ENZYMATIC HYDROLYSIS OF CELLULOSE

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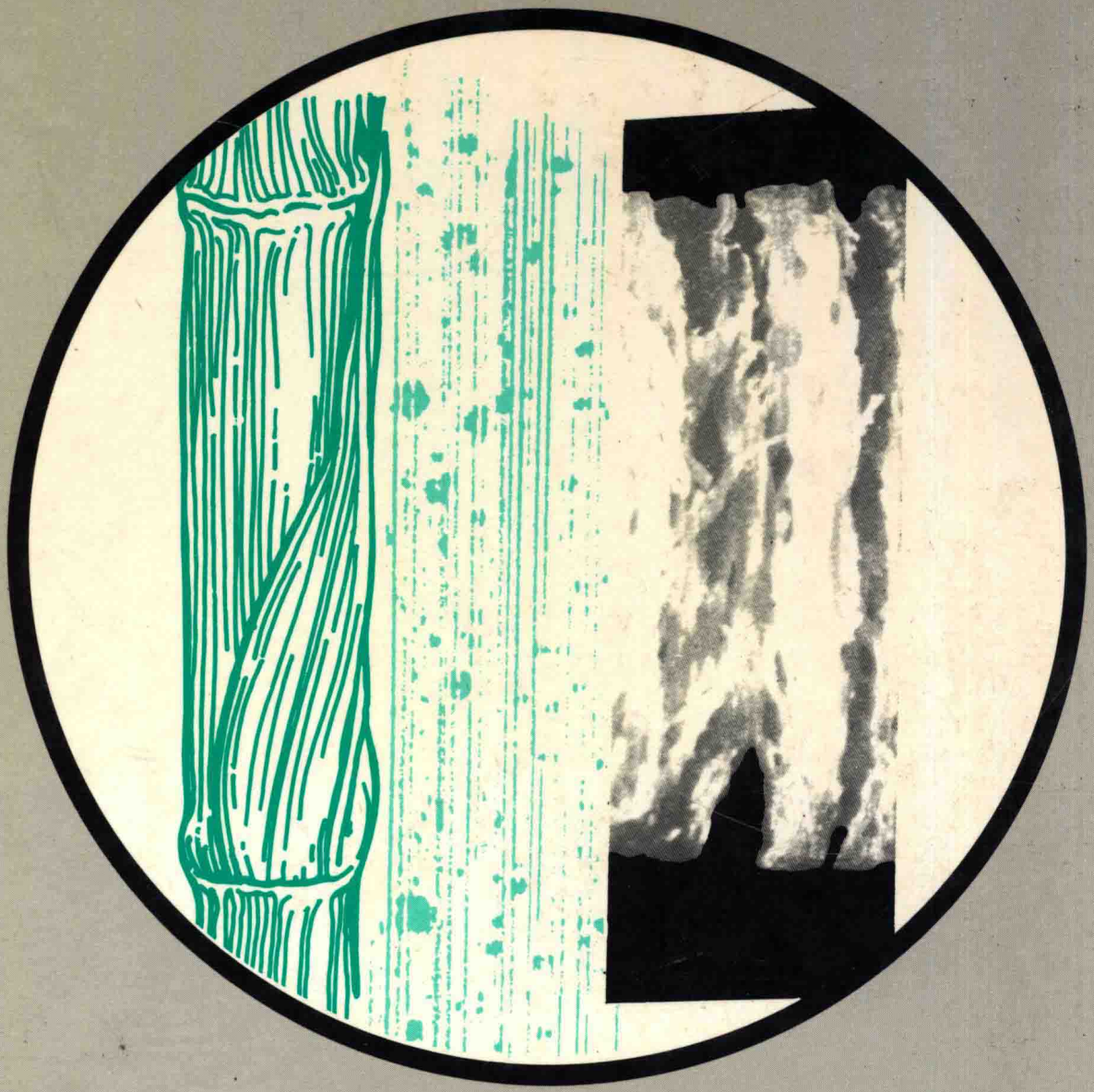

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Guest Editorial

Over the last decade considerable progress has been made toward understanding, manipulating and commercializing processes for enzymatically hydrolyzing cellulose to fermentable sugars. Research and development efforts have ranged from modifying cellulose to increase its digestibility to engineering cellulases, enzymes that degrade cellulose, to yield higher rates and extents of hydrolysis. This international research and development effort is critical for increasing the economic and technical viability of processes using enzymatic hydrolysis to convert cellulose to food, energy and chemicals. With an increasing world population and the associated demand for improved quality of life, this biomass option and others must be developed to their fullest potential.

The idea for this special issue of *Bioresource Technology* on enzymatic hydrolysis of cellulose grew from discussions with Dr Wayne Smith. There was a sense that the community of biomass researchers and developers would benefit from a comprehensive review of the scientific and engineering developments that have occurred in this field over the last decade. Of particular interest were breakthroughs in understanding the molecular mechanisms of cellulase–cellulose interactions and the assessing the role of biotechnology in manipulating these enzymes. We accepted this task with some trepidation and with much enthusiasm.

We thank the scientists who contributed to this special issue of *Bioresource Technology* and those who participated in the peer review process. We are grateful for the help that Dr Wayne Smith provided during the preliminary development of this issue and the final preparation of the publication. Finally, we want to thank the Elsevier editorial staff for their effort in processing the manuscripts in a timely fashion.

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Enzymatic Hydrolysis of Cellulose: An Overview

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Abstract

Despite the fact that the world community is no longer preoccupied with fossil fuel shortages, there is still considerable research and development directed toward understanding and commercializing enzymatic hydrolysis of cellulose. These efforts have ranged from applied work on bioreactors to basic research focusing on the detailed molecular mechanisms of hydrolysis. Studies on cellulose pretreatment have provided considerable insights into the influence of crystallinity and specific surface area on the rate and extent of hydrolysis. In addition these studies have demonstrated that the economics of enzymatic hydrolysis processes can be significantly improved by pretreatment. However, the major drawback to commercialization of enzymatic hydrolysis processes continues to be the relatively low hydrolysis rates achieved and the high cost of the enzymes.

Research efforts directed toward understanding and manipulating cellulase systems suggest that greater cellulase activity can be achieved through optimizing the mix of cellulases used in hydrolysis and through protein engineering of cellulases. Investigators are probing into the synergistic mechanisms observed in several cellulase systems in an effort to demonstrate that optimal combinations of cellulases can be obtained that will yield increased rates and extents of hydrolysis. These studies and other studies of cellulases have yielded considerable insights into the role that cellulase binding plays in synergism. Better understanding of the molecular mechanisms at work in hydrolysis will make it possible to use the revolutionary tools of cloning and site directed mutagenesis to modify cellulase systems so as to improve the conversion efficiency and economics of enzymatic hydrolysis processes.

Key words: Cellulose, cellulase, pretreatment, synergism, properties.

INTRODUCTION

Over the last 15 years there has been considerable progress in understanding the basic mechanisms by which enzymes hydrolyze cellulose, as well as in manipulating and exploiting enzymatic hydrolysis for the production of fermentable sugars. Much of this effort was spurred by concern over the escalating cost and reduced availability of fossil fuels. The global interest in cellulose conversion to energy and chemicals is not surprising since cellulose is the most abundant carbohydrate produced by the biosphere. In addition cellulose represents 40% (wt/wt) of the municipal solid waste generated in the United States (Walter, 1981). Although the world community is no longer preoccupied with fossil fuel shortages, there continues to be a strong international research and development effort aimed at the commercialization of biomass conversion.

In a study by Lee (1981) it was determined that cellulose, free of hemicellulose and lignin, could be produced for \$55 Mg^{-1} while the cost of cellulase was \$2665 dollars Mg^{-1}. For a plant using a CSTR (continuously stirred tank reactor), enzyme costs represent 43·7% of the total production cost (Fan *et al.*, 1987). One major long term goal of cellulase research is the development of more active enzymes that will reduce this major operating cost. Research and development efforts directed towards commercialization of enzymatic hydrolysis technologies have ranged from applied work on bioreactors, through pretreatment of cellulosic substrate and studies of crude cellulases to basic research on the detailed molecular mechanisms of cellulase activity. These topics will be addressed in this special edition of *Bioresource Technology*. The purpose of this paper is to provide an overview of the research and development effort directed towards the understanding and commercialization of the enzymatic hydrolysis of cellulose.

BASIC MECHANISM

Enzymatic hydrolysis of cellulose occurs by a complex system of reactions involving several steps (Reese *et al.*, 1950; Stone *et al.*, 1969; Wood & McCrae, 1979; Ladisch *et al.*, 1981; Lee & Fan, 1982). These steps are (1) transfer of enzymes from the bulk aqueous phase to the surface of the cellulose particles, (2) adsorption of the enzymes and formation of enzyme-substrate complexes (ES), (3) hydrolysis of cellulose, (4) transfer of the cellodextrins, glucose and cellobiose, from the surface of the cellulosic particles to the bulk aqueous phase, and (5) hydrolysis of cellodextrins and cellobiose into glucose in the aqueous phase. Adsorption of enzymes and the formation of enzyme–substrate complexes are considered to be critical steps in the enzymatic hydrolysis of cellulose (Mandels *et al.*, 1971; Huang, 1975; Maguire, 1977; Fan & Lee, 1983; Beldman *et al.*, 1988). These steps are influenced by the structural features of cellulose, the mode of interaction between the cellulases and the cellulose fiber, the nature of the cellulases employed and the enzymes' susceptibility to product inhibition (Fan & Lee, 1983; Ladisch *et al.*, 1983; Coughlan, 1985).

STRUCTURAL FEATURES

It has frequently been shown that the rate and extent of cellulose hydrolysis are influenced by the structural features of the cellulosic substrate (Stone *et al.*, 1969; Wood & McCrae, 1979; Fan *et al.*, 1980; Grethlein, 1985; Mandels, 1985). The two structural features considered most important are surface area and crystallinity (Fan *et al.*, 1980, 1981; Lee *et al.*, 1982; Gharpuray *et al.*, 1983; Bertran & Dale, 1985; Grethlein, 1985; Grous *et al.*, 1986).

Cellulose is a heterogeneous porous substrate with both external and internal surfaces (Cowling & Brown, 1969; Stone *et al.*, 1969; Ladisch *et al.*, 1981). The external surface area is determined by the shape and size of the cellulosic particles, while the internal surface area depends on the capillary structure of the cellulose fibers and the size of the penetrating reactant. External surface area can be measured with a particle counter (e.g., Coulter Counter) (Marshall & Sixsmith, 1974; Lee & Fan, 1982). Internal surface area can be measured by analyzing N_2 adsorption on a dry sample (see Table 1) (Fan *et al.*, 1980; Gharpuray *et al.*, 1983; Hernadi, 1984), or by the solute exclusion technique with a hydrated sample (Stone *et al.*, 1969; Grethlein, 1985). In recent years solute exclusion has become the preferred method for measuring specific surface area because it uses a sample that is submerged in an aqueous environment — the same environment present during enzymatic hydrolysis — as opposed to the dry environment during nitrogen adsorption which may promote the collapse or shrinkage of the pore structure (Stone *et al.*, 1969; Gharpuray *et al.*, 1983; Grethlein, 1985).

Cellulose fibers contain both amorphous and crystalline regions. Crystalline regions are considered to be more difficult to degrade than amorphous regions (Huang, 1975; Ladisch *et al.*, 1983; Coughlan, 1985). Thus, the fraction of the total cellulose that is crystalline is considered to be an important parameter affecting the rate and extent of enzyme hydrolysis. Crystallinity is usually measured with X-rays by the method of Segal *et al.* (1959). The crystallinity index is the percentage of total cellulose that is crystalline. Tabulated in Table 2 are measurements of the crystallinity index for several commercial celluloses.

A number of physical and chemical pretreatments have been developed to reduce the crystallinity and increase the surface area of cellulose

Table 1. Specific surface area of several commercial celluloses measured using the nitrogen adsorption method

Substrate	*Specific surface area* ($m^2\ g^{-1}$)	*Reference*
Avicel PH102	1·8	Ryu *et al.*, 1982
Avicel PH 102	5·4	Lee *et al.*, 1982
Sigmacell 50	1·84	Fan *et al.*, 1981
Solka Floc BW 40	2·13	Fan *et al.*, 1981
Solka Floc BW 40	1·89	Ryu *et al.*, 1982

Table 2. Crystallinity index for several celluloses

Cellulose	*Crystallinity Index (%)*	*Reference*
Avicel PH 102	80·8	Lee *et al.*, 1982
Avicel PH 102	81·0	Ryu *et al.*, 1982
Solka Floc BW 200	67·3	Ryu *et al.*, 1982
Solka Floc SW 40	74·2	Fan *et al.*, 1981
Solka Floc SW 40	76·7	Lee *et al.*, 1982
Sigmacell 50	84·5	Fan *et al.*, 1981

(Wilke *et al.*, 1976; Knappert *et al.*, 1980; Ryu *et al.*, 1982; Bertran & Dale, 1985; Grous *et al.*, 1986). Table 3 lists the effect of physical and chemical pretreatment on the specific surface area, crystallinity and relative extent of hydrolysis of wheat straw, as determined by Gharpury *et al.* (1983).

Grethlein (1985) conducted a series of experiments with samples of mixed hardwood and steam extracted pine, all pretreated with 1% sulfuric acid at various temperatures, using both continuous flow and batch reactors to determine the effect of pretreatment on the rate and extent of hydrolysis. He observed a very large increase in the pore volume accessible to a solute the size of a cellulase (a diameter of 51 Å) after pretreatment, thus causing an increase in the specific surface area of the substrate (see Table 4). The initial glucose yield in 2 h was linearly correlated with the calculated specific surface area of the substrate (see Fig. 1). He also observed a slight increase in the crystallinity of pretreated mixed hardwood and white pine. This would suggest that surface area has a greater effect on enzymatic hydrolysis than does crystallinity. The observation by Bertran and Dale (1985) that crystallinity increases in an aqueous environment and in the presence of cellulases would support this conclusion.

Table 3. Effect of physical and chemical pretreatment on the specific surface area of wheat straw[a]

Pretreatment	*Specific surface area* ($m^2 g^{-1}$)	*Crystallinity index* (%)	*Relative extent of hydrolysis*
Untreated	0·64	69·6	1·0
Physical treatment			
Fitz-milling	0·99	65·6	1·6
Roller-milling	1·2	57·6	3·3
Ball-milling			
4 h	2·3	23·7	4·0
8 h	1·8	54·5	4·0
16 h	1·9	17·5	3·6
24 h	2·0	19·4	4·4
Chemical treatment			
Caustic soda	1·7	53·3	8·00
Peracetic acid	1·7	28·4	13·25
Ethylene glycol	2·9	63·6	6·83

[a]Gharpuray *et al.*, 1983.

One pretreatment process that has received considerable attention over the last five years is steam explosion (Saddler *et al.*, 1982; Grethlein, 1985; Grous *et al.*, 1986; Holtzapple *et al.*, 1989). In this process cellulose is exposed to saturated steam at 3·86 MPa (260°C) for different durations and then is suddenly exposed to atmospheric pressure (Saddler *et al.*, 1982). This results in increases in surface area comparable to those obtained by Grethlein (1985) with dilute acid and high temperature without the expense and pollution caused by the use of acid (Grous *et al.*, 1986). The conventional mechanical method for increasing surface area requires 70% more energy than steam explosion to achieve the same size reduction (Holtzapple *et al.*, 1989).

Lee and Fan (1982) reported that the extent of enzyme adsorption increased approximately linearly as the specific surface area was increased, while the effectiveness of the adsorbed enzymes in producing reducing sugars was strongly dependent on the initial crystallinity index. Peitersen *et al.* (1977) also noted that higher rates and extents of hydrolysis were obtained as the surface area was

Table 4. Impact of pretreatment on glucose yield, pore volume accessibility and specific surface area[a]

Substrate	*Pretreatment condition*			*Glucose yield 2 h* (%)	*Pore volume accessible to solute of 51 Å* ($ml\ mg^{-1}$)	*Calculated specific surface area* ($m^2 g^{-1}$)
	Temperature (°C)	*Acid* (%)	*Time*			
Mixed hardwood	220	1·0	7·8 s	64·0	0·685	140
	220	1·0	7·8 s	41·8	0·51	112
	180	1·0	7·8 s	24·3	0·215	47·6
	100	1·0	5 h	14·7	0·190	37·1
	untreated			14·1	0·06	10·5
Poplar	200	0·41	6 s	39·0	0·45	88
	untreated			3·5	0·03	6·5
White pine	200	1·0	7 s	26·4	0·375	80·0
	untreated			3·2	0·095	13·7
Steam extracted pine	200	1·0	7 s	22·4	0·26	56·4
	untreated				0·07	13·9

[a]Grethlein, 1985.

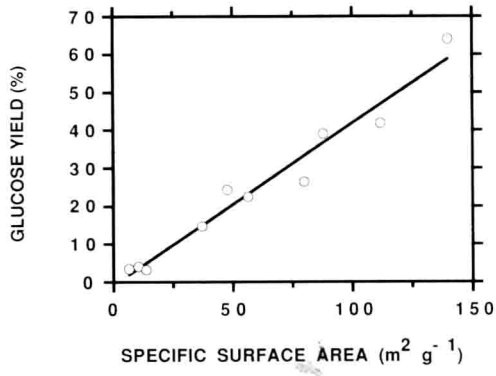

Fig. 1. Initial glucose yield at 2 h as a function of specific surface area available to solute the size of a cellulase molecule (51 Å) for several cellulose. Glucose yield reported as percentage of potential glucose in original cellulose (Grethlein, 1985).

increased. Lee *et al.* (1982) and Ooshima *et al.* (1983) observed that adsorption of cellulase was significantly affected by crystallinity. Bertran and Dale (1985) showed that the lower the initial crystallinity of cellulose, the higher the extent of conversion to soluble sugars.

The influence of the structural features of the substrate on the adsorption of cellulases has been explored by several investigators (Mandels *et al.*, 1971; Maguire, 1977; Peitersen *et al.*, 1977; Lee & Fan, 1982; Lee *et al.*, 1982; Ooshima *et al.*, 1983). Mandels *et al.* (1971) observed that the initial substrate concentration needed to give 50% adsorption in 30 min doubled when the mean particle size was increased from 6·7 μm to 50 μm. They also found that the adsorbed enzymes were not desorbed during hydrolysis. This last observation has been noted by several other investigators (Halliwell, 1965; Ohmine *et al.*, 1983).

Drawing definitive conclusions about the influence of the structural features on the rate and extent of hydrolysis is complicated because many pretreatment processes result in both a decrease in crystallinity and an increase in specific surface area. In addition, the two surface area measurement techniques (solute exclusion and nitrogen adsorption) are conducted in environments that are very different, as stated earlier. Drawing definitive conclusions is also complicated by changes in structural features during the course of hydrolysis. Cellulosic materials undergo a fragmentation process which increases the specific surface area (Halliwell, 1965; King, 1966; Marsh, 1966; Fan *et al.*, 1980; Kyriacou *et al.*, 1987; Walker *et al.*, 1990). Fan *et al.* (1980) observed a 300% increase in the specific surface area as a result of this fragmentation process combined with changes in particle size. The percentage of crystalline material also increases during the course of hydrolysis (Halliwell, 1965; Fan *et al.*, 1980; Bertran & Dale, 1985; Kyriacou *et al.*, 1987). Bertran and Dale (1985) observed that cellulose recrystallized after treatment with buffer alone or during enzymatic hydrolysis. Walker *et al.* (1990) observed that the rate of fragmentation is linearly dependent on the amount of bound crude cellulase from *Thermomonospora fusca.*

CELLULASE KINETICS

Much of what is known about the enzymatic hydrolysis of cellulose is based on kinetic studies using crude cellulases from *Trichoderma reesei* where a multiplicity of cellulases participate in the reaction. Given the multiplicity of cellulases, the observed changes in adsorption of enzymes and structural features with time, and the inhibition of crude cellulases by reaction products, it is not surprising that many of the mathematical models developed to predict the rates or extent of the enzymatic hydrolysis of cellulose are empirical (Ghose, 1969; Van Dyke, 1972; Lee *et al.*, 1980; Eigner *et al.*, 1985), and only a few of these models consider structural features (Van Dyke, 1972; Brandt *et al.*, 1973; Lee & Fan, 1983). For example Gharpuray *et al.* (1983) derived the following empirical model for the relative extent of hydrolysis (REH) of wheat straw given its specific surface area (SSA – m^2 g^{-1}), crystallinity index (CrI – %) and lignin content (%):

$$\mathrm{REH} = 2{\cdot}044(\mathrm{SSA})^{0{\cdot}988}(100-\mathrm{CrI})^{0{\cdot}257}(\mathrm{lignin})^{-0{\cdot}388} \quad (1)$$

Several kinetic studies that apply Michaelis–Menten kinetics to the cellulose–cellulase reaction have been conducted and these studies are reviewed by Lee *et al.* (1980). However, it is known that glucose and cellobiose inhibit the action of the crude cellulase of *T. reesei* (Mandels & Reese, 1963, 1965; Ladisch *et al.*, 1980, 1981; Fan & Lee, 1983; Marsden & Gray, 1986) and other cellulases (Wilson, 1988); therefore, the application of Michaelis–Menten kinetics to cellulose hydrolysis is inappropriate.

Cellobiose inhibits the cellobiohydrolase enzymes of the cellulase complex (Mandels & Reece, 1963, 1965; Lee & Fan, 1983; Marsden &

Gray, 1986) and glucose inhibits cellobiase (Ghose, 1969; Maguire, 1977; Ladisch *et al.*, 1980; Lee & Fan, 1983; Marsden & Gray, 1986). Cellobiose is a far more potent inhibitor than glucose (Ghose, 1969; Lee & Fan, 1982, 1983; Marsden & Gray, 1986). Two inhibition mechanisms have been proposed: one is competitive inhibition (Ghose *et al.*, 1971; Huang, 1975), and the other is noncompetitive inhibition (Okazaki & Young, 1978; Lee & Fan, 1983).

Since the rate and extent of hydrolysis are determined by the amount of enzyme adsorbed on the surface of insoluble cellulose, the kinetics of adsorption have been explored by several investigators (Huang, 1975; Maguire, 1977; Reese, 1977; Bisaria & Ghose, 1978; Ooshima *et al.*, 1983). Reese (1977) concluded that adsorption was a function of (1) the concentration of enzymes; (2) the nature and the amount of available surface area; (3) physical properties of the enzymes; and (4) the hydrolysis environment (i.e., pH, salt concentration, temperature). The presence of other substances, such as lignin, can also affect adsorption and hydrolysis (Deshpande & Eriksson, 1984; Marsden & Gray, 1986).

Langmuir adsorption kinetics have been applied to the adsorption of cellulase to cellulose by several investigators (Peitersen *et al.*, 1977; Lee *et al.*, 1982; Ooshima *et al.*, 1983; Beldman *et al.*, 1987; Kyriacou *et al.*, 1988, 1989). In this model the rate of adsorption is proportional to the number of free sites on the cellulose surface and the amount of cellulase in the supernatant. The rate of desorption is proportional to the amount of cellulase bound to the surface of cellulose. At equilibrium the following equation is obtained:

$$E_{ads} = \frac{K_p E_{ads,m}}{1{\cdot}0 + K_p E} E \qquad (2)$$

where E is the enzyme concentration in the supernatant in mg ml^{-1}; E_{ads} is the adsorbed enzyme in mg enzyme mg^{-1} cellulose; $E_{ads,m}$ is the maximum amount of enzyme adsorbed, mg enzyme per mg cellulose; K_p is a constant in ml mg^{-1}. Langmuir adsorption constants determined by several investigators are tabulated in Table 5. One problem with the application of Langmuir adsorption kinetics to cellulose hydrolysis is that the binding of cellulase to cellulose does not appear to be completely reversible (Halliwell, 1965; Mandels *et al.*, 1971; Ohmine *et al.*, 1983).

pH has been found to have little effect on adsorption in the range of 3·5 to 5·5 (Mandels *et al.*, 1971; Peitersen *et al.*, 1977; Marsden & Gray, 1986). Results from studies of the effect of temperature on adsorption are confusing. Mandels *et al.* (1971) found that the rate of adsorption on Solka Floc increased with temperature (Marsden & Gray, 1986). Subsequent work (Peitersen *et al.*, 1977; Ryu *et al.*, 1984) showed that the maximum level of adsorption decreased with increasing temperature (Marsden & Gray, 1986). In contrast, Bisaria and Ghose (1978), using alkali-treated bagasse, found that the specific adsorption increased with temperature up to a maximum at 50°C.

CELLULASES OF BACTERIAL AND FUNGAL SYSTEMS

Fractionation of culture filtrates of *T. reesei* has revealed that cellulases can be divided into three major classes (Shoemaker & Brown, 1978; Ladisch *et al.*, 1983; Wood, 1985). These are endoglucanases or endo-1,4-β-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21).

Table 5. Langmuir adsorption constants for several celluloses and crude cellulases

Cellulose	*Cellulase*	*Temperature (°C)*	K_p *(ml mg^{-1})*	$E_{ads,m}$ *(mg mg^{-1})*	*Reference*
Avicel PH 102	*T. viride*	30·0	0·43	0·088	Peitersen *et al.*, 1977
	T. viride	50·0	1·67	0·029	Peitersen *et al.*, 1977
Avicel	*T. viride*	5·0	3·21	0·056	Ooshima *et al.*, 1983
Solka Floc SW 40	*T. viride*	30·0	0·62	0·037	Peitersen *et al.*, 1977
	T. viride	50·0	1·56	0·019	Peitersen *et al.*, 1977
Sweco 270	*T. viride*	30·0	1·39	0·048	Peitersen *et al.*, 1977
	T. viride	50·0	1·19	0·049	Peitersen *et al.*, 1977

Endoglucanases, often called CM-cellulases (carboxymethylcellulose) or C_x enzymes, attack randomly along the cellulose fiber, resulting in a rapid decrease in the chain length of CM-cellulose or H_3PO_4-swollen cellulose and yielding glucose, cellobiose, cellotriose and other higher oligomers (Ladisch *et al.*, 1983; Wood, 1985). Cellobiohydrolyase, which is often called exoglucanase, is present as a major constituent of crude fungal cellulases and can degrade highly crystalline cellulose. Initially it was thought to be absent in bacterial cellulase systems (Ladisch *et al.*, 1983; Wood, 1985). However, Langsford *et al.* (1984) discovered that *Cellulomonas fimi* produces a cellobiohydrolyase, as do *Ruminococcus albus* (Ohmiya & Shimizu, 1988) and *Ruminococcus flavefacieons* (Gardner *et al.*, 1987). This classification of cellulases is not rigid. β-glucosidase, (cellobiase) hydrolyzes cellobiose and in some cases other cello-oligosaccharides to glucose (Ladisch *et al.*, 1983; Wood, 1985). Mixtures of endoglucanase and cellobiohydrolase account for most of the cellulase activity. However, β-glucosidase is needed to reduce the inhibitory effects of cellobiose (Wood, 1985). The endoglucanases and cellobiohydrolases from *T. reesei* appear to adsorb competitively and exert a synergistic effect during the hydrolysis of cellulose (Ryu *et al.*, 1984; Marsden & Gray, 1986).

Fractionation studies have demonstrated that each of the above categories consists of several enzymes adding further complexity to the cellulose–cellulase system. Shoemaker and Brown (1978) identified four electrophoretically distinct endo-1,4-β-D-endoglucanases from *Trichoderma viride*. Endoglucanases II, III and IV each yielded a single protein band when analyzed by gel electrophoresis. Carbohydrate staining indicated that these three enzymes are glycoproteins with average molecular weights of $37\,200 \pm 2400$, $52\,000 \pm 2600$ and $49\,500 \pm 2200$ for Endoglucanases II, III and IV. More recently Beldman *et al.* (1985) identified six endoglucanases (Endo I, II, III, IV, V and VI), three cellobiohydrolases (Exo I, II, and III) and a β-glucosidase from a commercial preparation of *T. viride* cellulase. The molecular weights and isoelectric points (pI) of these cellulases are presented in Table 6. Endo I, II and IV were more random in their attack on CM-cellulose than Endo III, V and VI. Exo II and III were cellobiohydrolases based on the products obtained from the hydrolysis of H_3PO_4-swollen cellulose. Exo I was able to remove a cellobiose molecule from a chain end and then hydrolyze this molecule in a second step to two glucose units.

Table 6. Molecular Weights, PIs and Langmuir Adsorption Constants for Pure Components of *Trichoderma viride* Cellulase[a]

Cellulase	M_r	*pI*	K_p ($ml\,mg^{-1}$)	$E_{ads,m}$ ($mg\,mg^{-1}$)
Endo I	50 000	5·3	0·88	0·126
Endo II	45 000	6·9	0·28	0·090
Endo III	58 500	6·5	11·67	0·026
Endo IV	23 500	7·7	2·50	0·0028
Endo V	57 000	4·4	0·89	0·105
Endo VI	52 000	3·5	3·44	0·0041
Exo I	53 000	5·3	ND[b]	ND
Exo II	60 500	3·5	4·96	0·0066
Exo III	62 000	3·8	6·96	0·063
β-glucoside	76 000	3·9	NA[c]	NA

[a]Beldman *et al.*, 1987.
[b]ND = no data.
[c]NA = not applicable.

Beldman *et al.* (1987) also determined the adsorption and kinetic constants (see eqn 2) for the pure components of the *T. viride* cellulase system. The adsorption constants are presented in Table 6. The adsorption isotherms for these cellulases indicated that Endo I, Endo III and Endo V adsorbed strongly on crystalline cellulose. Although the data followed a Langmuir adsorption isotherm, Beldman *et al.* (1987) were only able to obtain partial reversibility of adsorption after resuspension of cellulose carrying bound cellulase. Kyriacou *et al.* (1988) conducted similar binding experiments with pure component of *T. reesei*. In every case the affinity was reduced with increasing temperature; while the effects of pH and ionic strength on the saturation levels and affinity differed among the four components and depended on the adsorption temperature.

Several other investigators have observed the diversity of endoglucanases and exoglucanases (Gong *et al.*, 1979; Lee *et al.*, 1980; Woodward & Wiseman, 1982; Ladisch *et al.*, 1983; Odegaard *et al.*, 1984). Comparative studies are difficult because of the differences in the strains and the characterization procedures used. However, there are reports of two fungi, *Penicillium notatum* and *Stereum sanguindentum* which produce a single cellulase and still degrade cellulose (Erriksson & Patterson, 1968; Patterson, 1968).

In comparison with the numerous kinetic studies using fungal cellulases, very little research has been done with bacterial cellulases. The cellulases of the anaerobe *Clostridium thermocellum*

have been the most widely studied but are just beginning to be well characterized (Wood, 1985) as are the cellulases produced by thermophilic aerobic bacteria such as *T. fusca* (Calza *et al.*, 1985). Crawford and McCoy (1972) observed that cellulases from *Streptomyces thermodiastaticus* and *T. fusca*, act by internal hydrolysis at random points along the CMC (carboxymethylcellulose) chain, producing cellobiose, glucose, and intermediate length oligosaccharides. Cellobiase was not detected in culture filtrates produced under their experimental condition (Crawford & McCoy, 1972). Ferchak and Pye (1983) found that cellobiose was strongly inhibitory to the crude cellulase of *T. fusca*, reducing the activity against swollen cellulose to 25% at a concentration of 5%. However, more recent studies found significantly less inhibition of *T. fusca* cellulases by cellobiose (Wilson, D. B., unpublished data). Glucose had much less effect, reducing activity to 40% at a concentration of 20%. They also observed that the cellobiase activity had a very low product inhibition, whereas PNPGase (*p*-nitrophenyl-β-D-glucosidase) activity was more significantly inhibited (Ferchak & Pye, 1983).

Wilson (1988) working with a protease negative mutant strain of *T. fusca*, has isolated five different β-1-4-endoglucanases and one xylanase. All of the cellulases identified were endocellulases; each of them reduced the viscosity of CM-cellulose at a much greater rate than it released reducing sugars. The enzymes E_1, E_2 and E_5 are distinct antigenically, as rabbit antisera prepared against each enzyme specifically inhibit and react only with the enzyme they were prepared against. E_3 and E_4 do react with antisera prepared against each other but are antigenically distinct from the other enzymes. One major difference between the cellulases of *T. fusca* and *T. reesei* is that *T. fusca* does not appear to produce a cellobiohydrolase (Wilson, 1988). Information on the impact of structural features of cellulose on the rate and extent of hydrolysis using cellulases from *T. fusca* has not been reported.

SYNERGISM

One of the major challenges facing cellulase researchers is the elucidation of the synergistic interactions between individual components. Synergistic behavior between cellulase components has been studied by several investigators (Reese *et al.*, 1950; Gong *et al.*, 1979; Wood & McCrae, 1979; Haigeler *et al.*, 1980; Chanzy & Henrissat, 1985; Wood, 1985; Beldman *et al.*, 1986; Woodward *et al.*, 1988). Using cellulases from *T. reesei* and bacterial cellulose, Chanzy and Henrissat (1985) observed that cellobiohydrolase II by itself only attacked at one end of the microcrystal. However, when mixed with their endoglucanase (1,4-β-D-glucan glucanohydrolase II) several sites of attack on the crystal by cellobiohydrolase II were observed. These attacks were located at kinks or amorphous regions along the microcrystals. These observations along with those of other investigators (Haigeler *et al.*, 1980; Ladisch *et al.*, 1981; White & Brown, 1981*a*, *b*; Henrissat *et al.*, 1985) demonstrate that during the course of hydrolysis endoglucanases create a number of newly accessible non-reducing chain ends that are available for attack by cellobiohydrolase II. This is one of three models of synergism reviewed by Wood and McCrae (1979) and Wood (1985).

Chanzy and Henrissat (1985) and Chanzy *et al.* (1984) observed that cellobiohydrolase I does not show the specificity for crystal tips demonstrated by cellobiohydrolase II and that this enzyme can degrade highly crystalline cellulose without the help of endoglucanases. Berghem *et al.* (1976) also isolated a cellobiohydrolase from *T. viride* that could produce 45% degradation of Avicel when acting alone and 75% when inhibitory products were removed by ultrafiltration. In follow-up studies, Henrissat *et al.* (1985), observed synergism between cellobiohydrolase I and endoglucanase I or II. They also concluded that the synergism was determined by the structural features of the substrate. Cellobiohydrolase II behaves more like a typical exo-enzyme than cellobiohydrolase I, the latter having an endo-adsorption pattern on cellulose (Chanzy *et al.*, 1984). Henrissat *et al.* (1985) also observed that mixtures of cellobiohydrolases I and II synergistically degraded cellulose. They proposed a competitive adsorption model to explain the synergistic behavior. This is in agreement with the observations of Ryu *et al.* (1984).

As indicated previously, Wilson (1988) isolated five different endoglucanases from *T. fusca*. One enzyme, referred to as E_3, appears to be essential for synergism because a mixture of all five enzymes showed twice the activity on filter paper predicted from the sum of the individual activities, while a mixture of E_1, E_3 and E_5 give three times the activity predicted by the sum of the individual

Table 7. Degree of Synergism for Several Mixtures of Cellulases and Several Celluloses

Cellulase mixture	*Cellulose*	*Cellulase molar ratio*	*Degree of synergism*	*Reference*
Trichoderma viride				
Endo I/Exo III	Avicel SF	1·9	2·5	Beldman *et al.*, 1987
Endo II/Exo III	Avicel SF	5·2	2·5	—
Endo III/Exo III	Avicel SF	2·7	1·7	—
Endo IV/Exo III	Avicel SF	13·2	2·4	—
Endo V/Exo III	Avicel SF	2·2	3·5	—
Endo VI/Exo III	Avicel SF	3·3	3·8	—
Trichoderma reesei				
Endo I/Exo I[a]	Avicel 105	1·0	1·2	Woodward *et al.*, 1988
Endo I/Exo I[b]	Avicel 105	1·25	1·5	—
Thermomonospora fusca				
E_1/E_3	filter paper	2·13	1·2	Wilson, D. B., unpublished data
E_2/E_3	filter paper	0·18	1·8	Wilson, D. B., unpublished data
E_4/E_3	filter paper	0·75	1·2	Wilson, D. B., unpublished data
E_5/E_3	filter paper	0·63	1·5	Wilson, D. B., unpublished data

activities. All mixtures containing E_3 showed some synergistic behavior, while a mixture of E_1, E_2, E_4 and E_5 showed no synergism (Wilson, 1988).

Degrees of synergism for fungal and bacterial cellulase mixtures are tabulated in Table 7 (Wilson, D. B., unpublished data; Beldman *et al.*, 1987; Woodward *et al.*, 1988). The degree of synergism is defined as the ratio of the observed activity of the cellulase mixture to the sum of the individual activities (Okazaki & Young, 1978). Beldman *et al.* (1987) observed that the maximal degree of synergism occurred at molar ratios of Endo/Exo III much greater than 1 to 1 as indicated by Table 7. He also observed that the combinations of endoglucanases with Exo II result in lower degrees of synergism than combinations of endoglucanases with Exo III. Woodward *et al.* (1988) concluded that the greatest degree of synergism was obtained at total enzyme concentrations below that needed to saturate the available binding sites. This would suggest that there is a minimum amount of surface area needed for an individual cellulase to be effective in hydrolyzing cellulose.

Several mechanisms have been proposed for the synergism that occurs between cellulases (Reese *et al.*, 1950; Wood & McCrae, 1979; Ryu *et al.*, 1984). Wood and McCrae (1979) reviewed the sequential endo–exoglucanases hypothesis, where endoglucanases create sites for exoglucanases to cleave reducing sugars. They suggested that this hypothesis may be an oversimplification of the mechanism. They argued that if this hypothesis is correct then the logical expectation is for C_1 from one system to act synergistically with C_x from another system. Culture filtrates of *Stachybotrys atra* and *M. verrucaria* mixed with C_1 of *T. koningii* or *P. funiculosum* only yield small increases in the rate and extent of cotton hydrolysis (Wood, 1969; Wood & McCrae, 1979). On the other hand, culture filtrates of some strains of *Memnoniella echinata* and *Gliocladium roseum* give variable results when mixed with the C_x components from *T. koningii* or *P. funiculosum* (Wood & McCrae, 1979). Instead of totally rejecting the sequential endo–exoglucanases hypothesis, Wood and McCrae (1979) proposed that this model of synergism is one of two models of synergism that occur during the hydrolysis process. The second model, which Wood and McCrae admit is highly speculative, is the formation of an endo–exoglucanase complex on the crystalline surface of the cellulose chains.

Ryu *et al.* (1984) competitive adsorption model postulates three modes of synergism. In the first mode, the cellobiohydrolase speeds up the action of the adsorbed endoglucanase, accompanied by the desorption of endoglucanase. For the second mode, the two adsorbed enzyme components, endoglucanase and exoglucanase, simultaneously affect each other. In the third mode the adsorbed endoglucanase speeds up the desorption of cellobiohydrolase. The first mode is similar to the sequential endo–exoglucanase proposal presented previously. The second and third modes

could conceivably be lumped together to represent the enzyme–enzyme complex proposed by Wood and McCrae (1979). Analysis of the adsorption and desorption process for different mixtures of cellobiohydrolases and endoglucanases and the addition of components at different time intervals could provide a means of identifying which model is the most appropriate.

Wood (1985) also presented a hypothesis for the synergism between cellobiohydrolases I and II. He speculated that the observed synergism could be explained if one considers that the two enzymes exhibit substrate stereospecificity and have been synthesized to attack the two different non-reducing end groups. Removing cellobiose units successively from one type of non-reducing chain-end exposes a non-reducing end group on another chain with the correct configuration for attack by the other stereospecific cellobiohydrolase. A problem with this proposal is that there is no evidence for sterochemically different non-reducing ends of cellulose chains.

At present the actual mechanism responsible for synergism is not known for any cellulase nor is it known if the same mechanism is responsible for synergism seen with fungal and bacterial cellulases.

MOLECULAR PROPERTIES OF CELLULASES

Physical studies of the three dimensional structure of cellulases have been started in at least two laboratories (Joliff *et al.*, 1986; Aguja *et al.*, 1988). *Trichoderma reesei* Exo I has been studied by low angle X-ray scattering (Aguja *et al.*, 1988) and been shown to have a globular core of diameter 65 Å and a long tail of 120 Å. The native enzyme can be cleaved by proteolysis (Tomme *et al.*, 1988) to give an enzymatically active core fragment and a tail which lacks activity but can bind tightly to cellulose.

Studies of the proteolytic cleavage of other cellulases have shown that they also contain separate catalytic domains and cellulose binding domains (Ghangas & Wilson, 1988; Gilkes *et al.*, 1988; Tomme *et al.*, 1988). The order of the domains varies in different cellulases; but at present it is not known whether the order of the domains is unrelated to function or if different domain orders are associated with different cellulase activities. A *C. thermocellulum* endoglucanase has been crystallized and the crystals are suitable for X-ray crystallographic studies, which have been started (Joliff *et al.*, 1986).

There is only a little information available about the specific residues present in the active sites of cellulases. Early work on an *Aspergillus* cellulase showed that chemical modification of a single tryptophan residue completely inhibited activity (Hurst *et al.*, 1977). A *P. notatum* cellulase was inhibited by N-bromosuccinamide (NBS) oxidation with an average of 2·3 tryptophan residues being destroyed to give complete inhibition (Patterson, 1968). Two *T. koningi* cellulases were also inactivated by NBS oxidation (Iwasaki *et al.*, 1965). Many cellulases are inhibited by Hg^{2+} and by Ag^{2+}. Curiously, in several cases other sulfhydryl reagents such as iodoacetate and N-ethyl maleamide do not inhibit cellulase activity (Patterson, 1968; Calza *et al.*, 1985). Furthermore, some inhibited cellulases do not contain free cysteine residues. These results suggest that Hg^{2+} inhibits cellulases by interacting with some residue other than cysteine but that other residue (or residues) has not yet been identified in any cellulase. One possibility would be carboxyl groups; carboxyl groups have been implicated in the active site of a cellulase from *Schizoplyllum commune* by reaction with a water soluble carbodimide (Clarke & Yaguchi, 1985). In addition a number of workers have reported weak homologies between aspartic residues in the sequence of cellulases and the active site of lysozyme (Paice *et al.*, 1984; Benguin *et al.* 1987). At this time none of these homologies have been shown to be statistically significant.

The best procedure for identifying active site residues is affinity labelling. However the only reagent that has been used for this purpose, 4,5 epoxypentyl-β-cellotrioside has too low an affinity (0·1 M) for cellulases to specifically label active site residues (Legler & Bause, 1973).

CONCLUSIONS

It is apparent from this review and others (Ladisch *et al.*, 1983; Coughlan, 1985; Fan *et al.*, 1987) that considerable progress has been made toward understanding and manipulating enzymatic hydrolysis of cellulose. There is an array of pretreatment processes available that can significantly increase specific surface area and decrease crystallinity of celluloses, resulting in significant increases in the rate and extent of hydrolysis. Steam explosion of cellulose is particularly in-

triguing because it requires considerably less energy than other mechanical processes (Holtzapple *et al.*, 1989); it also does not have the recycling or environmental costs associated with chemical pretreatment.

Since enzyme cost is the major impediment to commercialization of enzymatic cellulose hydrolysis, the major challenge is to significantly increase the activity of cellulases. Several chromatographic procedures have been developed for acquiring pure cellulase components (Shoemaker *et al.*, 1983*b*; Bartley *et al.*, 1984; Wilson, 1988). Also, cellulase genes have been cloned into other microorganisms (Shoemaker *et al.*, 1983*a*; Benguin *et al.*, 1987; Ghangas & Wilson, 1988), opening the possibility of using cloning as a means of producing large quantities of pure cellulase. The ability to economically produce large quantities of pure cellulases makes it possible to explore synergisms at a more fundamental level and open up the possibility of blending cellulases to obtain better rates and extents of hydrolysis. The synergism studies reported in the literature (Wood & McCrae, 1979; Wood 1985; Beldman *et al.*, 1988) and the detailed molecular studies of cellulases will be useful in optimizing cellulase mixtures and improving the economics of the process (Erriksson & Pattersson, 1968; Patterson, 1968; Shoemaker & Brown, 1978; Woodward & Wiseman, 1982; Lee & Fan, 1983; Langsford *et al.*, 1984; Odegaard *et al.*, 1984; Ryu *et al.*, 1984; Beldman *et al.*, 1985, 1987; Calza *et al.*, 1985; Kyriacou *et al.*, 1987). Chemical modifications of cellulases (Hurst *et al.*, 1977) and site directed mutagenesis (Tao, 1989) will contribute much to the effort to improve cellulase activity.

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