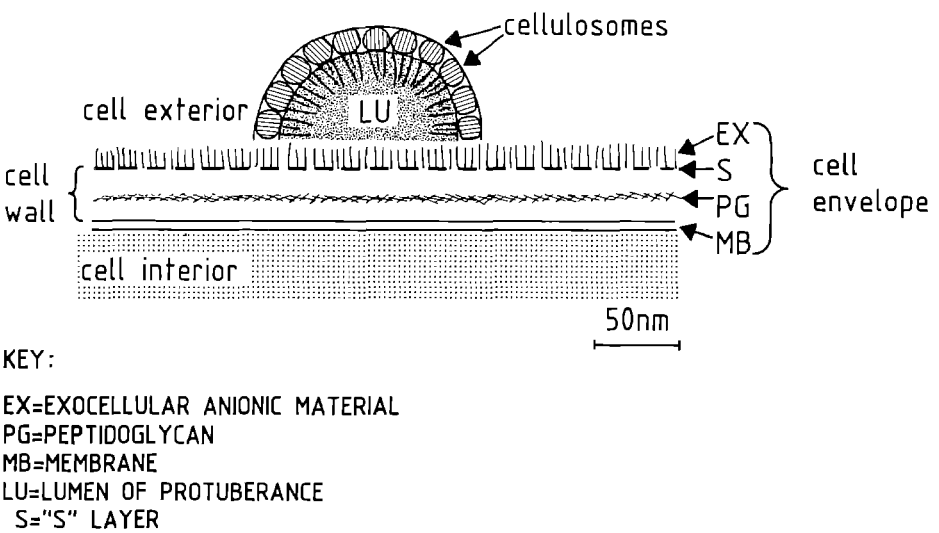

Fig. 6. Schematic description of the disposition of the polycellulosomal protuberances on the exocellular surface in the resting state. In the absence of the cellulosic substrate, the cellulosomes are fixed on the exterior of the protuberance in a compact state.

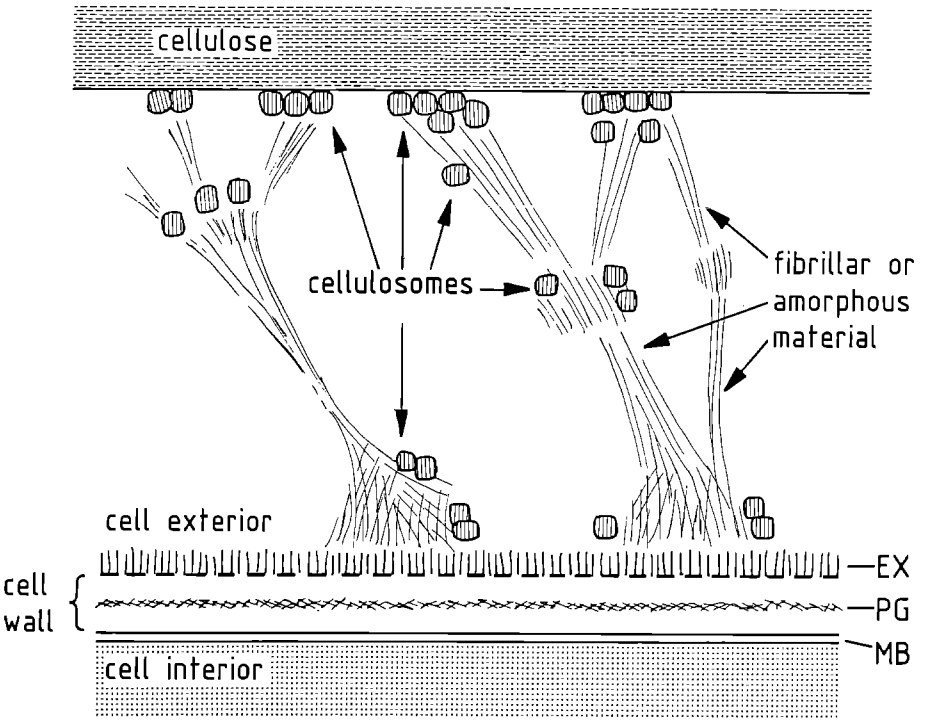

Fig. 7. Schematic description of the interaction of the polycellulosomal protuberance with cellulose. Upon contact with cellulose, the protuberance undergoes a dynamic suprastructural transformation in which the cellulosomes attach to the substrate but remain connected to the cell surface via protracted fibrous material. Thus, depolymerization of the substrate occurs via the synergistic action of a concentrated array of cellulosomal enzymes in the exocellular interface. The resultant soluble sugars, mainly cellobiose, are taken up by the cell by the appropriate transport apparatus.

associated cellulosomes (Bayer et al. 1983). To accomplish this task, the bacterial cells have been provided with a specific organelle, the polycellulosomal protuberance, which appears to contain multiple copies of the cellulosome (Bayer et al. 1985, 1986). In the absence of substrate, cells are inundated with large numbers of protuberances in contracted form (Fig. 6). Upon interaction with the substrate, however, the protuberances protract to form 'contact corridors' which appear to comprise fibrillar or amorphous material (Fig. 7). The cellulosomes which line the surface of the cellulose, begin to degrade the substrate extracellularly, producing large quantities of cellobiose, which is taken up by the cell via the appropriate transport system. Cell-associated β-glucosidase converts the cellobiose to glucose, and glucose is metabolized intracellularly via the usual pathways (Lamed & Bayer 1991). The major end-products in *C. thermocellum* are ethanol, lactic and acetic acids, molecular hydrogen and carbon dioxide.

The existence of the cellulosome in *C. thermocellum* appears not to be an isolated incident. Other unrelated cellulolytic bacteria, such as *Clostridium cellobioparum, Acetivibrio cellulolyticus, Bacteroides cellulosolvens,* and *Ruminococcus albus,* seem to harbor such multienzyme complexes on their cell surfaces, complete with protuberance-like organelles (Lamed et al. 1987). Moreover, S1-related antigenic determinants and similar sugar moieties seem to be shared among the various cellulolytic species. In this regard, the defining oligosaccharide residue of the S1 subunit has been elucidated in *C. thermocellum* (Gerwig et al. 1989, 1991). The sequence is shown in Fig. 8. The structure is characterized by a majority of galactosyl groups, one of which is in the very unusual furanose ring form. The terminal GalNAc residue is 3-0-

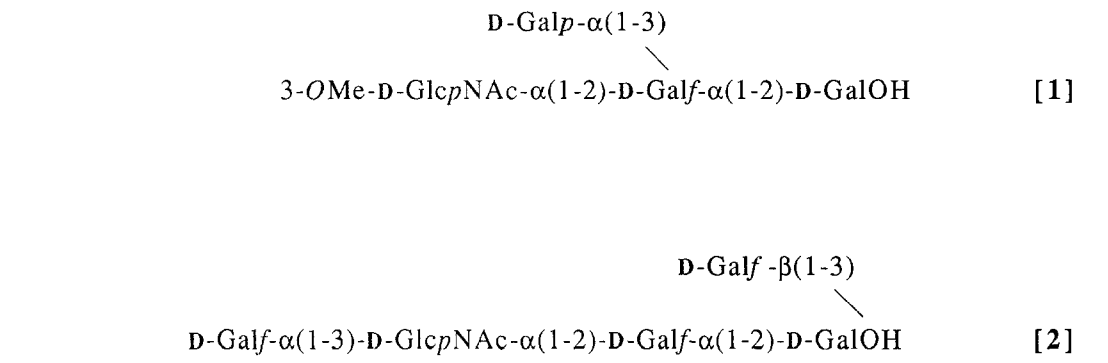

Fig. 8. The structure of the major galactose-rich cellulosomal oligosaccharide moieties from two different cellulolytic species of bacteria. [1] is from *Clostridium thermocellum* and [2] is from *Bacteroides cellulosolvens*. The oligosaccharide from *C. thermocellum* has been shown to be attached to the scaffolding S1 subunit. Note the similarities in the respective structures. The three initial sugars (from the nonreducing end) appear to be identical; the oligosaccharides differ in their substitutions, indicating species-specific variation.

methylated to form a unique naturally occurring sugar. In a very different cellulolytic bacterial species, a very similar structure was also found for its defining oligosaccharide, isolated from the cellulosome-like entity of *B. cellulosolvens* (Gerwig et al. 1992; Lamed et al. 1991a). The sequence contained several of the structural elements which characterized the S1-derived oligosaccharide from *C. thermocellum*. Although the precise role(s) of these oligosaccharide residues is currently unknown, the evidence implies that the S1 subunit and its sugars are critical to the cellulolytic action in such bacteria.

Solubilization of purified cellulose by a combined cellulosome-β-glucosidase system

Perhaps the major reason that cell-free enzyme systems have failed so dismally in attempts to efficiently degrade cellulosics is due to the strong inhibition by the major end product, cellobiose. Consequently, in designing a process for the enzymatic solubilization of cellullose, the major consideration is to rid the system of the resultant cellobiose so as to prevent feedback inhibition of the cellulase apparatus.

In nature, the cell precludes such inhibition by a variety of mechanisms (Lamed & Bayer 1991b). First, the substrate is degraded extracellularly by means of the polycellulosomal protuberances, after which part of the cellobiose formed is converted to glucose by the cell-associated β-glucosidase. The glucose and the residual cellobiose are transported into the cell by the corresponding transport systems. In addition, the large amounts of cellobiose formed in natural ecosystems would be further utilized by saccharolytic (but non-depolymerizing) satellite bacteria which occupy the same niche. In this way, cellobiose is effectively removed from the environment, and the cellulase system (i.e., the cellulosome) continues to degrade the substrate efficiently. In contrast, in the cell-free state, the cellulosome produces saturating levels of cellobiose, and cellulolysis is thus inhibited.

In a recent study (Lamed et al. 1991), we demonstrated that a purified β-glucosidase (from *Aspergillus niger*) can be combined with the purified cellulosome from *C. thermocellum*, in order to hydrolyze the accumulated cellobiose. Using this strategy, a dramatic enhancement of cellulose digestion could be achieved. The observed 10-fold enhancement was expressed both in the rate and degree of solubilization of the paracrystalline substrate, compared with that observed for the cellulosome alone (Table 3). Very dense suspensions of cellulose (at least 20%) could be converted to a glucose syrup with almost complete hydrolysis.

The combination of a β-glucosidase with a cellulose system has been reported earlier with varying success (for example, see Chakrabarti & Storey 1989; Kadam & Demain 1989). In general, these

processes were economically prohibitive, since catalytic efficiencies were extremely low, and very high enzyme-substrate levels were necessary. The major contribution, in using the cellulosome from *C. thermocellum* with the readily available β-glucosidase from *A. niger,* is that very high rates and levels of solubilization can be achieved with relatively low amounts of enzyme components. Preliminary results involving the hydrolysis of dried alfalfa using the combined enzyme system have shown that the approach is also suitable for natural cellulosic substrates.

Solubilization of citrus albedo using a combined cellulosome-β-glucosidase-pectinase system

As described above, cellulase systems in general are prone to feedback inhibition, and, in most cases, cellobiose is the predominant cause of such inhibition. In nature, cellular uptake of cellobiose and/or its hydrolysis to glucose serve to relieve the inhibitory effect, thus providing a regulatory mechanism to improve the economy of the process.

Pectin is another naturally occurring polymer, which is present in large quantities in nature, particularly in fruit-derived agricultural wastes. Pectin is a polymer consisting of methyl 1,4-α-D-galacturonic acid, wherein the extent of methylation varies. Pectin can be especially problematic in applied processes, due to its gelling properties which are responsible for the high volumetric nature of pectin-containing materials. Pectinase activity was not detected in our cellulosome preparations, and the cellulosome, either alone or in combination with β-glucosidase, was incapable of degrading such substrates. The cellulosome requires the presence of pectinase in order to reach its substrate.

Thus, a combined enzymatic system, which consisted of the cellulosome from *C. thermocellum,* the purified β-glucosidase preparation and a partially purified pectinase (both from *Aspergillus niger,* obtained from Novo Industries a/s, Bagsvared, Denmark), was designed to hydrolyze a model pectin-containing cellulosic substrate (citrus albedo, from pomelo, which contained 27% cellulose and 70% pectin).

We discovered that, in order to hydrolyze albedo, the addition of the cellulosome-β-glucosidase system failed to improve control systems containing the pectinase alone, unless the reaction was performed under conditions, whereby low-molecular-weight breakdown products (which served as inhibitors of the cellulosome) were removed (R. Kenig et al., in preparation). This could be achieved either by dialysis or by introduction of an

Table 3. Enhancement of cellulose solubilization by the cellulosome from *C. thermocellum* supplemented by β-glucosidase from *Aspergillus niger*.

Avicel concentration (mg/ml)	Solubilization (%)	
	Cellulosome alone	Cellulosome + β-glucosidase
20	53	100
50	38	100
200	12	96*

Data after Lamed et al. (1991b)
The assay was performed using 8 μg cellulosome per mg of substrate. β-Glucosidase (0.04 cellobiase units per mg substrate) was added when required. The degree of solubilization was determined following a 9-day reaction period.
* At the highest substrate concentration tested, a second addition of the enzyme mixture (administered 72-h after the onset of the reaction) was required to effect near-total hydrolysis.

Table 4. Inhibition of cellulosome action on microcrystalline cellulose by pectin breakdown products and related acidic sugars.

Additive	Solubilization (%)	
	60° C	50° C
None	95	95
Pectin hydrolysate	9	60
Galacturonic acid	22	88
Glucuronic acid	93	95

Data after Kenig et al. (unpublished results).
Solubilization (%) was determined in a turbidometric assay system, following a 72-h reaction period at the designated temperature, using 0.6 mg/ml of a suspension of Avicel as the substrate. The concentration of pectin hydrolysate used in the competition experiments was 5 mg/ml, and that for both uronic acid derivatives was 6 mg/ml. The concentration of the cellulosome used in the reaction was 8 μg/mg cellulose.

anion exchanger. Galacturonic acid also inhibited the degradation of pectin by the combined system but to a somewhat lesser degree (Table 4), and controls using a similar compound (glucuronic acid) were ineffective inhibitors.

Further experimentation indicated that the inhibitory effect of the pectin hydrolysates was determined to reflect a reduction in the stability of the cellulosome and not a strict example of competitive inhibition. Indeed, the S8 (the crucial cellobiohydrolase subunit of the cellulosome) was eventually found to undergo strong destabilization by the pectin degradation products, thus reducing the efficiency of the entire combined enzyme system.

In order to achieve optimum solubilization of the albedo, the destabilizing effect had to be eliminated. High concentrations of albedo (200 mg dryweight/ml!) were therefore subjected to the enzymatic action of the combined system at lower temperatures and in the presence of an anion exchanger to remove negatively charged pectin hydrolysis products as soon as they are formed. The results indicated that these conditions can effectively solubilize pectin-cellulosics such as citrus albedo, and that the coupling of extraneous enzymes to the cellulosome can be a valid approach for the degradation of naturally occurring mixed polymer systems. When a greater understanding is achieved concerning the composition of such native substrates and the effect of their breakdown products on the relevant enzyme systems, the design of cost-effective processes for waste disposal and utilization may eventually be realized.

Supercellulosomes – crosslinked enzyme systems

In the above-described systems, the enzymes were applied together in free soluble form. We also considered the possibility of coupling combined enzyme systems, either by chemical or biochemical means, in order to produce supercellulosomes which contain the relevant enzymes associated into complexes for more efficient hydrolysis and economy considerations. The idea behind this approach is that the cellulosome would serve to target the other enzymes to the relevant sites of hydrolysis, and the enzymes would achieve maximum effect where required.

As a model system, the purified cellulosome and β-glucosidase system were selected. Initial experiments in this direction were performed by mixing the desired enzymes in the presence of crosslinking reagents. For this purpose, several different reagents were examined: (1) glutaraldehyde was used to crosslink the enzymes via their lysine residues, (2) sodium periodate was used to oxidize the cellulosomal or β-glucosidase oligosaccharides to aldehydes which would interact secondarily with the lysines of either component, and (3) water-soluble carbodiimide was used to couple aspartate and glutamate residues of one enzyme component to lysines of another. Unfortunately, none of these covalent procedures worked, since in every case, the enzyme components were irreversibly inactivated.

In view of these results, we used a biochemical approach based on the avidin-biotin complex (Wilchek & Bayer 1990). Biotin was coupled to both the cellulosome and β-glucosidase; the addition of avidin (which has 4 biotin-binding sites per molecule) served to produce complexes which contained both enzyme species. In this case, both components could be biotinylated with over 70% of the enzymatic activity remaining. In the standard experiment, the biotinylated cellulosome was first applied alone to purified cellulose samples. Next, avidin was introduced to the resultant suspension, and the residue was washed. Finally, the biotinylated β-glucosidase was applied to the adsorbed cellulose sample, and again washed. In this manner, the combined enzyme system would comprise an active cellulolytic layer which produces glucose directly from microcrystalline cellulose on the surface of the insoluble substrate. The rate and extent of cellulolysis obtained using this targeted system was about three times more effective in its solubilization of cellulose than the cellulosome-absorbed system alone. These results were similar to those obtained using an excess of β-glucosidase (without washing). We are currently examining an *in situ* approach wherein the components are not applied in sequence, rather the crosslinked supercellulosome constructs are preformed prior to application to the insoluble substrate.

Other strategies for combining the appropriate enzyme species can also be explored in the future. One particularly appealing approach would be to use genetic engineering techniques, e.g., gene fusion or insertion of desired domains into a single entity. Thus, cellulose-binding domains can be combined at the gene level with catalytic domains of the desired enzyme species to enhance synergism and overall substrate hydrolysis.

As our knowledge of the structure and composition of natural cellulosic systems progresses, and our understanding of the various cellulolytic and related enzyme systems is broadened, it is hoped that we will be able to design and develop 'smart' cellulase systems for use in future economically viable industrial processes.

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