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ENZYMATIC AND STRUCTURAL FACTORS LIMI1.NG

HYDROLYSIS OF CELLULOSE BY

TRICHODERMA HARZIANUM E58 CELLULASES

by

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A thesis submitted to the Faculty of Graduate Studies in
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ABSTRACT

Trichoderma harzianum E58 extracellular cellulases can be
applied to a cellulose-to-glucose process. Optimal conditions
for long-term hydrolysis were found to be pH 4.8, 45°C. Enzyme
adsorption onto the commercial cellulose, Solka Floc BW300 was
examined. The enzymes that adsorb to the substrate in the
initial stage of hydrolysis had a strong role in governing the
overall reaction. The enzyme loading at which the substrate was
saturated with the adsorbed cellulases corresponded to the
optimal enzyme loading for hydrolysis.

Enzyme-related and structural-related factors responsible
for the decline in the hydrolysis rate were studied.
Exoglucanase and β-glucosidase were resistant to thermal
inactivation at 45°C over a 4 day period, while endoglucanase
lost 20% of its activity. Site blockage of adsorptive or
catalytic sites on the substrate did not appear to limit
hydrolysis. End-product inhibition of β-glucosidase and endo-
and exocellulase by glucose and cellobiose, respectively, is a
major problem in hydrolysis. Inhibition can be alleviated by
supplemental β-glucosidase or use of a combined hydrolysis and
fermentation process. T. harzianum E58 produced sufficiently
high levels of β-glucosidase to cope with glucose inhibition, at
low substrate concentrations.

The relationship between initial crystallinity of the
substrate and its hydrolysis appeared to be bimodal. No clear

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pattern between accessible surface area of the substrate and its
hydrolysis emerged. Changes occurring to the crystallinity and
the accessible surface area during hydrolysis were followed. The
change in crystallinity was negligible, while the accessible
surface area increased drastically in the initial phase of
hydrolysis, then slowly declined. The extent to which the
substrate becomes resistant to enzymatic attack was examined. A
70% drop in its accessibility during 4 days hydrolysis was
observed, indicating that the substrate became more recalcitrant
during long-term hydrolysis.

ACKNOWLEDGEMENTS

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INTRODUCTION

I. HISTORY

Research on optimizing cellulose hydrolysis, ironically, started out in an attempt to prevent microbial decomposition from occuring. During the 1940's, American troops stationed in the South Pacific were dismayed at the rapid deterioration of their cotton goods, so the Army requested help in solving the problem. A long range basic research laboratory was set up at the U.S. Army Natick Development Center to investigate the nature of the rotting, the causal organisms, their mode or action, and a method of control. The laboratory collected deteriorated items, and isolated thousands of microorganisms. The cultures were grown on cotton duck, and the strips tested for loss in tensile strength.

Some interesting facts began to emerge. Most of the cellulolytic fungi grew well on cellulose and were able to degrade it. Filtrates from these cultures contained enzymes that released sugars from amorphous cellulose, but only rarely produced sugars from crystalline cellulose. It was deduced that several enzymes are required to hydrolyze crystalline cellulose, but not all cellulolytic micro-organisms manufacture, or secrete these enzymes.

The strong interest in cellulose degradation waned in the post-war years, but due to the concerted efforts of a small group of scientists, led by Mandels and Reese, research on cellulolytic micro-organisms continued, and much of the ground-work was laid for later researchers.

In the 1970's interest in cellulose degradation was renewed due to concern over the energy shortage, environmental protection and waste utilization. Bioconversion of lignocellulosic residues to their component sugars and then to chemicals was thought to be the solution to many of the problems, and research into this area expanded. The prime target for most of this research is the hydrolysis of cellulose to glucose, which could then be streamed into a multitude of other products.

Although enzymatic hydrolysis is technically promising, a better understanding of the mechanisms and limitations involved in hydrolysis would help to develop and optimize the process.

The major problem in studying enzymatic hydrolyis of cellulose is the complexity of the system. Wood, the usual substrate, is insoluble and heterogenous - composed of cellulose, hemicellulose, lignin - the nature and concentration depending on the source. Cowling and Kirk (1976) compared the composition of a number of sources of cellulose (i.e. hardwoods, softwoods and grasses) with that of cotton, which contains about 90% cellulose. There was a wide variation in the amounts of cellulose (10-55%), hemicellulose (25-50%) and lignin (10-35%). "Cellulase" itself is a complex of enzymes acting synergistically. In addition, both the structural features of the substrate and the mode of attack of the cellulase complex are constantly changing during the course of the reaction. Since the reaction is long-term, inactivation of the enzyme must also be considered. Given the immense complexity of this system, it is not surprising that the mechanisms of the reaction are still not elucidated.

Figure 1.

Antiparallel arrangement of the cellulose chains.
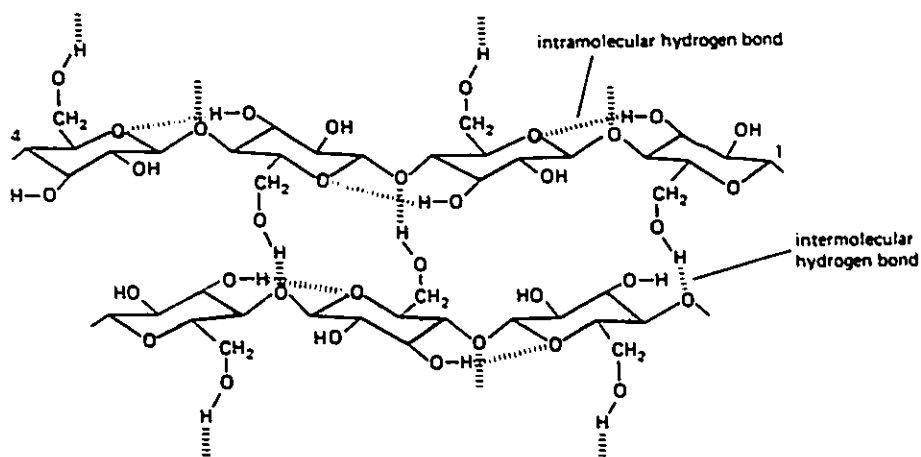
Alberts et al., 1983

(Courtesy of Garland Publishing Inc.)

II. CELLULOSE STRUCTURE

Cellulose is a linear polymer consisting of anhydroglucose units held together by β-1,4 linkages. This linkage rotates each glucose unit 180 degrees about its main axis in relation to its neighbour (Figure 1). This results in intramolecular hydrogen bonding between adjacent glucose molecules which helps to maintain rigidity of the glucose chain. Glucose chains are arranged in parallel, and are held together by intermolecular hydrogen bonding. Individually, the hydrogen bond is not strong, but this extensive network of inter and intra-hydrogen bonding contributes to the strength of the cellulose molecule.

Many models have been proposed to explain the arrangement of the glucose chain within the cellulose molecule. There are two basic types of models, the extended chain or the folding chain (Figure 2). The most widely accepted model is the Ellefson fibrillized, or folding chain model which proposes that the cellulose molecule folds over on itself.

In the cellulose molecules there are crystalline and amorphous regions. The term, "crystalline regions" relate to the areas of tight hydrogen-bonding, where the chains are arranged in a highly ordered manner (Figure 2). Areas where looser binding occurs are termed "amorphous" or "paracrystalline" regions.

Bundles of the cellulose molecules pack together to form the microfibril. The microfibers are cross-linked with lignin, hemicellulose and protein to form the cell wall of higher plants (Figure 3).

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Figure 2.

Chain models of the elementary fibril:
a) fringe-micellar model; b) folding chain model.

(Width~40 Å)

Folds

Amorphous

Crystalline

Regions

(~500 Å)

Fold
Length

Amorphous

(a)   (b)
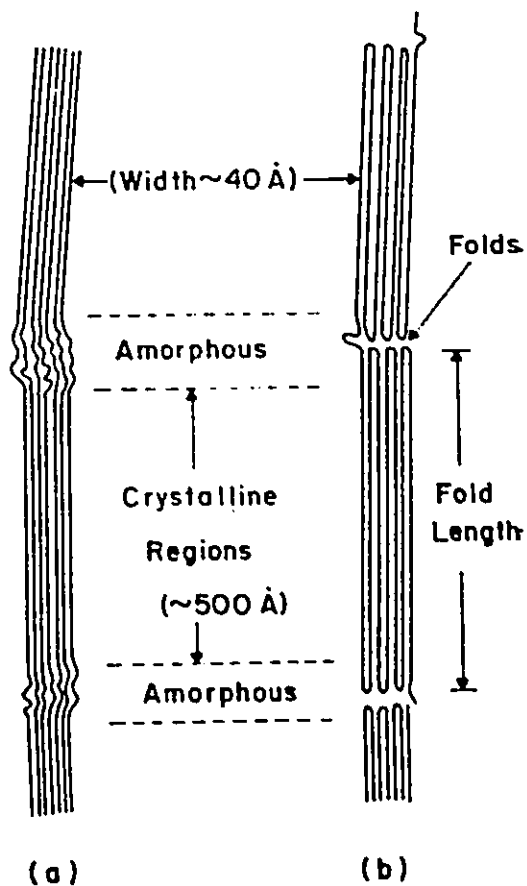
Chang and Tsao, 1981

Figure 3.

Organization of cellulose molecules in the cell wall of a higher plant.

cellulose molecule is a
β-1, 4 glucose polymer

3.5 nm

a crystalline array of hydrogen-bonded
cellulose molecules forms a cellulose microfibril

microfibrils are
cross-linked
with other
polysaccharides
and protein
molecules to
form the cell wall
of higher plants
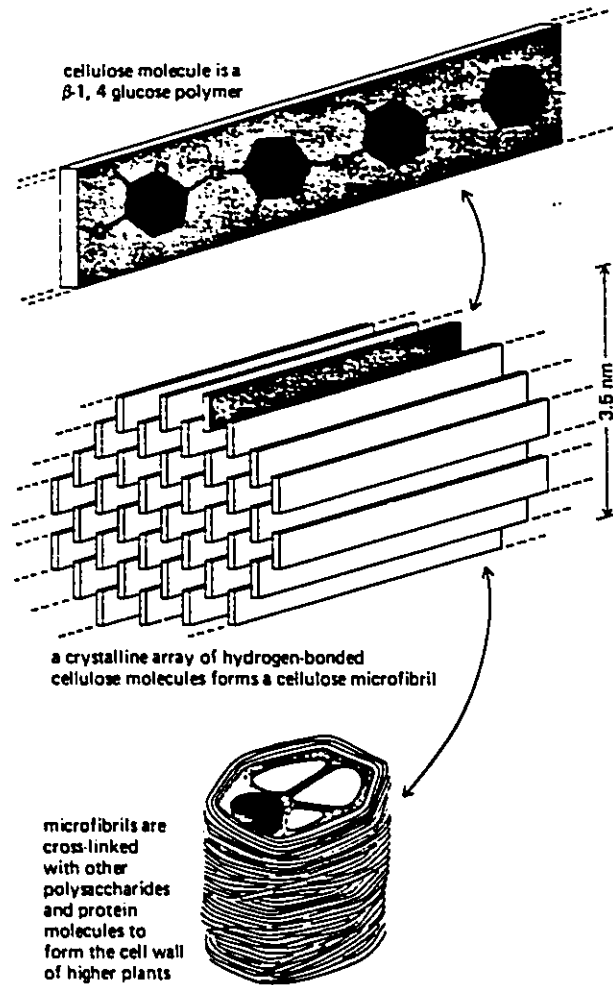
Alberts et al., 1983

(Courtesy of Garland Publishing Inc.)

III. STRUCTURAL FEATURES RESTRICTING ENZYMATIC HYDROLYSIS

Native lignocellulosics are virtually inaccessible to enzymatic attack without some form of pretreatment. It is now widely accepted that the crystallinity of a lignocellulosic material and its association with lignin are the major factors restricting enzymatic hydrolysis. These and other structural features that limit its susceptibility to enzymatic attack have been outlined by Cowling (1975): 1) degree of crystallinity of the cellulose, 2) moisture content of the fibres, 3) unit cell dimensions of the crystallites, 4) degree of polymerization, 5) conformation and steric rigidity of the anhydroglucose units, 6) nature, concentration, and distribution of substituent groups and 7) nature of the associated substances.

Currently, Klesov (1986) includes surface area, while Stone et al. (1969), and Grethlein (1985) list pore size of the substrate as major factors limiting hydrolysis. Both these factors relate the size and diffusibility of the enzyme molecule to the size and surface properties of the cellulosic material.

In order to reduce the complexity of the substrate, and to reduce the number of variables involved in examining hydrolysis, this thesis will focus primarily on purified cellulose substrates, with the lignin and hemicellulose fractions extracted.

A. Surface Area

The voids in wood and agricultural crops can be divided into two types: 1) gross capillaries, such as the cell lumina, pit

apertures and pit-membrane pores (these range from 20 to 10,000 nm in diameter); and 2) the cell-wall capillaries, such as the spaces between microfibrils and the cellulose molecules in the amorphous regions. The cell wall capillaries are closed when the material is dry, but can swell up to 20 nm when fully saturated.

The total surface area of the gross capillaries is roughly 2.0 m^2g^{-1}, while the total surface area within the cell-wall capillaries is up to 300 m^2g^{-1}. Much of the confusion regarding the relationship between surface area and hydrolysis is due to the methodologies employed to determine surface area. Many surface area measurements are made on the basis of the adsorption of much smaller molecules, such as nitrogen gas, which overestimate the area accessible to the enzyme.

The accessible surface area of the cellulose fibers is defined by the size, shape and surface properties of the capillaries within the fiber in relation to the size, shape, and diffusibility of the cellulase enzyme. Some surface area measurements use an enzyme exclusion approach: using an enzyme probe (peroxidase) comparable in size to the cellulase enzyme to measure available surface area. A more direct approach is to measure the amount of cellulase enzyme able to adsorb to the substrate.

Klesov and Sinitsyn (1981) measured the accessible surface area of a wide range of substrates using an enzyme-exclusion technique. They found the initial rate of hydrolysis to be proportional to the surface area of the substrate available to a 4.0 nm spherical enzyme.

On the other hand, Fan et al. (1980) found that the specific

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surface area of refined cellulose, as measured by the nitrogen adsorption technique, had only a small effect on the initial rate of hydrolysis.

B. Crystallinity (CrI)

The fraction of crystalline material in the total cellulose is expressed in terms of its crystallinity index. Crystallinity is commonly determined by the method of Segal et al. (1959) with an x-ray diffractometer.

The relationship between crystallinity and enzymatic hydrolysis of refined substrates was first reported in the 1950's in work by Walseth (1952) and Baker et al. (1959). Walseth, using phosphoric acid swollen cellulose, found substrates with low crystallinity were more susceptible to hydrolysis by Aspergillus niger. Baker et al. found an inverse linear relationship between in vitro digestibility and the crystallinity index of cotton linters and wood celluloses.

Later work using more precise experimental techniques continued to support this relationship. Klesov and Sinitsyn (1981), using an extensive range of substrates and pretreatments found a decrease in the crystallinity of a substrate corresponded with an increase in the initial rate of hydrolysis by Trichoderma reesei and Aspergillus niger cellulases. A strong correlation between increase in surface area and extent of hydrolysis was also demonstrated.

Fan et al. (1980) found an inverse relationship between the crystallinity index and glucose production using T. reesei.

cellulases. They worked out a mathematical relationship between the amount of glucose produced after 8 hours (X_8) and the initial crystallinity (CrI) and specific surface area (SSA) of the substrate, as seen in Equation 1:

$$X_8 = 0.380(SSA)^{0.195} (100 - CrI)^{1.04} \qquad (Eqn\ 1)$$

Crystallinity is held to be a stronger determinant of hydrolysis than specific surface area, as demonstrated by its larger exponent.

Klesov and Sinitsyn (1981), Fan et al. (1980) and Bertran and Dale (1985) noted recrystallization of the pretreated substrate occurs when it is moistened, with the more amorphous substrates recrystallizing to a greater extent. Fan and co-workers obtained a stronger correlation between enzymatic hydrolysis and initial crystallinity index by first moistening, then solvent-drying the pretreated substrates prior to its crystallinity determination.

Information on the effect of enzymatic hydrolysis on the crystallinity of the substrate during long term hydrolysis is conflicting.

Norkrans (1950) speculated that the more amorphous regions of the substrate are selectively hydrolyzed. She hydrolyzed filter paper for 8 hours using Trichoderma nudum enzymes and noted an increase in the crystallinity of the residue.

Similarily, Ohmine et al. (1983) found that the CrI increased during the hydrolysis of Avicel by T. reesei cellulases, and concluded that there was preferential attack of

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the amorphous region.

Lee and Fan (1983) found a slight increase in crystallinity during hydrolysis of hammer-milled Solka Floc (from 78.1 to 80.0) with T. reesei cellulases. For more amorphous substances the change in CrI during hydrolysis was more significant. When the substrate was ballmilled to an initial crystallinity of 34.9 the CrI was reported to increase to 71.6 after hydrolysis. This was explained by the presence of a large amount of amorphous material that could be more easily hydrolyzed.

Chang et al. (1981) reported finding constant crystallinity during the enzymatic hydrolysis of both native and regenerated cellulose. This led them to conclude that both crystalline and amorphous regions are attacked concurrently.

Caulfield and Moore (1974) using ball-milled cellulose and the commercial cellulase "Onozuka" (from T. reesei) noted a relationship between the initial crystallinity and digestibility which they believe to be more a parallel correlation than a causal one. During grinding crystallite size and particle size are reduced, increasing the accessibility of both amorphous and crystalline cellulose to the enzyme molecules. The accessibility of the crystalline component is enhanced to a greater extent than that of the amorphous component.

Schurz (1986), using spruce sulfite pulp, found no significant change in the crystallinity, the degree of polymerization, the specific inner surface, the void fraction or the molecular weight distribution during 48 hours of hydrolysis by T. reesei enzymes. On the basis of these results they proposed an "all or nothing" mechanism for degradation: a chain

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is either fully degraded or not at all.

C. Cellulase Dimensions

The dimensions of the cellulases produced by various micro-
organisms have been estimated to range from 2.5 to 7.7 nm for
spherical enzymes and 1.3 x 7.9 nm to 4.2 x 25.2 nm for
ellipsoids (Cowling, 1975). Trichodermal enzymes have dimensions
at the upper end of these ranges, and would be primarily limited
to attacking the gross capillaries of the fibers. Only a small
fraction of the cell-wall capillaries in water-swollen fibers are
large enough for the enzymes to penetrate.

Schmuck et al. (1986) determined the molecular structural
parameters of the major cellobiohydrolase purified from the
culture filtrate of T. reesei. The distance distribution
function measured by the small angle x-ray scattering technique
indicated that the enzyme has a tad-pole like shape. The maximum
length is 18 nm and the largest diameter 4.4 nm.

D. Pore Size

Pore size distribution is a very refined method of
determining the surface area of a substrate. Stone and Scallan
(1968) developed the solute exclusion technique, to measure the
accessible volume, using a range of dextrans as inert probes.

The importance of pore size distribution of a substrate on
its hydrolysis was first noted by Stone et al. (1969), who
demonstrated a linear relationship between the initial reaction
rate and the surface area within the cellulose accessible to a

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molecule of 4.0 nm.

Grethlein (1985) found the initial glucose yield, after 2 hours hydrolysis with T. reesei, was linear to the pore volume available to a spherical molecule of 5.1 nm.

Weimer and Weston (1985) found that rate of degradation of native celluloses by Trichoderma cellulases is strongly related to both the total pore volume and the initial crystallinity of the substrate. The rate-limiting component for the fungal cellulase system was found to have a size of 4.3 nm along one axis.

E. Degree of Polymerization

The degree of polymerization (number of anhydroglucose units in a cellulose molecule) varies with the source and pretreatment of the substrate, and ranges up to 10,000-14,000 for wood or cotton. Klesov and Sinitsyn (1981) and Schurz (1986) found no correlation between the degree of polymerization (DP) and hydrolysis.

F. Pretreatment

In nature cellulose is mostly crystalline, and is very resistant to hydrolytic agents, including enzymes. Removal of the lignin and hemicellulose fractions by physical and/or chemical means does not guarantee that the cellulose will become more susceptible to enzymatic attack.

The cellulose can be made more susceptible to the cellulases by reducing its crystallinity. This can be done by subjecting the cellulose to intensive milling or by treatment with cellulose

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solvents.

Attrition methods reduce particle size and severely disrupt the crystalline order of the cellulose. Hammer-milling, ball-milling, compression-milling, vibratory-milling have been investigated by various groups.

There are a number of cellulose solvents, such as concentrated mineral acids, nonaqueous organic solvents such as dimethyl sulfoxide, and transition metal complexes e.g. chelating metal caustic swelling solvent (CMCS). These agents have a similar mode of action. They disrupt the hydrogen bonding, causing the cellulose molecule to pass into solution. Excess water is added to re-precipitate the amorphous cellulose.

Most of the attempts to correlate cellulose structure with degradability have involved pretreating cellulosic materials with physical or chemical agents that drastically alter several aspects of the cellulose fine structure. The data from these studies must be interpreted cautiously as several structural features are often altered simultaneously by a treatment.

There is evidence that some structural characteristics may be inter-dependent. Klesov claims a relationship between the degree of crystallinity of cellulose and its specific surface area as they were found to affect the hydrolysis rate to the same extent. The cellulose molecules are packed together more tightly in a crystalline substrate than in an amorphous substrate. Consequently, the specific surface area (i.e. surface area/weight substrate) is smaller for the crystalline substrate, and less surface area is available to the cellulase enzyme.

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IV. CELLULASE COMPLEX

A. Characterization of the Enzymes

The three major cellulolytic components produced by Trichoderma spp. are: endo-β-1,4-glucanase, exo-β-1,4-glucanase and β-1,4-glucosidases. A multiplicity of each of these enzymes exists - it is not yet known if the individual forms are genetically determined, or if they arise from differential modification by proteolysis or glycosylation. The enzymes are best described by their mode of action, or the products of their reaction (Fig 4).

The endoglucanases (1,4-β-D-glucan glucanohydrolase (EC 3.2.1.4), C_x, CMC'ase), hydrolyze cellulose molecules in a random fashion, with a preference for larger molecules. Water soluble cello-oligosaccharides are the intermediate products of attack, but once formed these are rapidly hydrolyzed to cellobiose and glucose by the exoglucanases and β-glucosidase, respectively. Endoglucanases are typically characterized by their high activity on substituted celluloses e.g. carboxymethylcellulose (CMC), and low specific activity on Avicel (on the order of 10^3 times lower). These enzymes exist in the largest number of multiple forms, possessing significantly different properties (e.g. in the randomness of their attack, degrees of synergism with the cellobiohydrolase, molecular weight, isoelectric point (Beldman et al., 1985, 1987; Bhikhabhai et al., 1984)).

Two classes of exoglucanase exist, exo-cellobiohydrolase (CBH) and exo-glucohydrolase. The main exoglucanase is exo-

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Figure 4.

Schematic representation of the synergistic enzyme hydrolysis of cellulose.

ENZYMATIC HYDROLYSIS OF CELLULOSE
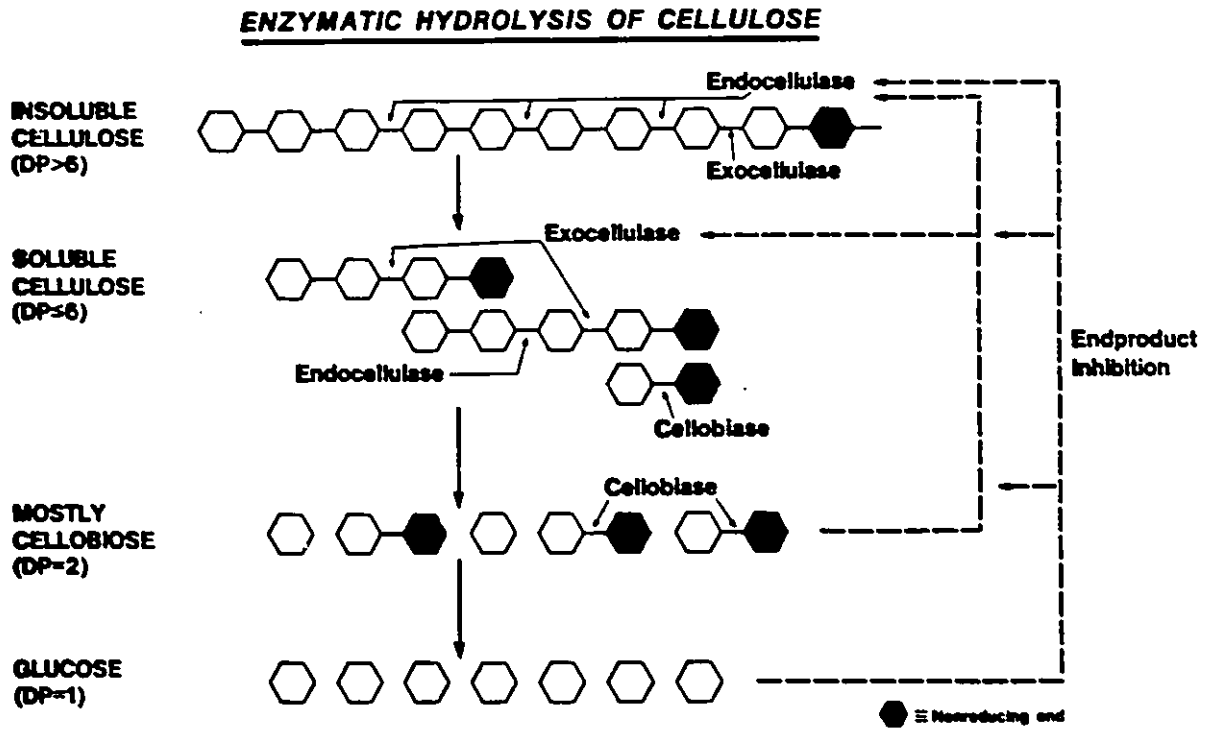
Saddler, 1986

(Courtesy of Forintek Canada Corp.)

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cellobiohydrolase (1,4-β-D-glucan cellobiohydrolase (EC 3.2.1.91) which removes cellobiose units from the non-reducing end of the cellulose molecule. It interacts synergistically with endoglucanase to break down crystalline cellulose, against which neither enzyme acting individually has significant activity. This CBH is currently equated with the old C_1 enzyme by many researchers. The existence of an exo-glucohydrolase (1,4-β-D-glucan glucohydrolase, EC 3.2.1.74), able to cleave off glucose units from the non-reducing end of the cellulose molecule, has been reported for some fungi, e.g. T. reesei (Marsden et al., 1982) and Penicillium funiculosum (Wood and McCrae, 1982).

Cellobiohydrolases are generally characterized by their ability to hydrolyze amorphous (phosphoric-acid swollen) cellulose, with cellobiose as the main product, and by their inability to attack substituted cellulose (e.g. CMC). Likewise, glucohydrolases act on amorphous cellulose to release glucose, but are not active against substituted celluloses.

β-Glucosidase (EC 3.2.1.21) is responsible for formation of the final product, glucose. While the terms cellobiase and β-glucosidase are used interchangeably, β-glucosidase is able to hydrolyze cellodextrins to glucose.

One of the main difficulties with the assay of cellulolytic enzymes is the choice of a suitable substrate. As described above, cellulase is a complex of enzymes. For the complete hydrolysis of an insoluble cellulose, a synergistic action between these components is required. Assay of the cellulase components has usually been tailored to meet the requirements of the researcher. This has resulted in a large array of

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substrates, enzyme actions and nomenclatures (Table 1).

B. Synergism

Wood (1975) elegantly demonstrated that the cellulases in the culture filtrate of T. koningii act synergistically to hydrolyze fibrous cotton to glucose. The proteins in the culture filtrate were separated on DEAE Sephadex and identified as different enzymes: C_1 (exoglucanase), C_x (endoglucanase), and cellobiase. Individually the enzymes had little or no effect on cellulose, but when recombined at levels equal to those of the original filtrate they were able to hydrolyze the substrate to an extent equivalent to that obtained with the original culture filtrate.

When one considers synergism, a description of the substrate must also be included. The structural characteristics of a substrate determine the relative importance of synergism in the enzymes acting against it. Degradation of amorphous cellulose can be effected by either exo- or endoglucanase acting independently, whereas crystalline celluloses require the synergistic interaction of these enzymes (Coughlan, 1985). When incubated with amorphous cellulose the combined pure components show only a simple addition of the independent enzyme activities, rather than a synergism (Wood and McCrae, 1979). Obviously, the co-operative action between the two enzymes is low on amorphous substrates. Synergy operates at a maximum when the components are used in the ratios in which they occur in the fermentation filtrate (Wood, 1975).

Table 1. Cellulase assays.

ENZYME	SUBSTRATES	PRODUCT(S) MEASURED
Cellobiase/	Cellobiose	Glucose
B-glucosidase	Cellodextrins	Glucose
Aryl-B-glucosidase/	o-Nitrophenyl-B-glucoside	o-Nitrophenol or Glucose
B-glucosidase	Salicin	Saligenin or Glucose
Exo-B-1,4-glucanase		
Cellobiohydrolase	Avicel	Cellobiose
(CBH, C_1)	Crystalline cellulose (cotton)	Cellobiose
	p-Nitrophenyl-B-D-lactoside (pNPL)	o-Nitrophenol or Disaccharide
	p-Nitrophenyl-B-D-cellobioside (pNPC)	o-Nitrophenol or Disaccharide
	4-Methylumbelliferyl-B-D-cellobioside (MUC)	4-Methylumbelliferone or Disaccharide
Glucohydrolase	Avicel	Glucose
	Crystalline cellulose (cotton)	Glucose
	Cellodextrins	Glucose
Endo-B-1,4-glucanase	Carboxymethylcellulose (CMC)	Reducing sugars or loss in viscosity
(EG, C_x)	Amorphous cellulose	Reducing sugars or loss in viscosity
"Complete cellulase"	Filter paper (FP)	Reducing sugars or loss in weight
(FPase, Avicelase)	Avicel	Reducing sugars or loss in weight
	Cellodextrins	Reducing sugars
	Dyed cellulose	Release of dye
	Thread	Breaking strength

The mechanism of synergistic interaction on crystalline substrates is not fully understood.

An endo-exo sequential mode of action has been proposed, with the endoglucanase initiating sites of attack for the exoglucanase. This is supported by the work of Streamer et al. (1975), who demonstrated synergistic hydrolysis is obtained.when the substrate is first incubated with endoglucanase, prior to addition of the exoglucanase. Incubation with exoglucanase, followed by the endoglucanase, produced only low levels of sugars.

Ryu et al. (1984) studied the adsorption of the individual cellulolytic components of T. reesei filtrates on Avicel. They postulated that the endoglucanase and cellobiohydrolase components adsorb to different sites on the substrate, corresponding to the sites of hydrolysis. Addition of cellobiohydrolase to a substrate containing bound endoglucanase caused an acceleration in the action of the latter enzyme, and brought about its desorption. Similarly, endoglucanase on binding to cellulose increased the rate of action of the bound cellobiohydrolase and caused its desorption. This phenomenon, termed "competitive adsorption", is believed to explain the synergistic interaction of these components in cellulose hydrolysis. To further support this theory, they found the synergistic interaction was most evident when both components were present in the proportion found in the crude filtrate.

For efficient hydrolysis of amorphous cellulose the quantity of enzyme is the limiting factor. With crystalline substrates it appears that both the "quality" of the endoglucanase (discussed

below) and the "quantity" of the exoglucanase components are important.

Klesov et al. (1986) investigated the adsorption of endoglucanase, produced by different organisms, on a number of pure cellulose substrates varying in their crystallinity and surface area. The endoglucanases fell into two classes; those that adsorbed "strongly" and those that adsorbed "weakly", as determined by the partition coefficient (K_p) of the enzyme between the substrate surface and the bulk solution. They found that the decisive factor in effective hydrolysis of amorphous cellulose is the amount and activity of enzyme bound to it. Hydrolysis of the crystalline substrate by the complete system of exo- and endoglucanase depended not only on the extent but also on the "quality" of binding of the endoglucanase. Strongly adsorbed endoglucanases were found in the cellulase systems that are strongly active against crystalline cellulase, while the weakly adsorbed enzymes were found in systems that were relatively inactive against these substrates.

Wood (1982) studied this phenomenon from a different angle. He examined "cross-synergism" of cellobiohydrolase and endoglucanase components, from "strong" and "weak" cellulose degraders. The cellobiohydrolases were purified from fungi capable of degrading crystalline cellulose extensively (e.g. T. koningii, P. funiculosum, and F. solani), while the endoglucanases were purified from a number of fungal and bacterial sources. The source of endoglucanase had a decisive role in determining the extent of hydrolysis: endoglucanase

derived from "strong" degraders highly solubilized the cotton, while endoglucanase purified from "weak" cellulose degraders caused little or no solubilization. Apparently, there is a lack of synergy between the cellobiohydrolase and the "weak" endoglucanases. The major difference between the two types of endoglucanase is that cellobiohydrolase is not produced by the weak degraders.

In hydrolysis the endoglucanase nicks the cellulose chain generating a non-reducing end. The exoglucanase attacks this site, and then proceeds along the chain cleaving off cellobiose units. From a practical viewpoint, a single endoglucanase generates a large number of sites for a large number of exoglucanase components to act on. It would be expected that for an efficient hydrolysis the ratio of exoglucanase to endoglucanase would be high.

This postulation is supported by the work of Henrissat et al. (1985) who evaluated the action of cellobiohydrolases I and II (CBHI and CBHII) and endoglucanase I and II (EGI and EGII) purified from T. reesei in the degradation of pure cellulose substrates. No synergism between the exoglucanases and endoglucanases was noted on amorphous cellulase. Degradation of crystalline cellulose required the concerted action of both CBHII and EGI or EGII; with the reaction limited by the amount of the exo-enzyme present. Apparently, small amounts of endocellulase provide sufficient chain-ends to saturate the exo-enzymes present. The maximum amount of hydrolysis was observed with an enzyme mixture containing mainly exoglucanase with a small amount of endoglucanase.

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A model for the mechanism of hydrolysis of crystalline cellulose was formulated by Coughlan (1985). The first step involves amorphogenesis, whereby the substrate is made more accessible to the cellulases. There is much debate as to the nature of this phenomenon. Reese et al. (1950) proposed that a "swelling factor", C_1, initiates attack on the substrate, facilitating subsequent attack by the cellulase C_x. Later researchers identified this C_1 component as cellobiohydrolase, and the idea of a swelling factor lost favour. More recently, Griffin (1984) isolated a "microfibril-generating factor" in the culture filtrate of T. reesei. The factor was characterized as non-enzymatic, due to its low protein content, and low molecular weight. The factor, requiring the presence of iron for its activity, displayed synergism with the cellulase to degrade filter paper.

C. End-Product Inhibition

Both endoglucanase and exoglucanase are strongly inhibited by cellobiose, the product of their concerted action, and moderately inhibited by glucose. There is much variation in the product inhibition models proposed for the exo- and endo-glucanases. Exoglucanase purified from T. reesei was competively inhibited by its products, cellobiose and glucose (Hsu et al., 1980), while a non-competitive product inhibition was reported for the T. viride enzyme system (Howell and Stuck, 1975).

The sensitivity of the cellulase enzymes to the inhibitors varies drastically with the organism under study. Inhibition

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constants, K_m, ranging from 0.06 to 1.1 mM have been reported for cellobiose acting on exoglucanase (Ladisch et al., 1983). Exoglucanase, derived from T. reesei QM9414 was inhibited 10 times more strongly by cellobiose, than by glucose (Hsu et al., 1980).

β-Glucosidase is strongly inhibited by its product, glucose. Kinetic studies on β-glucosidase purified from T. reesei are more consistent than for the cellulases, with non-competitive inhibition reported (Ladisch et al., 1977; Gong et al., 1977). Glucose inhibits cellobiase less strongly than cellobiose inhibits the cellulases with K_m values from 1.2 to 4.3 mM reported for the former (Ladisch et al., 1983).

Nature, in its economy, provided T. reesei with sufficient β-glucosidase to support its growth on cellulose, where sugars do not accumulate. Trichoderma reesei cellulase preparations have roughly 0.2-0.3 β-glucosidase units per filter paper unit (Ryu and Mandels, 1980). In a hydrolysis reactor, however, glucose levels are high, ranging from 5 to 10%. End-product inhibition is held accountable as one of the major factors limiting hydrolysis. As hydrolysis proceeds glucose accumulates and inhibits the β-glucosidase. In turn, cellobiose builds up and inhibits the cellulase. Therefore, an adequate level of β-glucosidase is critical for cellulose hydrolysis.

There are a number of ways to circumvent end-product inhibition. Supplemental β-glucosidase, derived from a high β-glucosidase producer, such as Aspergillus phoenicis can be added (Sternberg et al., 1977). Addition of the Aspergillus cellobiase to Trichoderma cellulase reduces cellobiose accumulation and

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greatly enhances the glucose yield. The effect of supplemental β-glucosidase on enhancing hydrolysis reaches a maximum at a ratio of approximately 1 to 1.25 IU β-glucosidase per filter paper unit (Dekker et al., 1987). A combined hydrolysis and fermentation (CHF) or simultaneous saccharification and fermentation (SSF) has been investigated by several groups (Blotkamp et al. 1978; Saddler et al. 1982; Takagi et al. 1977). The cellulase system is incubated with a microbe that ferments the liberated glucose to the desired product, directly upon its release. The end result is reduction of glucose inhibition, so the enzymes are able to operate at a faster rate for a longer period of time.

D. Enzyme Inactivation

Enzymatic hydrolysis of cellulose is a long term process during which time the enzymes are subject to shearing, and thermal inactivation (Mukataka et al., 1983; Herr, 1980). As the reaction time for hydrolysis increases, the amount of active enzyme decreases, imposing a restraint on efficient hydrolysis. For a practical process the enzyme must remain stable for extended periods of time (at least 2 days, and much longer if recycling is to be considered). But hydrolysis operates at fairly high temperatures (45-50°C) with agitation, factors that are not conducive to longevity of the enzymes.

The effect of long-term incubation on enzyme stability at a wide range of temperatures has been investigated by numerous researchers. There is a wide range in the half-life of the

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different components of the cellulase system. The results of these experiments must be interpreted cautiously as biocides, e.g. thimerosal, sodium azide, are sometimes used in these studies. These biocides inactivate the cellulase components to different degrees, depending on the concentration at which they are used (Reese and Mandels, 1980).

The enzyme complex derived from some Trichodermal strains are more sensitive to temperature than others. After 2 days incubation at 50°C, at moderate shaking conditions (120 rpm), the Trichoderma strains, QM9414, MCG77 and C30 lost 26, 44, and 61% of their filter paper activity, and 0, 0 and 11% of their endoglucanase activity (Mandels et al., 1981). There was little difference in the loss of activity between shaken or unshaken conditions for the above strains.

In general, β-glucosidase of T. reesei strains appears to be tolerant of high incubation temperatures under both shaken or unshaken conditions (Mandels et al., 1981) .

Andren (1978) demonstrated that, for a long-term incubation the faster rate of hydrolysis obtained at high temperatures must be weighed against the effects of thermal inactivation. As the time of saccharification increased, the maximum extent of hydrolysis was found to occur at lower temperatures.

Mukataka et al. (1983) studied the effect of agitation on the overall hydrolysis. They found the rate of hydrolysis was enhanced with agitation up to about 200 rpm, then declined. The drop in the rate was reported due to greater shear at the higher agitation.

These experiments on stability were carried out at pH 4.5-

5.0, and at 45-50°C, but in the absence of substrate, and as such
do not duplicate the hydrolytic conditions. There is some
indication that the enzymes are more stable in the presence of
substrate, due to adsorption onto the substrate (e.g.
immobilization effects). The extent to which thermal
inactivation hinders the enzymatic reaction, however, is mainly
dependent on the source of the enzyme system.

V. INTERACTION BETWEEN ENZYME AND SUBSTRATE

Lee and Fan (1982) divided the hydrolytic reaction into the following steps: 1) mass transfer of the enzyme molecule from the bulk aqueous phase to the surface of the cellulose particle; 2) enzyme adsorption to the surface of the cellulose and formation of the enzyme-substrate (E-S) complex; 3) transfer of water molecules to the active site of the E-S complex; 4) surface reaction between water and cellulose, promoted by the E-S complex; 5) transfer of the soluble products, glucose and cellobiose, from the cellulose particles to the bulk aqueous phase and 6) decomposition of cellobiose into glucose in the aqueous phase by β-glucosidase.

A. Adsorption

As the first step in the enzymatic reaction is adsorption of the enzyme onto the surface of the substrate, it is important to understand the nature of the process and how it may relate to the overall hydrolysis.

Adsorption of cellulase onto a cellulosic material was first observed by Halliwell and Griffin (1973). Enzyme adsorption was accompanied by the rapid liberation of sugars, so it appeared that adsorption was an integral part of the hydrolytic process.

Factors affecting the adsorption of cellulases to cellulose include; 1) the amount of enzyme present and the enzyme/substrate ratio, 2) the accessible surface area and the affinity of the various cellulase components for the substrate,

3) the physical properties of the enzyme (e.g. charge, size, solubility), 4) the environment (pH, temperature and salt concentration), 5) the nature of the substrate (e.g. purity, pretreatment), and the changing topography of the substrate during hydrolysis (Reese, 1977).

1. Enzyme Concentration

Many investigators have found that the extent of adsorption increases as the cellulose concentration or enzyme concentration is increased, the adsorption behaviour obeying the Langmuir adsorption isotherm (Peitersen et al. 1977, Lee et al. 1982, Goel and Ramachandran, 1983).

Using T. reesei MCG77 Lee and co-workers (1982) demonstrated that the amount of protein adsorbed onto Solka Floc SW40 parallelled its initial rate of hydrolysis.

2. Accessible Surface Area, Affinity and Crystallinity

The initial extent of protein adsorption has been seen to depend on the specific surface area of the cellulose (Lee and Fan, 1982; Mandels et al., 1971; Bisaria and Ghose, 1977). In contrast, Goel and Ramachandran (1983) found that the substrate particle size i.e. surface area, and crystallinity had only a limited effect on the adsorption of cellulases in T. reesei D1-6 culture filtrates.

Adsorption was reported to be identical on both amorphous and crystalline cellulose (Klesov, 1982). The degree of crystallinity of cellulose did not influence the adsorption of

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endoglucanases from T. reesei.

Similarily, Lee et al. (1982) found that the extent of adsorption onto Avicel (purified cellulose) was similar to that on Sweco-270 (purified cellulose that was milled in a Sweco vibratory mill and passed through a 270-mesh screen), although the respective SSA (specific surface area) and CrI values of Avicel (5.4 m^2/g and 80.8%) and Sweco-270 (24.2 m^2/g and 12.5%) were very different. As a result of these findings they concluded that the adsorption affinity, as well as the structural properties have a significant effect on adsorption.

The different affinities of the components of the cellulase complex towards cellulose has been the basis for their fractionation and purification (Nummi et al., 1981; Halliwell and Griffin, 1978). The binding mechanism is very specific, with non-cellulolytic proteins such as albumin unable to bind. The endo- and exo-glucanases both adsorb to pure cellulose substrates. Ghose and Bisaria (1979) report that the endo- and exoglucanase components of the T. reesei system are adsorbed to the same extent, while Wilke and Mitra (1975) state that the exoglucanase fraction of T. viride filtrates adsorb more strongly than the endoglucanase to Solka Floc. Tan et al. (1986) report that the cellobiohydrolase component of T. harzianum E58 filtrates adsorbed more strongly to Solka Floc BW300 than endoglucanase in a column reactor, and had much lower levels of leaching.

Ryu et al. (1984), using partially purified cellulases, found that the cellobiohydrolase component of T. reesei had a stronger adsorption affinity towards Solka Floc SW40 than the

endoglucanases; with the endoglucanases consisting of adsorbable and non-adsorbable fractions. The role of the unadsorbed endoglucanases in hydrolysis has been debated. One thought is that they hydrolyze the long-chain, soluble cello-oligosaccharides released during saccharification.

Recent work using highly purified enzymes shows the adsorption behavior is very complex. Beldman et al. (1987) studied the adsorption of six endoglucanases and two exoglucanases, derived from T. viride, onto Avicel cellulose. Three of the endoglucanases and one of the exoglucanases adsorbed strongly; adsorption of the remaining glucanases was much lower. It appeared that a portion of the enzyme molecules was irreversibly adsorbed on the cellulose, even after prolonged desorption time. Kinetic measurements indicated that only a part of the adsorbed enzymes were hydrolytically active. This indicates that some of the adsorbed enzymes may not be bound at their catalytic site, or they may not be able to express their hydrolytic action e.g. as an endoglucanase unless another enzyme, such as an exoglucanase, is present.

Woodward et al. (1988) purified the enzymes from T. reesei L27 and found that the binding of CBH protein to cellulose was much stronger than that of endoglucanase.

The β-glucosidase component of T. reesei does not adsorb to pure celluloses (Mandels et al., 1981) whereas it can adsorb to native lignocellulosics (Puls et al., 1985).

The adsorption affinity of the enzyme for the substrate has a significant influence on hydrolysis. This claim is supported

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by the work of Castanon and Wilke (1981) who reported that the adsorption of filter paper activity on newspaper was hindered by Tween 80. The surfactant caused a more reversible binding between the enzyme and the substrate resulting in the enhancement of the saccharification.

Similarily, Ooshima et al. (1986) reported addition of Tween 20 decreases the amount of endoglucanase able to adsorb to the substrate, while enhancing its hydrolysis. Surfactants may act by shifting the adsorption balance between the exoglucanase and endoglucanase to the side preferable to the reaction.

Work by Henrissat et al. (1985) has demonstrated the relative proportions of endo- and exoglucanase in a reaction mixture determines the degree to which they are adsorbed onto the substrate, which in turn decides the extent of their synergistic action in hydrolysis.

3. Enzyme Properties

The dimensions of the cellulase molecules determine the surface area of the substrate available to it, which in turn determines the amount of enzyme able to adsorb to the substrate. The interrelationship between cellulase size and structural features of the substrate have been discussed in Section III.

4. Temperature, pH and Adsorption

There is a wide variation in the reported data on the effect of temperature on adsorption. Part of the reason may be the rapid rate of hydrolysis as the temperature is increased, resulting in a decrease in available surface area per weight

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substrate.

Goel and Ramachandran (1983) and Peitersen et al. (1977) reported that adsorption of T. reesei cellulases onto a cellulosic substrate was slightly decreased by increasing the temperature from 5 to 50°C. Kim et al. (1988) observed adsorption of T. viride cellulases was highest at 5°C and greatly decreased with increasing temperature.

Conversely, Mandels et al. (1971) found the rate of adsorption on Solka Floc increased with temperature, but also reported the maximum levels of adsorption decreased with increasing temperature.

Mandels et al. (1971) and Peitersen et al. (1977) observed adsorption of the cellulases onto cellulose is largely independent of pH in the range of 3.5 to 6.0. Bisaria and Ghose (1977) found adsorption declined slightly at pH's greater than 4.8, but in no way comparable to the drastic drop observed for catalytic activity. Conversely, Kim et al. (1988) found that pH had a significant influence on the adsorption behaviour of cellulase, with maximum adsorption of cellulase on microcrystalline cellulose occuring at pH 4.8.

B.   Alteration in Structural Features

During hydrolysis the structural features of the substrate are changing along with the composition and concentration of the adsorbed enzymes. The drastic decrease in the initial rate of hydrolysis is attributed to several factors, including the transformation of the substrate into a less digestible form,

inhibition of the enzyme action, and a decrease in the amount of adsorbed enzyme. As adsorption of the enzyme to the surface of the substrate is a prerequisite for hydrolysis, a decline in the number of binding sites would retard the rate of hydrolysis.

In the initial phase of hydrolysis the specific surface area (SSA) dramatically increased, then slowly declined throughout the remainder of hydrolysis (Lee and Fan, 1983). The initial increase in SSA was explained by endoglucanase attacking the substrate, generating many new adsorption sites for the exoglucanase. As hydrolysis proceeded the number of binding sites decreased, and small portions of the enzyme were released back into solution. Although the SSA decreased during hydrolysis there were sufficient binding sites for the enzyme to adsorb to ensure efficient hydrolysis.

C. Role of Initially Adsorbed Cellulases in Hydrolysis

The enzymes initially adsorbed to the substrate are primarily responsible for the overall hydrolysis. Lee and Fan (1983) compared hydrolysis of Solka Floc with T. reesei QM9414 cellulases in two systems. In the control system the substrate was incubated with enzyme; while in the second system the substrate was recovered after one hour hydrolysis, then resuspended in fresh buffer. The reducing sugars yields were comparable for the two systems, supporting the assumption that the course of hydrolysis is mainly set by the amount of enzyme initially adsorbed.

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D. Kinetics of Enzyme Adsorption and Cellulase Action

In cases where the soluble enzyme concentration is much greater than the amount of substrate surface area accessible to the enzyme, Langmuir Adsorption kinetics may be applied (Appendix A). The basic Langmuir assumptions are as follows: 1) there is no interaction between adsorbed molecules 2) all the surface of the solid has the same activity for adsorption 3) all adsorption occurs by the same mechanism and each adsorbed complex has the same structure and 4) the extent of adsorption is less than one complete monolayer on the surface. The applications of the Langmuir equation are limited to use with purified substrate and purified enzymes. The equation will provide information on the maximum amount of enzyme able to adsorb onto the substrate, and the affinity of the enzyme towards a given substrate.

There have been many excellent reviews on the mechanisms and kinetics of cellulase action (Chang et al., 1981, Gilbert and Tsao, 1983, 1985). These are mainly based on studies using purified celluloses as the model substrate and Trichoderma spp. enzymes.

E. Mechanism of Adsorption

The mechanism of adsorption of the cellulases onto cellulose are not fully understood. The adsorbent is the substrate of the enzyme, and there is a preferential adsorption of the cellulases; with the other enzymes left in solution. Ionic bonding, hydrogen bonding, hydrophobic interactions and permeation effects are all involved in the adsorption of cellulases onto cellulose (Reese,

1982; King, 1965; Kim et al., 1988).

The cellulases appear to have both catalytic and adsorptive sites on the substrate, referred to as "productive" and "non-productive" binding (Beldman et al., 1987; Rabinovich et al., 1982).

It has been postulated that the glycosylated regions of the endo- and exoglucanases are involved in the binding to insoluble cellulose. Van Tilbeurgh et al. (1986) partially proteolyzed CBH I from T. reesei into 56 kDa and 10 kDa portions. The core (56 kDa) protein retained its activity against soluble substrates, but completely lost its activity against insoluble cellulose, concomitant with a decrease in adsorption. The 10 kDa peptide was identified as the heavily glycosylated carboxy-terminal of the native CBH I. They proposed a bifunctional organization of the enzyme; one domain containing the active site, while the other, corresponding to the carbohydrate-rich carboxy-terminal containing the cellulose adsorption site.

VI. OBJECTIVE OF THESIS

Cellulases used for enzymatic hydrolysis have been mainly derived from different strains of Trichoderma, especially T. reesei. The yield of β-glucosidase from these systems is low, limiting the usefulness of these cellulases for commercial applications. During the screening of the Forintek Canada Corp. culture collection it was found that Trichoderma harzianum E58 gives higher amounts of β-glucosidase than most other cellulolytic fungi (Saddler, 1982). In addition, the fungus produced high yields of the other components of the cellulase

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system, exoglucanase and endoglucanase. In order to use the cellulases derived from T. harzianum E58 in a cellulose conversion process the hydrolytic properties of the system must be better defined. This thesis examined the ability of T. harzianum E58 cellulases to degrade the commercial cellulose, Solka Floc BW300, to glucose.

Cellulose bioconversion requires high levels of enzyme to counteract factors limiting hydrolysis, such as end-product inhibition, enzyme inactivation, substrate recalcitrancy and enzyme adsorption. The major advantages of high enzyme loading are faster rates of hydrolysis and increased sugar yields - the main drawback is the high cost of the enzyme (Perez et al., 1980; Wilke et al., 1981; Becker et al., 1982). Judicious use must be made of the enzyme to cut costs and avoid waste. The first objective of this thesis was to identify conditions for efficient hydrolysis. Temperature and pH both impact on enzyme activity in a process. The relationship between enzyme loading and hydrolysis was examined, and various methods of determining the minimal amount of enzyme for efficient conversion were studied.

Enzymatic hydrolysis of crystalline cellulose is biphasic: it is characterized by an initially rapid rate of reaction, followed by a dramatic reduction in the rate. This decline is a function of both the changing behavior of the enzyme towards the substrate, and alteration in the substrate. This thesis addresses the factors limiting hydrolysis. My approach has been to examine the relative importance of enzyme-related and structural-related factors in determining a) the initial rate of hydrolysis and

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b) long-term hydrolysis.

Enzyme-related factors limiting hydrolysis that were investigated include; thermal stability, site blockage by denatured enzymes and end-product inhibition. The role of the structural features, crystallinity and surface area, in determining the susceptibility of the substrate to enzymatic attack, and in governing long-term hydrolysis were also examined.

MATERIALS AND METHODS

Microorganisms

All the fungi used were obtained from the Forintek culture collection. Trichoderma harzianum E58 was originally isolated from a lumber yard in Ontario, and is a wild type organism. It was identified as T. harzianum in 1982 by Dr. Bissett (Agriculture Canada) and later verified as such, by our curator Dr. K. Seifert.

Saccharomyces cerevisiae C495 (NRCC 202001) was grown aerobically at 28°C, shaken at 110 rpm, using the medium of Rainbow (1970).

Cellulase Production

Spores maintained on potato dextrose agar (Difco) slants at 4°C were used to inoculate shake flasks (300 mL, 100 mL working volume) containing Vogel's medium (Montenecourt and Eveleigh, 1977) with glucose as the carbon source. Each flask was inoculated with a spore suspension to give a final concentration of 5-8 x 10^6 spores/mL of the medium. After two days incubation (28°C, 110 rpm) the culture was centrifuged (10,000 rpm, 40 minutes), the pellet resuspended in 100 mL of distilled water and used as the inoculum. The mycelial inoculum was added to the enzyme production vessels at a final concentration of 0.3-0.6 mg biomass (dry weight)/mL medium. Enzyme production was carried out in shake flasks (500 mL, 150 mL working volume), or in a 30-L New Brunswick Microferm fermentor, containing Vogel's medium with

1% Solka Floc BW300 as the carbon source. The fermentation vessels were incubated at 28°C, 110 rpm and harvested after peak activity had been reached. The culture filtrate was collected by filtration through Whatman 934-AH glass microfiber filters.

The fermentor-grown culture filtrates were concentrated by ultrafiltration using the Millipore Pellicon Cassette system fitted with a polysulfone membrane, with a molecular weight exclusion limit of 10,000 daltons. The retentate was freeze-dried at -70°C to produce a free-flowing powder which was stored at 4°C. No loss in cellulase activity was noted after one year storage under these conditions.

See Table 2 for information on the cellulase preparations used for the various experiments.

Purified Exoglucanase and β-glucosidase

The purified exoglucanases and β-glucosidase used in this study were kindly donated by Dr. L.U.L. Tan.

Fractionation of cellulase activities: Crude cellulase from the culture filtrate of T. harzianum E58 was fractionated by ion-exchange chromatography on DEAE-Sephadex A50 into 4 major protein peaks. The exoglucanase, with a molecular weight of 67,000 daltons, was found to be homogeneous as judged by a single protein band on SDS-PAGE (Tan et al., 1986).

β-Glucosidase was purified following the method of Tan et al. (1987).

Table 2. Cellulase activity and protein concentration of T. harzianum E58 enzyme preparations.

Figure Number	Endoglucanase Activity IU/mg protein	β-glucosidase Activity IU/mg protein	Filter paper Activity IU/mg protein	Protein Concentration mg/mg lyophilized powder or mg/mL enzyme solution	Comments
5	14.91	0.84	0.73	0.86 mg/mg	E_{act} at optimal pH
6	14.91	0.84	0.73	0.86 mg/mg	E_L=15 FPU/g substrate
7	11.98	0.66	0.73	1.20 mg/mL	E_{act} at optimal temperature
8	11.53	0.64	0.74	0.72 mg/mg	E_L=30 FPU/g substrate
9	11.65	0.66	0.68	0.91 mg/mg	
10	12.32	0.51	0.73	0.79 mg/mL	For pH range 3.0-6.0; E_L=11.5 FPU/g subs.
	12.24	0.52	0.82	0.84 mg/mL	For pH range 6.0-7.5; E_L=13.8 FPU/g subs.
11	16.21	0.61	0.90	0.98 mg/mL	E_L=17.6 FPU/g substrate
12	15.60	0.59	0.73	0.93 mg/mg	
14	15.60	0.59	0.73	0.93 mg/mg	
15	15.60	0.56	0.79	0.93 mg/mg	E_L=25.6 FPU/g substrate
	0.89	22.51	N.D.	0.55 mg/mL	E_{act} profile for purified β-glucosidase
16	N.D.	N.D.	0.08	5.70 mg/mL	E_{act} profile for purified exoglucanase
17	19.07	0.55	0.85	0.82 mg/mg	E_L=25.7 FPU/g substrate
	0.89	22.51	N.D.	0.55 mg/mL	E_{act} profile for purified β-glucosidase
18	19.87	0.84	0.87	78.3 mg/mL	Ultra-filtered enzyme preparation
19	13.30	0.45	0.67	1.12 mg/mL	E_{act} profile for cellulase preparation
	0.37	N.D.	0.09	4.17 mg/mL	E_{act} profile for purified exoglucanase
20	See Figure 19 for E_{act} profile for cellulase preparation				E_L=30 FPU/g substrate at t=0
21	15.60	0.59	0.73	0.93 mg/mg	
22	See Figure 21 for E_{act} profile for cellulase preparation				
23	17.00	0.59	0.73	0.93 mg/mg	E_L=30 FPU/g substrate
24	10.94	0.65	0.70	0.92 mg/mg	
25	11.56	0.63	0.69	0.90 mg/mg	
26	11.65	0.66	0.68	0.91 mg/mg	E_L=10 FPU/g substrate
27	15.60	0.59	0.73	0.93 mg/mg	E_L=20 FPU/g substrate (for hydrolysis)
	11.53	0.63	0.73	0.72 mg/mg	E_L=24.7 FPU/g substrate (for adsorption)
28*	N.D.	N.D.	0.084	6.13 mg/mL	E_{act} profile for purified exoglucanase E_L=30.6 mg protein/g substrate
29*	15.60	0.59	0.73	0.93 mg/mg	E_L=20 FPU/g "spent cellulose"

E_{act} = Enzyme activity

E_L = Enzyme loading

N.D. = None detected

* Substrate generated in Figure 27 (for hydrolysis) was used for the adsorption experiments (Figures 27,28) and the recalcitrancy study (Figure 29).

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Substrates

Avicel pH 101 (FMC Corp., PA, USA) is a microcrystalline cellulose derived from fibrous plants. It is a particulate, free-flowing powder.

CF 11 Cellulose Powder (Whatman medium length fiber)

Sigmacell Type 50 (Sigma Chemical Co., St. Louis, MO, USA) is a microcrystalline cellulose with an average particle size of 50 μm.

Solka Floc AS1040 (Brown and Co., NH, USA) was made from purified cotton linters.

Solka Floc BW300 (Brown and Co., NH, USA) is manufactured from ball-milled sulfite wood pulp; average fiber length of 22 μm. It was used as the standard cellulose substrate for cellulase production, hydrolysis and adsorption studies.

Solka Floc BW300 (General Filtration, Division of Lee Chemicals Ltd., Toronto, Ont., Canada) is manufactured from ball-milled sulfite wood pulp; average fiber length of 22 μm.

Solka Floc SW 40 (Brown and Co., NH, USA) was made from hammer-milled wood pulp and was a fibrous grade of cellulose.

Assays

Soluble protein was determined after precipitation with 7% trichloroacetic acid (TCA) by the method of Lowry et al. (1951), using bovine serum albumin (Sigma Chemical Co.) as a standard.

Total reducing sugars were measured colorimetrically using the dinitrosalicylic acid reagent (Miller, 1959).

Glucose was estimated colorimetrically by the glucostat

(Sigma Chemical Co.) enzyme assay (Raabo and Terkildsen, 1960).

Ethanol was determined on a gas-liquid chromatograph (Varian

4600) fitted with a 6 ft. x 1/8 in. Chromosorb 101 column

(Chromatographic Specialities) and a flame ionization detector.

The detector was kept at 250°C, the column oven at 180°C and the

injector port at 200°C. Helium, saturated with formic acid

(Ackman, 1972), was used as the carrier gas at a flow rate of 30

mL/min.

Enzyme assays

The different cellulase activities were measured using the

method of Mandels et al. (1976). Enzyme unit values were

calculated in the dilution range where approximately 0.3-0.6

mg/mL of reducing sugars were detected at the end of the assay.

Therefore, the absorbance read at 575 nm was in the range of 0.4-

0.8.

Endoglucanase activity [1,4-β-D-glucan-4-glucanohydrolase,

EC 3.2.1.4] was determined by incubating 1 mL enzyme solution

with 10 mg carboxymethyl cellulose (Sigma Chem Co.; medium

viscosity, D.P. 1100, D.S. 0.7) in 1 mL 0.05 M citrate buffer, pH

4.8, at 50°C for 30 minutes. The reaction was terminated by

addition of 3 mL dinitrosalicylic acid reagent. The tubes were

placed in a boiling water bath for 5 min then cooled to room

temperature and the absorbance read at 575 nm.

Filter paper degrading activity determines the "complete

cellulase" (endocellulase and exocellulase) system. Filter paper

activity was measured by the method of Mandels et al. (1976).
One mL of enzyme suspension was added to 1 mL 0.05 M citrate
buffer, pH 4.8, containing a 1 cm x 6 cm strip (50 mg) Whatman
No. 1 filter paper. After incubation for 1 h at 50°C the
reaction was terminated by the addition of 3 mL dinitrosalicylic
acid reagent.

β-Glucosidase activity [EC 3.2.1.21] was determined by
incubating 1 mL of enzyme suspension with 10 mg salicin (Sigma)
in 1 mL 0.05 M citrate buffer, pH 4.8, at 50°C for 30 min. The
procedure followed was the same as for the endoglucanase assay
described above.

One unit of endoglucanase, filter paper and β-glucosidase
activity was defined as 1 μmol glucose equivalents released per
minute.

Duplicates

All experiments outlined in Figures 5-29, and Table 3 were
performed in duplicate, and the values averaged. The variability
between duplicates was within 5%.

Effect of pH on Enzyme Activity

Three parts culture filtrate was combined with one part of
0.2 M citric acid-sodium citrate buffer, pH range 3.0-6.2, or one
part of 0.2 M citric acid-Na_2HPO_4 (McIlvaine) buffer, pH range
2.6-7.6 (McKenzie, 1979). The enzyme assays were performed using
the same buffer.

Saccharification Studies

The majority of the saccharification studies were carried out using Solka Floc BW300 (Brown & Co.) as the standard substrate.

The substrate and buffer were added to the reaction flasks and autoclaved 15 minutes at 121°C prior to addition of the enzymes. The enzyme solutions were filter-sterilized through a 0.45 μm nylon membrane (Nalgene) to prevent microbial contamination, then aseptically added to the reaction flasks.

The substrates used for hydrolysis in the experiments outlined in Figures 15, 23, 25 and 29 could not be autoclaved, as this would alter the substrate and prevent proper interpretation of the results. To avoid contamination, a bactericide, 0.001% thimerosal, was included in the systems. The enzyme solutions were filter-sterilized, as outlined above.

Hydrolysis was carried out at 45°C, with an agitation speed of 140 rpm. The systems were maintained at pH 4.8 using 0.05 M sodium acetate buffer. The enzymatic reaction was terminated by boiling the systems for 10 minutes.

The following equation was used to determine the percent conversion of cellulose (anhydroglucose) to reducing sugars or glucose:

$$\% \text{ Conversion} = \frac{\text{reducing sugars or glucose (mg/mL)}}{\text{initial substrate conc. (mg/mL)} \times 180/162} \times 100$$

$M.W._{Glucose} = 180$

Base Molecular $Weight_{Anhydroglucose} = 162$

Combined Hydrolysis and Fermentation (CHF) Procedure

The conditions for the saccharification studies were
modified slightly in the CHF to satisfy the fermentative
requirements of the yeast. Serum vials containing 5% (1.0 g)
Solka Floc BW300 and 0.5% (0.1 g) yeast extract (Difco) in 5.0 mL
of 0.05 M sodium acetate buffer, pH 4.8, were flushed with N_2,
sealed with butyl stoppers and aluminum caps, and autoclaved at
121°C for 15 minutes. The cellulases were dissolved in 0.05 M
sodium acetate buffer, filter sterilized, and 14 mL volumes were
aseptically injected into the vials. A 5% (1.0 mL) inoculum of a
2 day old S. cerevisiae culture containing approximately 10^6
cells/mL was added immediately after. The vials were incubated
at 37°C, 140 rpm for four days, with samples removed at the
designated times. The contents of the flask were assayed for
reducing sugars, glucose and ethanol. The percent conversion
based on the ethanol yield was calculated as follows:

$$\% \text{ Conversion} = \frac{\dfrac{EtOH\ (mg/mL)}{46.07} \times \dfrac{180}{2} \times \dfrac{1}{\text{Biomass factor*}}}{\text{Conc. of initial substrate} \times \dfrac{180}{162}} \times 100$$

$M.W._{EtOH} = 46.07$

*Biomass factor accounts for the fraction of produced glucose
which is used to fulfill the energy requirements of the organism.
It was determined by running a 5% glucose control system in
parallel to the CHF (5% cellulose) system and calculated as
follows:

$$\text{Biomass factor} = \frac{\text{actual ethanol production}}{\text{theoretical ethanol production}}$$

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Adsorption Studies

Routine Methodology

The cellulosic substrates were suspended in 0.05 M sodium acetate buffer at pH 4.8 and left to stand overnight at 28°C to ensure complete water saturation of the substrate. The cellulase enzyme solution, at a temperature of 28°C, was added to the vials. Adsorption experiments were routinely carried out at 28°C, 150 rpm for 10 minutes, after which time the system was vacuum filtered for 15 seconds to remove the cellulose residue. Whatman 934 AH glass microfiber filters were used instead of cellulose filters as the former allowed complete recovery of the cellulases. Longer filtration times, of up to one minute, did not alter the amount, or activity of enzyme recovered from the system (Data not shown). Controls for these experiments consisted of enzyme incubated without cellulose for 10 minutes under the same temperature, pH, and agitation conditions.

Enzyme adsorption could not be measured directly and was determined by difference. The supernatant was assayed to determine recovered protein concentration and enzyme activity. Adsorbed protein and enzyme were calculated by subtracting the recovered protein and enzyme in solution from the control.

Adsorption Studies: Effect of pH

Sodium hydroxide adjustment and the Clark-Lubbs buffer system were used to study the adsorption of the cellulases under various pH's. For the pH range of 4.0-6.0 the culture filtrate

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was adjusted to the desired pH and added to the substrate vials.
The Clark and Lubbs (KH_2PO_4-NaOH) system was used for studies
ranging from 5.8-7.6. After filtration of the system to separate
the adsorbed fraction from the unadsorbed enzymes the filtrates
were immediately adjusted back to pH 4.8.

Adsorption Studies: Effect of Temperature

The methodology outlined above was used, except the
substrate vials and enzyme preparation were preincubated at
either 4, 28, 37, or 45°C before mixing.

Adsorption Studies: Various Proteins

Bovine serum albumin (Sigma Chemical Co.) and the proteases,
chymotrypsin (Sigma Chemical Co.) and trypsin (Sigma Chemical
Co.) were dissolved in cold (5°C), 0.05 M sodium acetate buffer,
pH 4.8. The routine methodology outlined above was used, except
the experiment was run at 4°C to avoid proteolytic activity. The
protein preparations were added to the substrate at final protein
concentrations of 2,4 and 10 mg/g Solka Floc BW300. After
filtration the solutions were immediately assayed for protein
content.

Initially Adsorbed Cellulases

A 5% concentration of Solka Floc BW300 was incubated in 0.05
M sodium acetate buffer, pH 4.8 for 24 h to allow complete
saturation of the cellulose. T. harzianum E58 cellulases, at
25.6 FPU/g substrate, were added. The system was incubated at

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4°C, 150 rpm, for 1 h to allow the enzymes to adsorb to the substrate. The substrate-enzyme complex was collected by vacuum filtration, rinsed with distilled water to remove the unadsorbed proteins, then lyophilized. A substrate control, with no enzymes added, was prepared in parallel.

The lyophilized substrates at a 2% concentration (see below) were prepared in 0.05 M sodium acetate, pH 4.8:

a) substrate-enzyme complex only

b) substrate-enzyme complex and supplemental purified β-glucosidase at 20 IU β-glucosidase/g substrate

c) substrate (control) and cellulases at 25.6 FPU/g and 20 IU β-glucosidase/g substrate (original preparation).

The systems were incubated for 4 days at 45°C, 150 rpm and assayed for release of sugars.

Determination of Crystallinity

The water-swollen cellulose was solvent exchanged in order to preserve its swollen state as closely as possible. Air drying results in collapse of the cellulose fibers, changing its structural characteristics. The expanded capillary structure is maintained if the water is replaced by a polar organic solvent, e.g., methanol, which in turn, is replaced by a nonpolar organic solvent, e.g. benzene. When the nonpolar organic solvent is removed by drying, the water-swollen nature of the cellulose remains largely intact (Fan et al., 1980).

Accordingly, the water-swollen cellulose was recovered by vacuum filtration and immediately mixed with methanol to obtain a 5% (w/v) slurry. The mixture was stored at 4°C for 24 hours, then the substrate was filtered. This methanol wash was repeated

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twice, then the methanol was replaced with benzene, using three consecutive 24 hour soakings with fresh benzene. The samples were air dried for 48 hours prior to analysis to remove traces of the benzene.

Crystallinity Indexes

Crystallinity indexes were determined on a Norelco diffractometer, under the supervision of R. Hartree, Geology Dept., University of Ottawa, following the methodology of Segal et al. (1959).

The crystallinity was measured using the powder method of x-ray diffraction, and scanning for a range of 2θ from 10° to 29°. From the diffractograms the crystallinity indices (CrI) were determined by the following equation:

$$CrI=[(I_{002}-I_{am})/I_{002}] \times 100$$

where I_{002} is the intensity of the 002 peak (at about $2\theta=22^{\circ}$), and I_{am} is the intensity at $2\theta=18^{\circ}$. The I_{002} peak corresponds to the crystalline fraction and the I_{am} intensity to the amorphous fraction.

Preparation of Amorphous Cellulose

Acid-swollen Cellulose

Phosphoric-acid swollen cellulose was prepared following the method of Walseth (1952). In a 1 L beaker, 30 g of Whatman crystalline cellulose powder was slowly added with mixing to 400 mL of cold, 85% phosphoric acid. The mixture was worked to a

smooth consistency. The acid-swollen cellulose gel was
maintained at 4°C for 2 hours in an ice-water bath, and stirred
intermittently. Cold distilled water was added to the sample,
with mixing, to disperse the gel. The system was vacuum filtered
through a sintered glass filter of coarse porosity. The sample
was transferred to a 1.5 L beaker containing 1 L of cold,
distilled water and blended to form a suspension using a motor-
driven, stainless steel stirring blade. This process was
repeated until the washings were neutral. After the last wash
the sample was vacuum filtered, lyophilized and stored at -25°C.

Chelating Metal Caustic Swelling Solvent (CMCS)

The cellulose solvent, ferric tartrate/sodium hydroxide
(FeTNa) was prepared following the methods of Hamilton et al.
(1984). To a 2.0 L glass beaker, surrounded with aluminum foil
207.1 g sodium tartrate and 400 mL water were added. The mixture
was gently heated to dissolve the crystals. The solution was
cooled to room temperature, then 81.1 g ferric chloride
hexahydrate, dissolved in 160 mL water, were added. These
solutions were vigourously mixed for 45 minutes while being
protected from the light then cooled in an ice water bath to
10°C. A solution of 98 g sodium hydroxide dissolved in 200 mL
water was slowly added, with the temperature of the system
maintained below 14°C. During the addition of the sodium
hydroxide, the colour of the solution shifted from reddish-brown
to yellowish-green. At this point 50 g sodium sulfite was added
for stabilization. The solution was made up to 1 L with water

then filtered to remove any ferric hydroxide precipitate. The

solvent was stored in a polyethylene bottle at room temperature

until use.

The following cellulose substrates were treated with the

cellulose-swelling agent: Solka Floc BW300, Solka Floc AS1040,

Solka Floc SW40, Avicel, Sigmacell 50, and CF11. A 2.0 g

quantity of the substrate was slowly added, with vigourous mixing

to the solvent. The volume of solvent used varied with the

solubility of the substrate. Solka Floc AS1040, Avicel, Sigmacell

50 and CF11 dissolved in 50 mL solvent per gram substrate, while

Solka Floc BW300 and Solka Floc SW40 required 100 mL of solvent

per gram substrate.

The CMCS solvent was vigorously stirred while the substrate

was slowly added, to avoid clumping. To avoid oxidation of

glucose, a problem inherent in the caustic swelling systems, the

solutions were protected from light during the dissolving

procedure. Some of the substrates did not completely dissolve as

indicated by a "cloudy" appearance; and the solutions were left

at 4°C overnight until these fine particles dissolved. The

solutions were dark green, translucent and very viscous. The

cellulose was reprecipitated by slow addition to a 2 L volume of

water, while stirring vigorously. The precipitate was recovered

by filtering the mixture through glass filters of medium

porosity. The precipitate was washed with 2 L of water, then

with 1.0 N HCl until all traces of the iron tartrate complex were

removed and a fluffy white residue was left. The precipitate

(amorphous cellulose) was washed with distilled water until

neutral washings were obtained, lyophilized, and stored in a

dessicator.

Ball-milling

A 40.0 g quantity of Solka Floc BW300 (Brown and Co.) was
milled dry in a 5 L laboratory porcelain jar following the
modified procedure of Brownell (1968). Each jar was charged with
4.5 kg of small burundum grinding cylinders (1.25 cm. in diam.,
1.25 cm. in length, 207 to the kg). The jars were rotated at 60
rpm for a two week period.

Spent Cellulose

A series of Erlenmeyer flasks containing Solka Floc BW300
were enzymatically hydrolyzed by T. harzianum E58 cellulases.
Flasks were removed at 0, 2, 4, 8, 12, 24, 48 and 96 hours. The
flask contents were vacuum filtered and the residual substrate
collected. The supernate was assayed for liberated reducing
sugars, and glucose. The residual substrate was treated with
0.1% NaOH for 30 minutes to extract the adsorbed proteins. The
substrate was sequentially extracted with methanol and benzene,
air-dried, then stored in a dessicator. This substrate, termed
"spent cellulose" was investigated in Figures 26-29.

RESULTS AND DISCUSSION

I. ENZYMATIC HYDROLYSIS

A. Enzyme Assays

For the enzymatic process to be optimized, the process
parameters for the enzyme system must be defined. Both the
cellulase assays and hydrolysis of cellulose substrates are
performed under the same conditions of temperature and pH. Most
Trichodermal cellulases have a pH optimum of 4.5-5.5 and a
temperature optimum of 50-60°C (Coughlan, 1985). The first step
was to establish the optimum pH and temperature for detection of
cellulase activity in T. harzianum E58.

The components of the cellulase preparation were assayed and
found to have pH optima in the range 4.6-5.0 (Figure 5). As a
hydrolytic process involves an interaction of the enzyme
components, the pH optima for the complete enzyme system acting
on crystalline cellulose should also be determined. The pH of
the cellulase preparations were adjusted between pH 4.0-6.1, in
0.05 M sodium citrate buffer, and incubated with Solka Floc BW300
for 8 hours. Based on the maximum reducing sugars and glucose
yields obtained the optimum pH for hydrolysis was 4.8 (Figure 6).

The optimum temperature for the short-term assays was
determined to be 50°C for endoglucanase and filter paper activity
and 55°C for β-glucosidase (Figure 7). Cellulase activity is
traditionally measured by relatively short-term assays (30-60

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Figure 5.

Effect of pH on the endoglucanase (◊), β-glucosidase (▲) and filter paper (o) activities of T. harzianum E58.
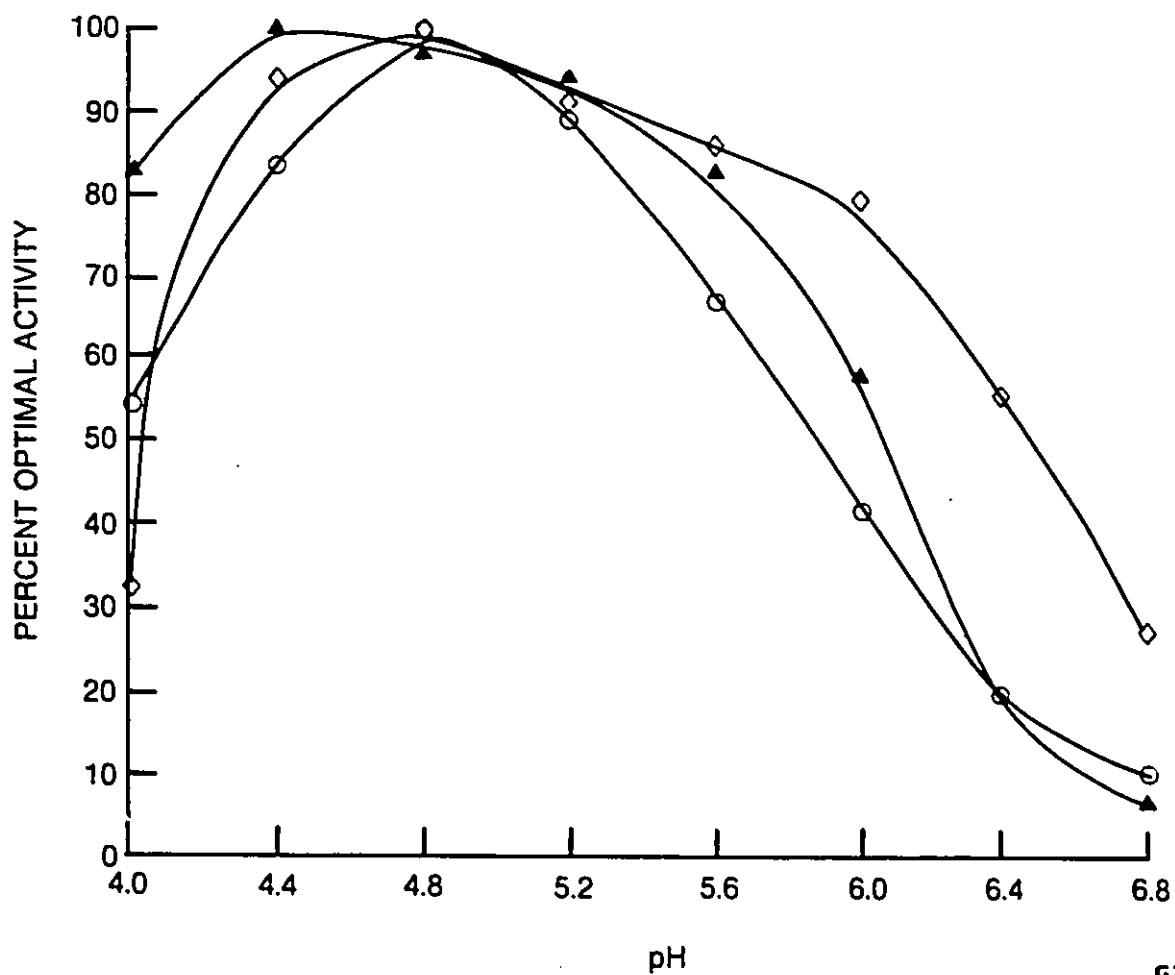
Enzyme activity (1U/mg protein) at
the optimal pH was:
Endoglucanase, 14.91
β-glucosidase, 0.84
Filter paper activity, 0.73

Incubation times of 30 minutes (endoglucanase and β-glucosidase) or 60 minutes (filter paper activity) were used for the assays.

Figure 6.

Effect of pH on the hydrolysis of a 5% (w/v) concentration of Solka Floc BW300 to reducing sugars (●) and glucose (■) after 8 hours incubation at 45°C.

Enzyme preparation from Figure 5 was used at an enzyme loading of 15 FPU/g substrate.

Figure 7.

Effect of temperature on the endoglucanase (◊), β-glucosidase (▲) and filter paper (○) activities of T. harzianum E58.
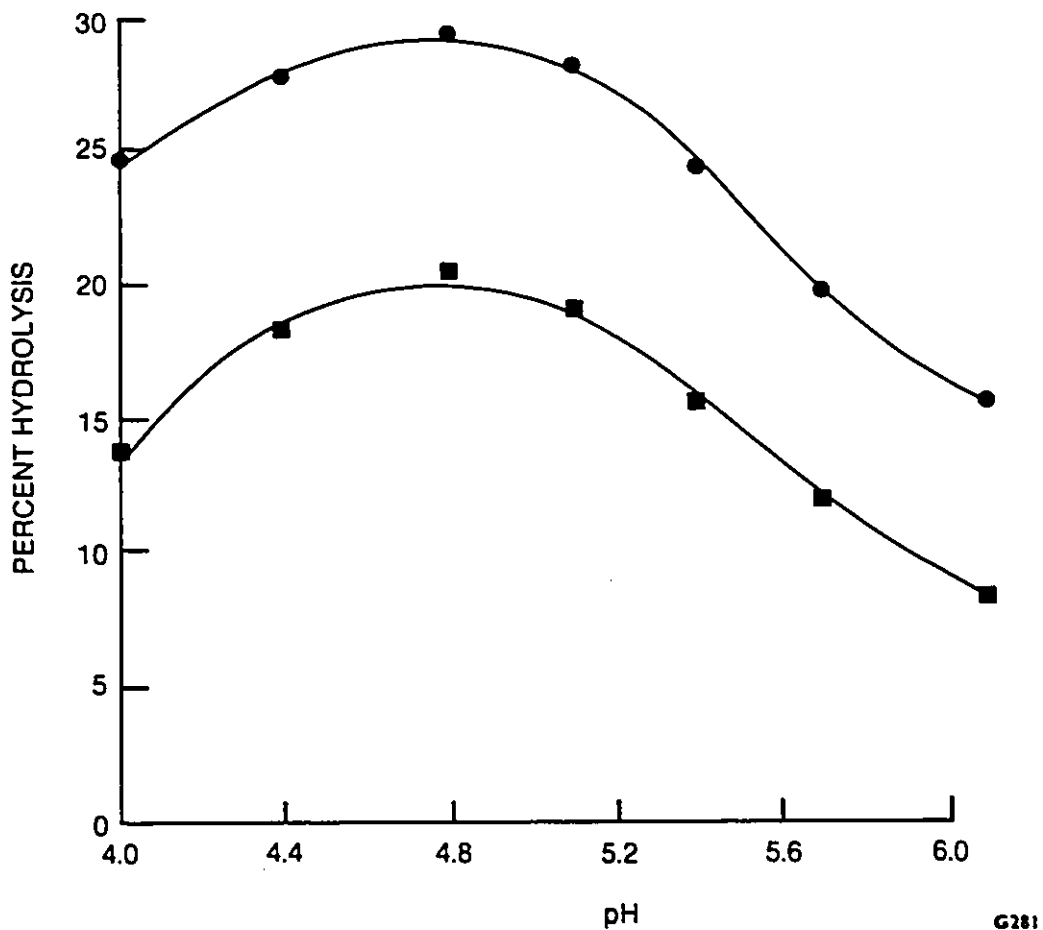

Enzyme activity (IU/mg protein) at the optimal temperature was:
Endoglucanase, 11.98
β-glucosidase, 0.66
Filter paper activity, 0.73

Incubation times of 30 minutes (endoglucanase and β-glucosidase) or 60 minutes (filter paper activity were used for the assays.

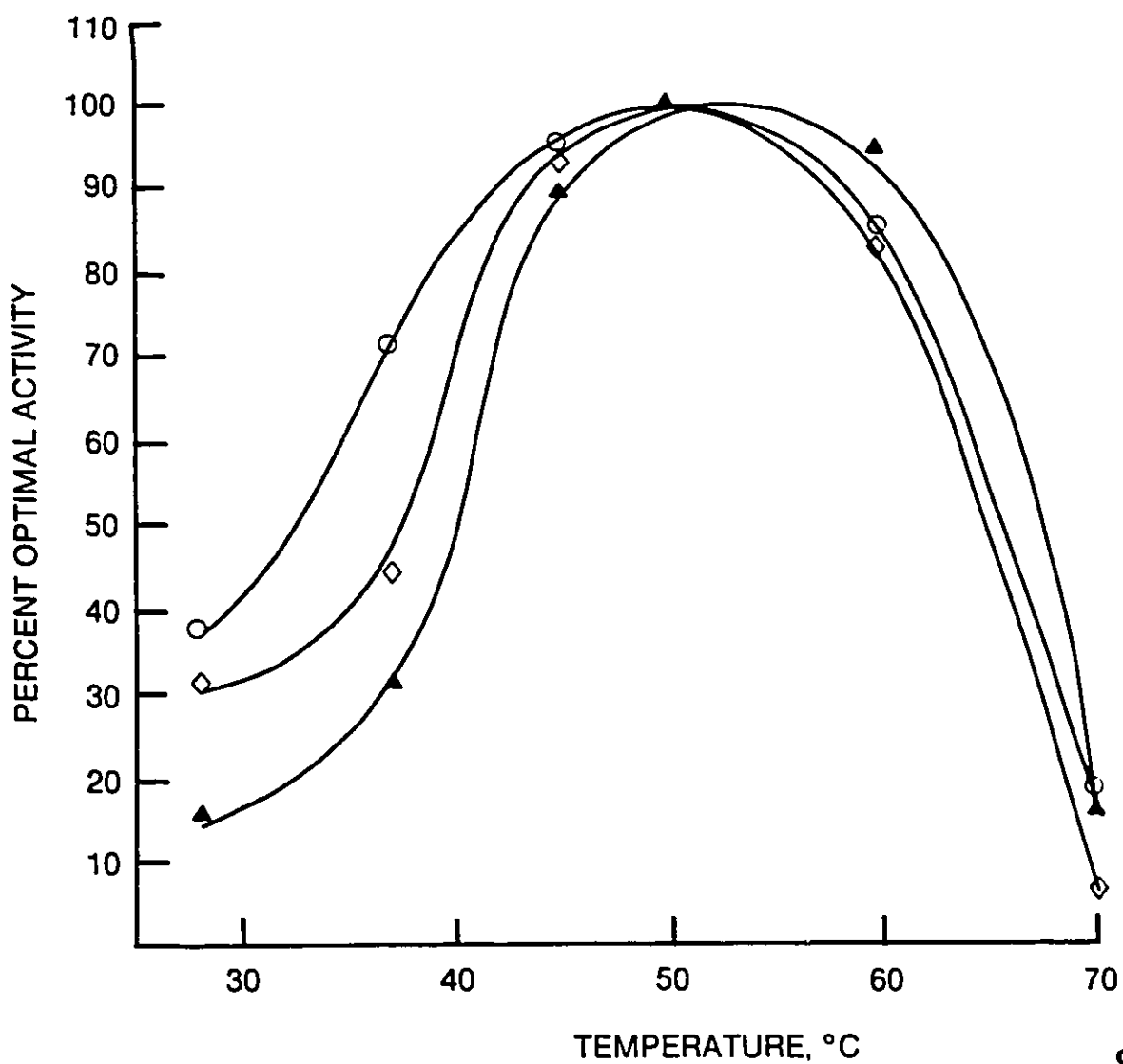minutes) using soluble or fairly amorphous substrates. These assays give a fast estimation for the activity of the individual components of the cellulase system and their concentration. Their major flaw is they can not be extrapolated to measure the ability of the enzymes to act against crystalline cellulose, which requires a synergistic interaction of the enzymes. Crystalline cellulose would be a more representative substrate to examine the phenomena of hydrolysis. Accordingly, a crystalline cellulose, Solka Floc BW300 was selected as the model cellulose substrate to study long-term hydrolysis.

Hydrolysis of crystalline material requires longer incubation times of 1 to 4 days. As the cellulases are fairly labile at high operating temperatures the thermal stability of the enzyme system is a prime consideration. The optimal temperature for enzyme activity must be balanced against greater inactivation at higher temperatures. The optimal temperature for long-term hydrolysis was determined by incubating the cellulases with cellulose at various temperatures and comparing the amount of hydrolysis at different times (Figure 8). Although higher temperatures initially resulted in higher sugar yields, as the incubation time increased the differences in hydrolysis for the different temperatures decreased. This agrees with the work of other researchers (Andren, 1978) who found as the time of saccharification increased the maximum extent of hydrolysis occured at lower temperatures. The best temperature for long-term hydrolysis appears to be 45°C, based on its fast initial rate combined with its maintenance of high yields throughout the process.
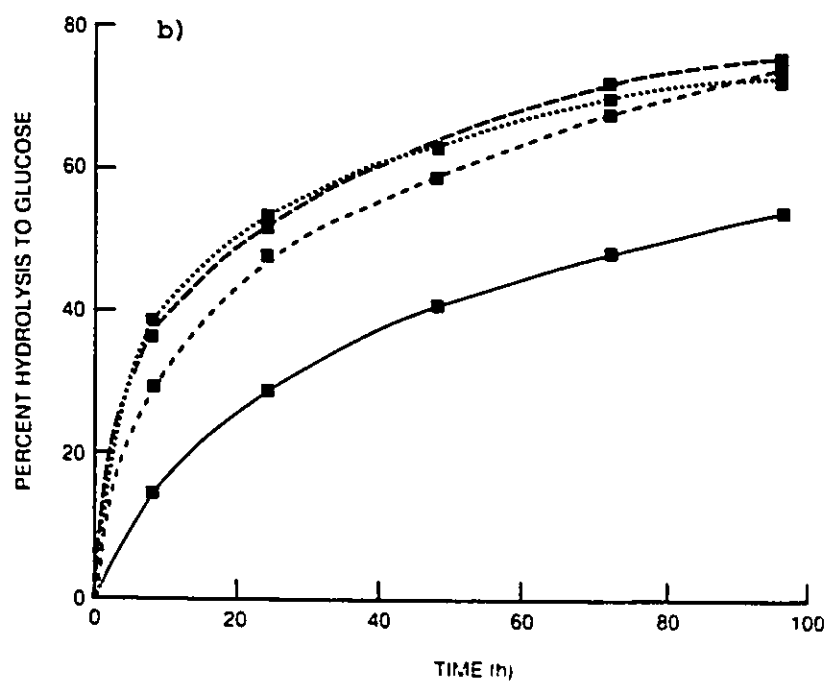
Figure 8.

Hydrolysis of a 2% (w/v) concentration of Solka Floc BW300 by T. harzianum E58 cellulases at 28 (——), 37 (---), 45 (—–) or 50 (·····)°C to a) reducing sugars and b) glucose.
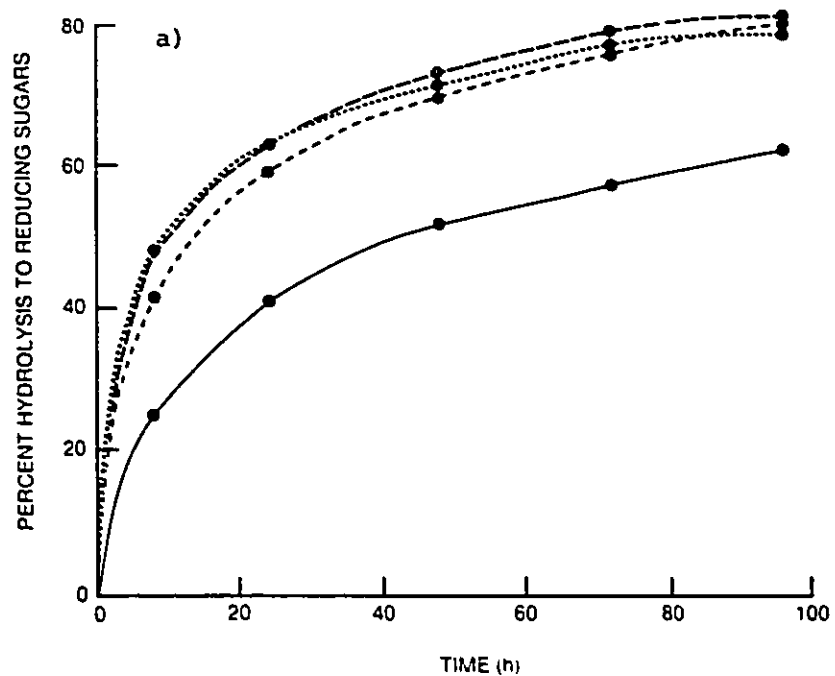
An enzyme loading of 30 FPU/g substrate was used.

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B. Initial Rate

Cellulose hydrolysis by cellulases is characterized by an initially rapid rate, which levels off quite drastically after about 8 hours. The initial rate and the extent of hydrolysis is strongly dependent on the susceptibility of the substrate, and the composition and concentration of the cellulase system. The hydrolysis profile of T. harzianum E58 on Solka Floc BW300 was examined to establish its pattern of degradation. Two different enzyme loadings were investigated and their effects on the initial rate and extent of hydrolysis were compared.

The initial hydrolysis rates were rapid, but these rates declined as hydrolysis proceeded (Figure 9). The initial rate of hydrolysis was higher with the higher enzyme loading, as was the levelling off value. A higher enzyme loading gives the system an added advantage against debilitating factors such as end-product inhibition and thermal inactivation which are serious limitations in long-term hydrolyses. The ratio of the initial rate at high loading to the initial rate at low loading was much greater than the corresponding ratio of final yields. This implies that the enzyme faces an increasingly resistant substrate as hydrolysis proceeds that imposes a limit on the ability of the enzyme to hydrolyze it.

Complete hydrolysis of the substrate was a prolonged process. Although the hydrolysis rate slowed down after 8 hours, there was a steady release of sugar throughout the 4 days of incubation at both enzyme loadings. As the objective of a

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Figure 9.

Hydrolysis profile of a 1% (w/v) concentration of Solka Floc BW300 incubated with T. harzianum E58 cellulase at enzyme loadings of 50 (——) or 10 (—–—) FPU/g.
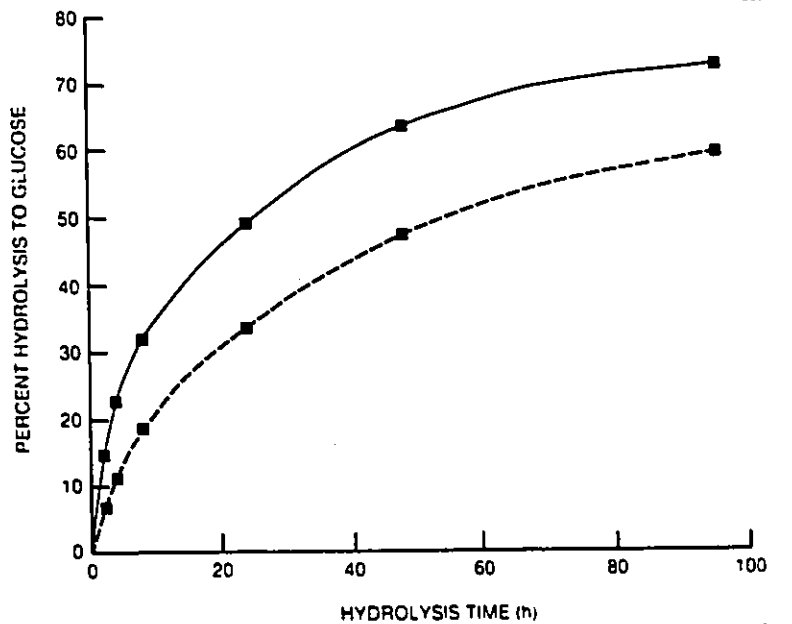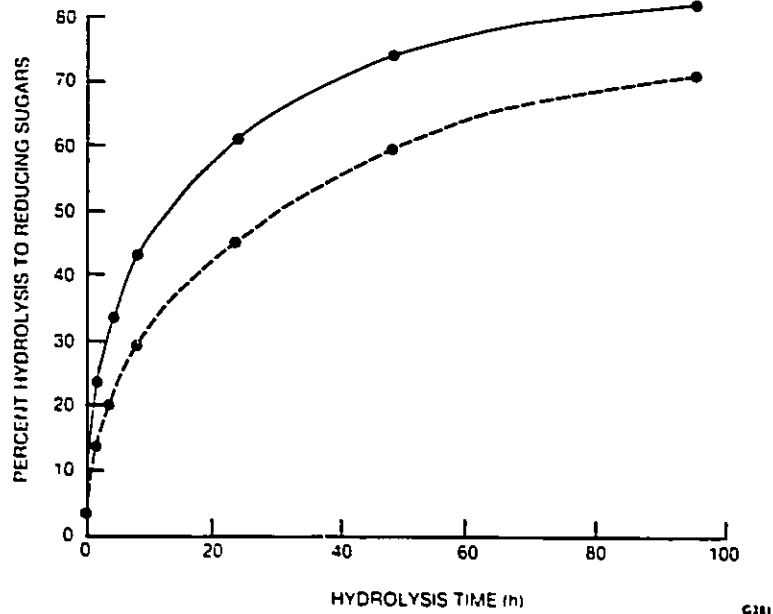
hydrolytic process is to obtain high (>85%) levels of conversion to glucose as quickly as possible, the region where the reaction slows down is of importance in hydrolysis. By defining the factors responsible for the decline in the rate it may be possible to reduce their effect.

C. Adsorption and Hydrolysis

Contact between the enzyme and the insoluble substrate is essential for hydrolysis. The first step in cellulose hydrolysis is adsorption of the enzyme onto the surface of the substrate (Lee and Fan, 1982). Once adsorbed, the enzyme can hydrolyze the substrate. The substrate has only a given number of binding sites available to the enzyme; so hydrolysis is at first limited by the amount of enzyme able to adsorb to the surface, or penetrate into the substrate. Although hydrolysis is routinely performed at pH 4.8 and 50°C, this may not correspond to the optimal conditions for adsorption. To study the relationship between adsorption and hydrolysis, the effect of pH and temperature on adsorption was determined. The pH in the range of 4.0-7.0 had no effect on the adsorption of endoglucanase, filter paper activity or protein (Figure 10).

Neither β-glucosidase nor the protein standard, bovine serum albumin (at 10mg/g cellulose), adsorbed to the substrate under the pH range studied (Data not given). Mandels et al. (1971) and Peitersen et al. (1977) reported the same observation using the T. reesei enzyme system.

Not all the added endoglucanase or filter paper activities

Figure 10.

Effect of pH on the adsorption of a) endoglucanase and b) filter paper activities and c) protein onto a 5% (w/v) concentration of Solka Floc BW300.
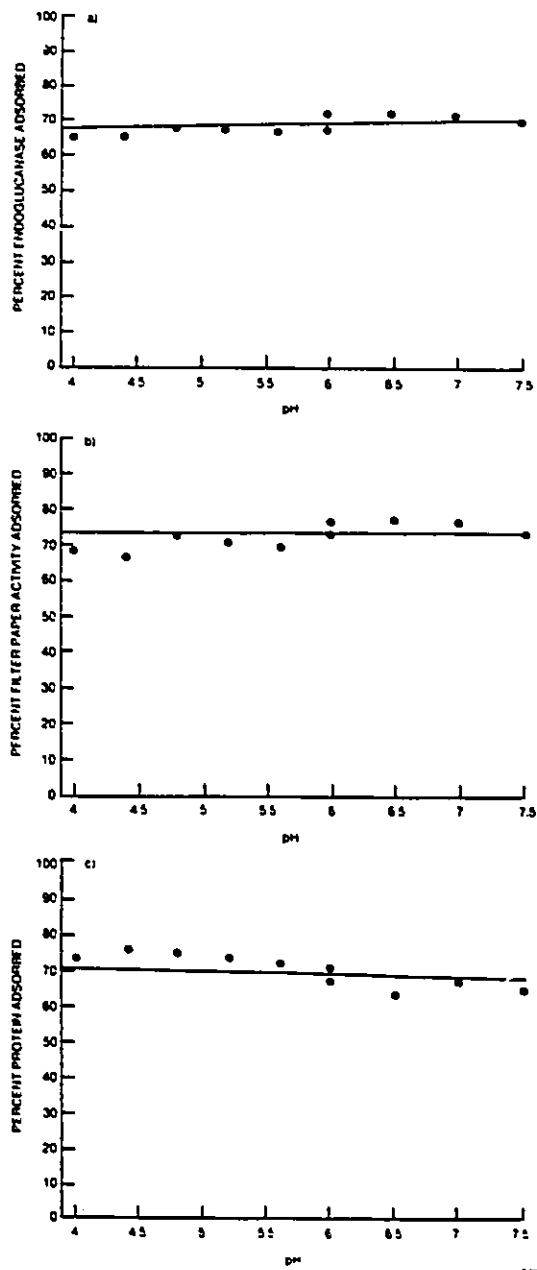
The enzyme loading for the pH range 3.0-6.0 was 11.5 FPU/g substrate.
The enzyme loading for the pH range 6.0-7.5 was 13.8 FPU/g substrate.

The % adsorption at a given pH was based on a pH (minus Solka Floc) control to account for any enzyme inactivation.

bound to the substrate, although low enzyme concentrations were used to avoid saturation of the substrate. Approximately 35 percent of the endoglucanase remained in solution. In general Trichoderma spp. secrete multiple forms of endoglucanase. Beldman et al. (1987), Rabinovich et al (1983) and Ryu et al. (1984) reported a wide variation in the affinity of the different forms of the enzyme towards cellulose, with some components non-adsorbable. Rabinovich and co-workers (1983) suggest the function of the low-affinity, unbound endoglucanase is to act on the soluble, intermediate hydrolysis products of cellulose.

The effect of temperature on adsorption of cellulases derived from T. harzianum E58 onto Solka Floc BW300 was investigated (Figure 11). There was a slight desorption of the endoglucanase at higher temperatures, probably due to hydrolysis of the substrate concomitant with enzyme release. Filter paper activity also exhibited a slight drop in adsorption with increasing temperature. Adsorption onto cellulosic substrates was reported to be only slightly affected by variations in the temperature from 0-60°C (Goel and Ramachandren, 1983; Mandels et al., 1971; Peiterson et al, 1977). Certainly, the slight changes noted in cellulase adsorption with increasing temperature can not be compared to the drastic increase in activity.

Previous workers have found hydrolysis of the substrate is a major problem in studying adsorption at higher temperatures. Hydrolysis of the substrate alters its structural properties, and interferes with the assessment of cellulase adsorption. The problem of hydrolysis can be reduced by using lower incubation temperatures when examining adsorption. A temperature of 28°C

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Figure 11.

Effect of temperature on the adsorption of endoglucanase (◊), and filter paper (○) activities and protein (□) onto a 5% (w/v) concentration of Solka Floc BW300.

TEMPERATURE, °C
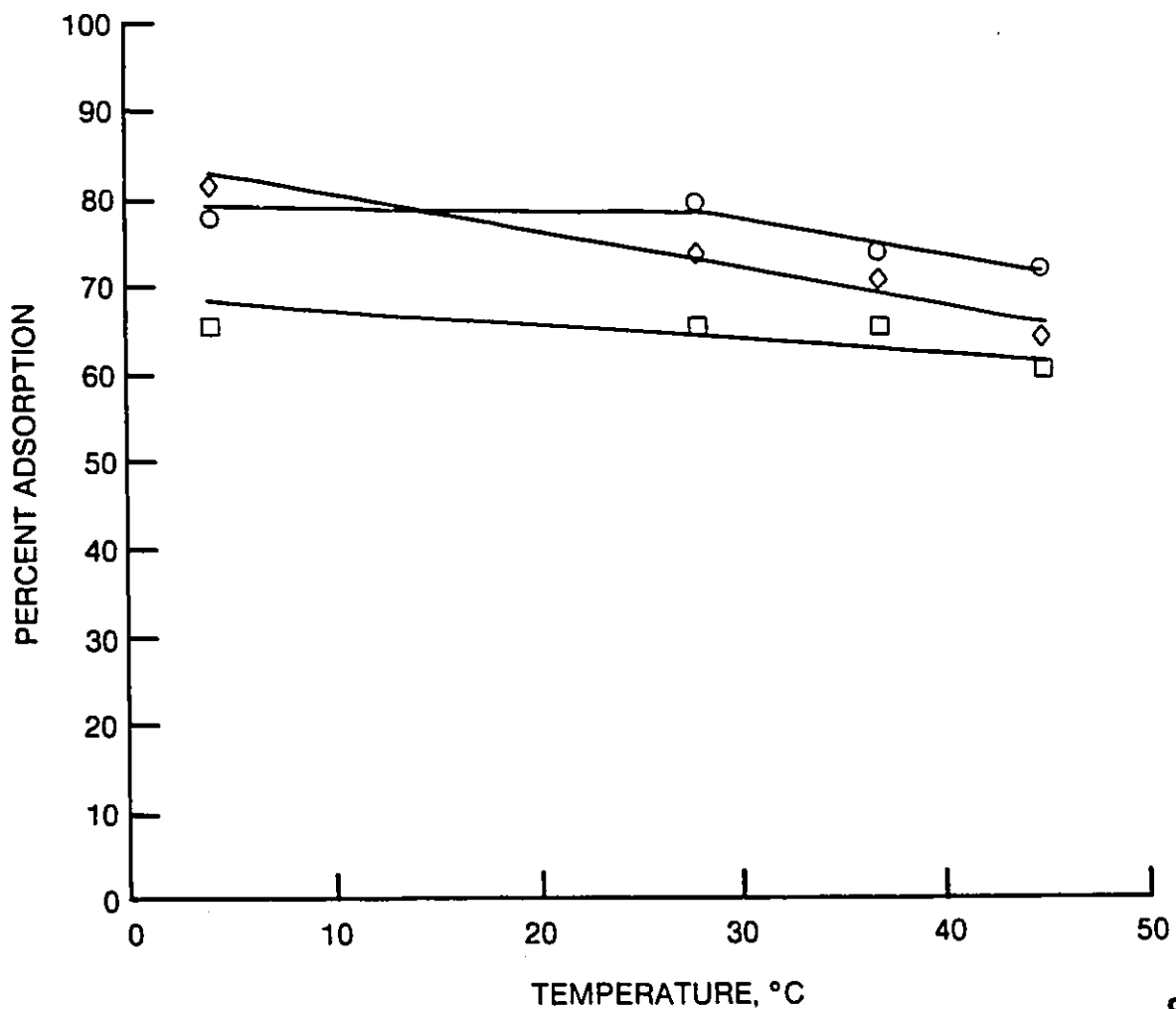
G281

Enzyme activity (IU/mg protein) profile was:
Endoglucanase, 16.21
β-glucosidase, 0.61
Filter paper activity, 0.90
Enzyme loading was 17.6 FPU/g substrate

Incubation time for the adsorption study was 10 minutes.
Thermal controls (minus substrate) were run in parallel to the test systems. No enzyme inactivation was noted for the above temperature range.

was sufficiently low to avoid hydrolysis of the substrate, and so was used throughout this thesis to examine adsorption.

Neither β-glucosidase nor the protein standard, bovine serum albumin (at 10 mg/g cellulose) adsorbed to the substrate in the temperature range examined. Bovine serum albumin, and the proteases, trypsin and chymotrypsin when incubated with Solka Floc BW300 at 4°C did not adsorb (Data not given). This suggests the substrate is specific as to the nature of the molecules able to bind to it.

The optimal temperature and pH range for adsorption was much broader than for hydrolysis, with high levels of cellulase adsorption onto the substrate occuring at the optimal conditions for hydrolysis. Although an enzyme has to be adsorbed to the substrate for it to effect hydrolysis, this does not guarantee that hydrolysis will occur. The appropriate pH (i.e. 4.8) and thermal (i.e. 37-55°C) conditions must be present for catalysis of the substrate to occur.

The substrate appeared to specific as to the nature of molecule able to bind to it; only enzymes acting on the insoluble substrate could be adsorbed.

Adsorption does not limit hydrolysis, as the optimal conditions for hydrolysis lie within the range for optimal adsorption of the cellulases. Hydrolysis, however, can interfere with the assessment of cellulase adsorption. Based on the above results it was decided to use pH 4.8 to study hydrolysis and adsorption – a pH optimal for both processes. Further hydrolyses were run at 45°C, the optimal temperature for a long-term

reaction. Alternately, adsorption studies were carried out at 28°C to avoid alteration of the substrate due to hydrolysis.

D. Enzyme Loading

A hydrolysis curve for T. harzianum E58 cellulases at various enzyme loadings was established using Solka Floc BW300 (Figure 12). The amount of hydrolysis increased with the enzyme-loading, so that at sufficiently high enzyme levels hydrolysis was virtually complete. Glucose yields were on average only 10% less than the reducing sugar yields, which is high in relation to other Trichoderma species (Saddler et al., 1985). This is due to the high levels of extracellular β-glucosidase produced by T. harzianum E58. This suggests that supplemental β-glucosidase may not be necessary in processes converting high levels of cellulose to glucose using T. harzianum E58 cellulases.

The prime objective of a cellulose-to-glucose process is to obtain high conversion rates using the the least amount of enzyme as possible. As seen in Figure 12, at low enzyme loadings the amount of hydrolysis obtained per filter paper unit was high. At enzyme loadings over 50 FPU/g little additional hydrolysis was achieved with additional enzyme. The maximum amount of enzyme that should be used for hydrolysis appeared to lie roughly in the range of 20-50 FPU/gram.

Why did enzyme loadings over 50 FPU only slightly enhance hydrolysis when there was plenty of substrate left for the enzyme to attack? One possible answer was there were no binding sites available on the substrate. As illustrated in Figure 13, there

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Figure 12.

Effect of enzyme loading on the hydrolysis of a 2% (w/v) concentration of
Solka Floc BW300 to reducing sugars (●) and glucose (■) after 4 days
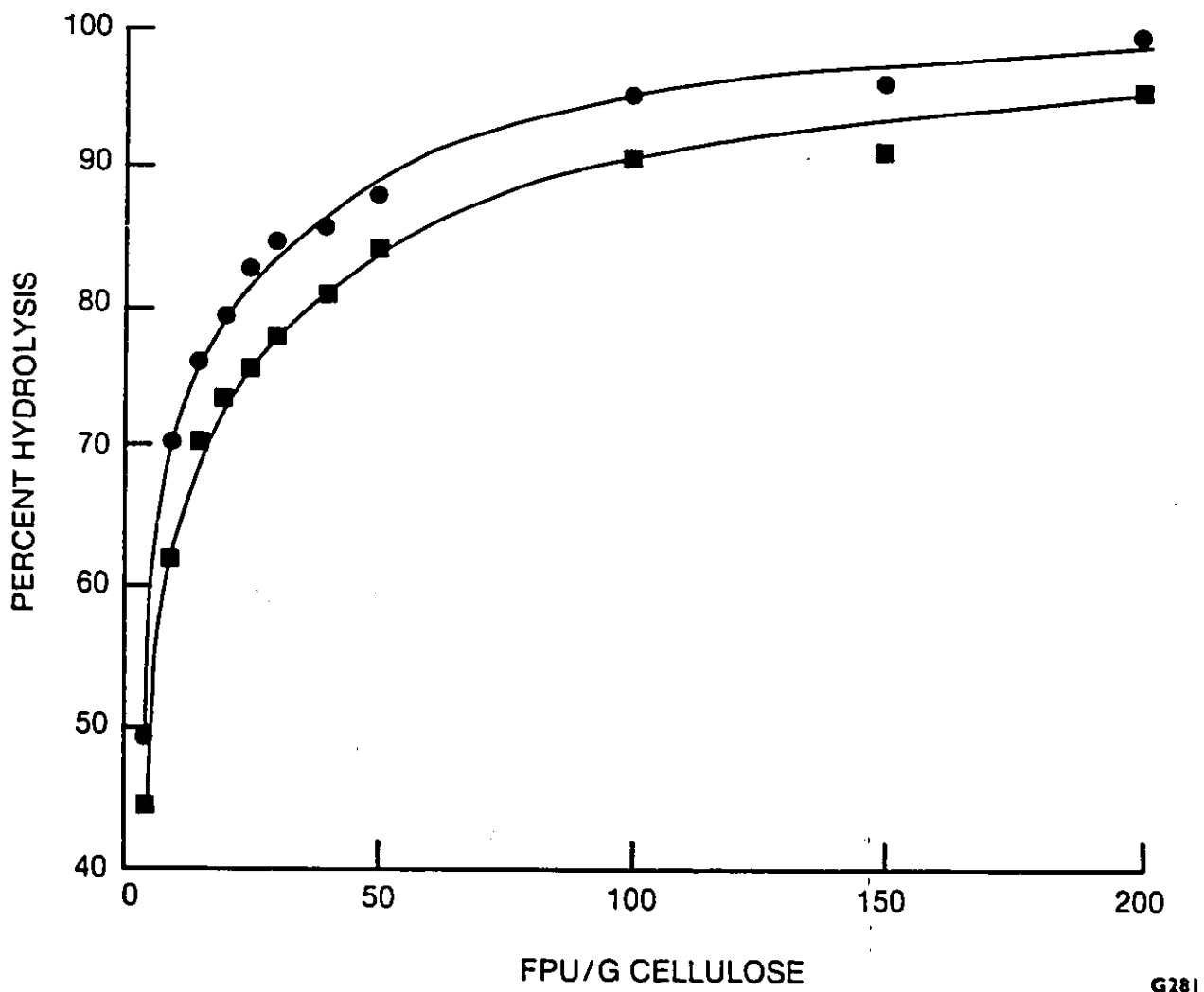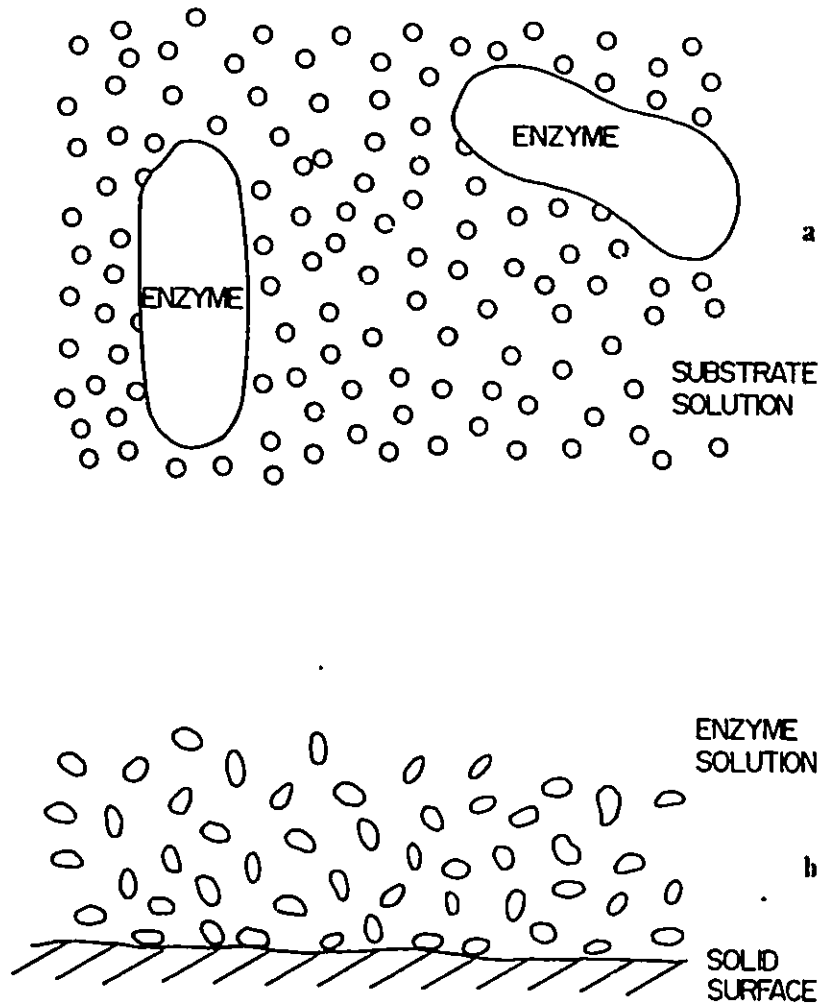incubation at 45°C.

Figure 13.

Pictorial representation when a) substrate (soluble)>>enzyme (soluble) and b) enzyme>>accessible substrate.

Gilbert and Tsao, 1985

are a limited number of sites accessible to the enzyme at a given

time due to the insoluble, crystalline nature of the substrate

(Lee and Fan, 1983; Gilbert and Tsao, 1985). Once these sites

are filled the rest of the enzyme remains in solution, until an

accessible site is available. The most efficient amount of

enzyme to add for hydrolysis would be the concentration at which

all the sites accessible to enzymatic attack are filled with

enzyme. The above data suggested the substrate may be saturated

with enzymes at an enzyme loading between 20-50 FPU/gram. Enzyme

adsorption may be a means of determining the optimal enzyme

loading for hydrolysis.

E. Substrate Saturation

The critical problem in relating adsorption to hydrolysis is

the difference in the nature of the processes. In adsorption one

captures the substrate at a given moment and examines it; while

in hydrolysis one is looking at a continual process.

The first step to examine the relationship between the

amount of enzyme adsorbed to the substrate, and the hydrolysis of

the substrate is to study the initial adsorption. The capacity

of the model substrate, Solka Floc BW300, to adsorb T. harzianum

E58 cellulases was determined and compared with its hydrolysis at

a range of enzyme loadings (Figure 14). The amount of adsorbed

filter paper activity and protein increased with added enzyme up

to 25 filter paper units (FPU). After this, the amount of

adsorbed protein and filter paper activity levelled off. In

parallel to this, hydrolysis of the substrate to reducing sugars

increased up to 25 FPU, above which the amount of sugars released

70

Figure 14.

Relation between a) the amount of enzyme adsorbed onto 5% (w/v) Solka Floc
BW300 and b) its hydrolysis to reducing sugars (●) and glucose (■).
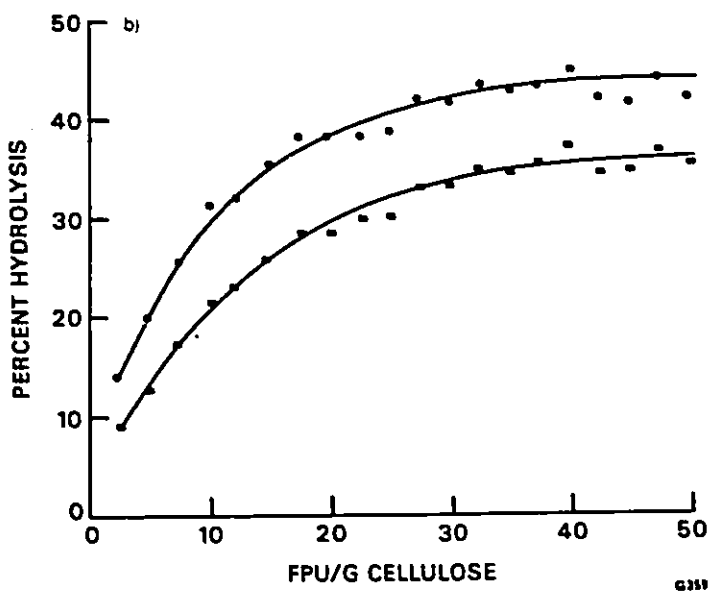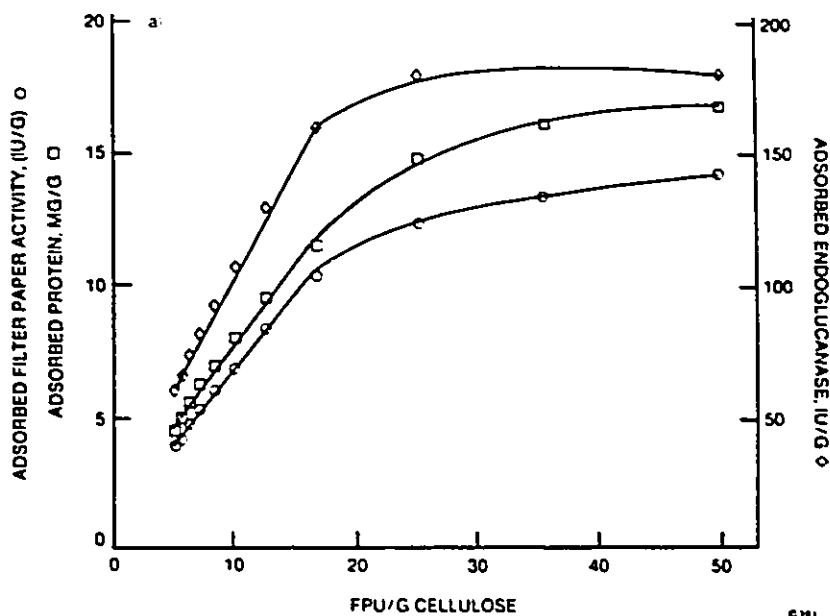
Enzyme activity (IU/mg protein) profile was:
Endoglucanase, 14.51
β-glucosidase, 0.55
Filter paper activity, 0.68

Incubation time for the adsorption study was 10 minutes.
Incubation time for hydrolysis was 8 h.

with additional enzyme started to level off. The enzyme loading

at which saturation of the substrate occurs appears to correlate

with the optimum loading for hydrolysis. These results are in

accord with those of other researchers, who have demonstrated a

relationship between the amount of cellulase enzyme initially

adsorbed to the substrate, and the hydrolysis of the substrate.

Lee et al. (1982) reported that the adsorption of cellulases onto

cellulose is one of the most important parameters governing the

enzymatic rate of hydrolysis. Similarly, Wald and co-workers

(1984) found glucose yield reached a maximum when the substrate

became saturated with enzyme. Additional enzyme did not increase

the yield, as the adsorption process was controlling the overall

rate of hydrolysis.

F. Role of Initially Adsorbed Cellulases in Hydrolysis

Can initial enzyme adsorption be used to determine the

miniumum amount of enzyme needed for hydrolysis? To do so it

must be established that the initially adsorbed enzyme has a

strong role in the overall hydrolytic process, and is not just

representative of the initial phase.

Mandels et al. (1971) found that the enzyme adsorbed

initially was sufficient to digest cellulose with no

replenishment of enzyme, even though the liquid phase containing

the sugars was continually removed during hydrolysis. Similarly,

Lee and Fan (1983) obtained comparable sugar yields after either

incubating the substrate with the same enzymes for the entire

process or removing the liquid phase after one hour and replacing

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it with buffer.

In the work cited above the researchers removed the liquid phase and replaced it with buffer, thereby removing the β-glucosidase along with the unadsorbed cellulases. The ability of the bound cellulases to promote hydrolysis could be handicapped by cellobiose inhibition (Ladisch et al., 1983). To measure the extent to which the initially bound cellulases control hydrolysis, cellobiose inhibition should be removed by supplementing the system with β-glucosidase. For comparative purposes, a β-glucosidase loading equal to the control should be added.

The role of the cellulases initially adsorbed to the substrate in effecting hydrolysis was examined. An enzyme loading of 26 FPU/gram cellulose was used, as this appeared to correspond to the maximum protein adsorption level for the substrate as indicated by Figure 14. T. harzianum E58 cellulases were incubated at 4°C for one hour with Solka Floc BW300 to allow maximum enzyme adsorption (See Materials and Methods; Initially Adsorbed Cellulases). After removal of the liquid phase the substrate was washed to remove unadsorbed proteins, then lyophilized. Part of the enzyme-substrate complex was resuspended in buffer; the other part was resuspended in buffer and supplemented with purified β-glucosidase at levels corresponding to those of the original mixture. A control containing the original enzyme activity and substrate was also prepared.

As shown in Figure 15 the reducing sugars and glucose released by the adsorbed cellulases supplemented with β-

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Figure 15.

Hydrolysis of Solka Floc BW300 by adsorbed cellulases after removal of the unadsorbed protein remaining in solution: control (——); supernatant removed and replaced with buffer (——); supernatant removed, replaced with buffer and supplemented with purified β-glucosidase (---).

G231
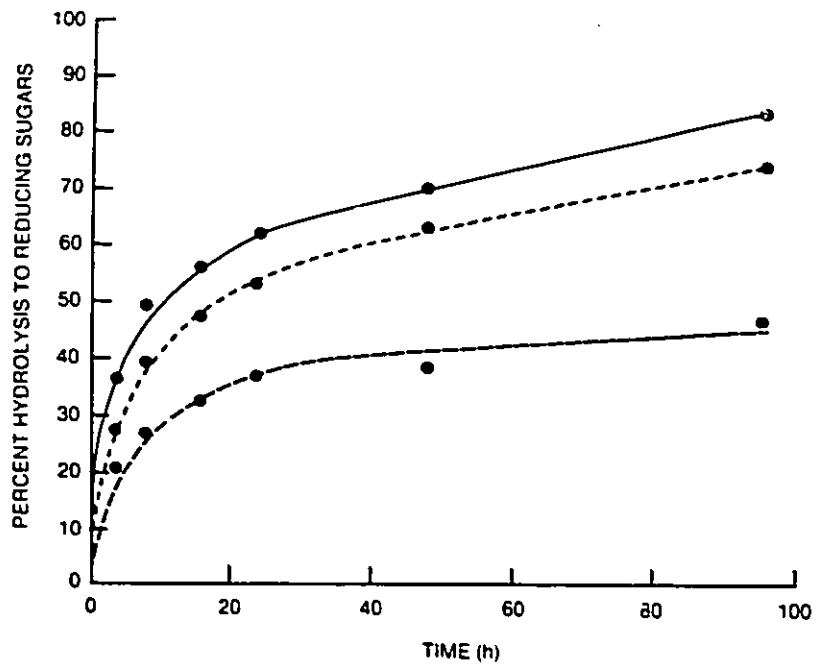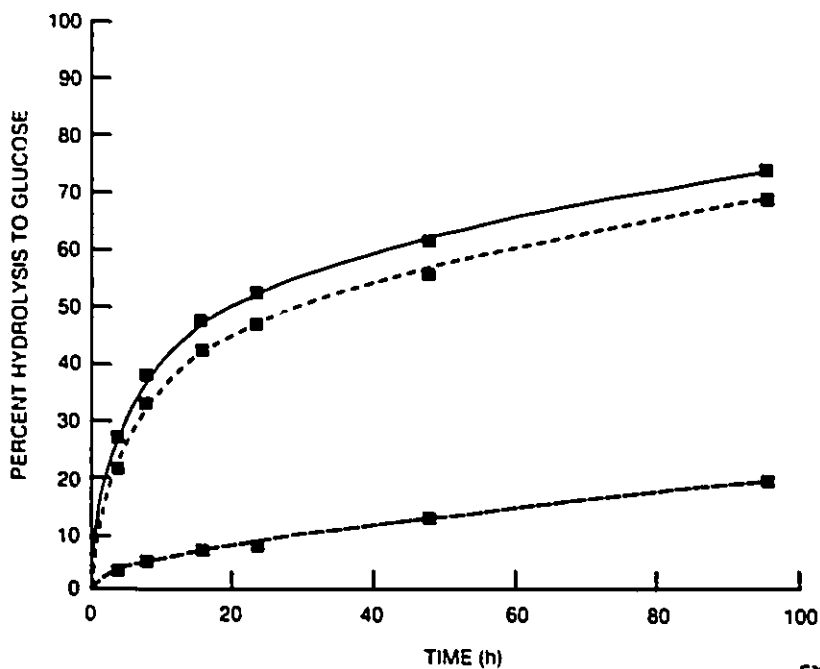
G231

Enzyme loading of 25.6 FPU/g substrate was used.

The enzyme activity (IU/mg protein) profile of the T. harzianum E58 cellulase preparation was:
Endoglucanase, 15.60
β-glucosidase, 0.56
Filter paper activity, 0.79

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glucosidase was roughly 90% of that obtained by the control. The reducing sugars and glucose yields obtained by the adsorbed cellulases-alone were only 50 and 25% respectively of the control. As no β-glucosidase was present in the latter system the low sugars yields are largely due to end-product inhibition.

It was very interesting to note that the cellulases which initially adsorbed to the substrate were able to hydrolyze the substrate to an extent comparable to the control. The enzymes that do not bind apparently have only a minor role in the hydrolysis of the substrate, or are in excess. This established that the enzymes initially adsorbed to the substrate were primarily responsible for its hydrolysis. The minimal enzyme requirements for effective hydrolysis may then be determined by examining the adsorption of the cellulases to the substrate to be hydrolyzed.

G. Substrate Saturation/Langmuir Adsorption Isotherm

A potential problem in using adsorbed enzymes to determine optimal enzyme loading for hydrolysis is the heterogeneous nature of the cellulase system. The components of the cellulase complex are adsorbed in different proportions to the substrate, and have different affinities towards it. For hydrolysis to be feasible, however, all the components of the cellulase system must be present.

Tan and co-workers (1986) have investigated the enzyme system of T. harzianum E58, and studied its pattern of adsorption onto Solka Floc BW300. They found the major enzyme secreted by

this fungus is exoglucanase. In addition, this enzyme has a stronger affinity for the cellulose, than does the endoglucanases. Using culture filtrate the majority of the adsorbed proteins were the exoglucanases.

Purified exoglucanase, derived from T. harzianum E58, was used as a binding probe to determine the substrate saturation level (See Materials and Method; Purified Exoglucanase). Varying concentrations of purified exoglucanases were added to Solka Floc BW300, and the amount of adsorbed protein determined (Figure 16). Adsorption increased with added protein up to 15 mg adsorbed protein/g substrate, after which little additional protein was adsorbed. The substrate is saturated with enzyme at this point, any excess enzyme added either remains in solution or is only loosely associated with the substrate.

The adsorption behaviour obeys the Langmuir Adsorption Isotherm, with the amount of enzyme adsorbed increasing with the amount of enzyme added. As a homogeneous macromolecule and purified cellulose were used, the Langmuir Adsorption Isotherm Equation could be applied to predict the maximum amount of protein able to adsorb to the substrate, and the affinity of the enzyme towards the substrate (Appendix A). According to the Langmuir Adsorption equation, the inverse of the free enzyme plotted against the inverse of the adsorbed enzyme will give a measure of the maximum amount of enzyme able to adsorb to the substrate (1/b) and the affinity of the enzyme towards the substrate. The maximum amount of exoglucanase able to adsorb was calculated as 14.4 mg/g substrate. This corresponds to an enzyme loading of 35 mg protein per gram Solka Floc BW300.

Figure 16.

Adsorption of varying loadings of purified exoglucanase, derived from T. harzianum E58, onto Solka Floc BW300.
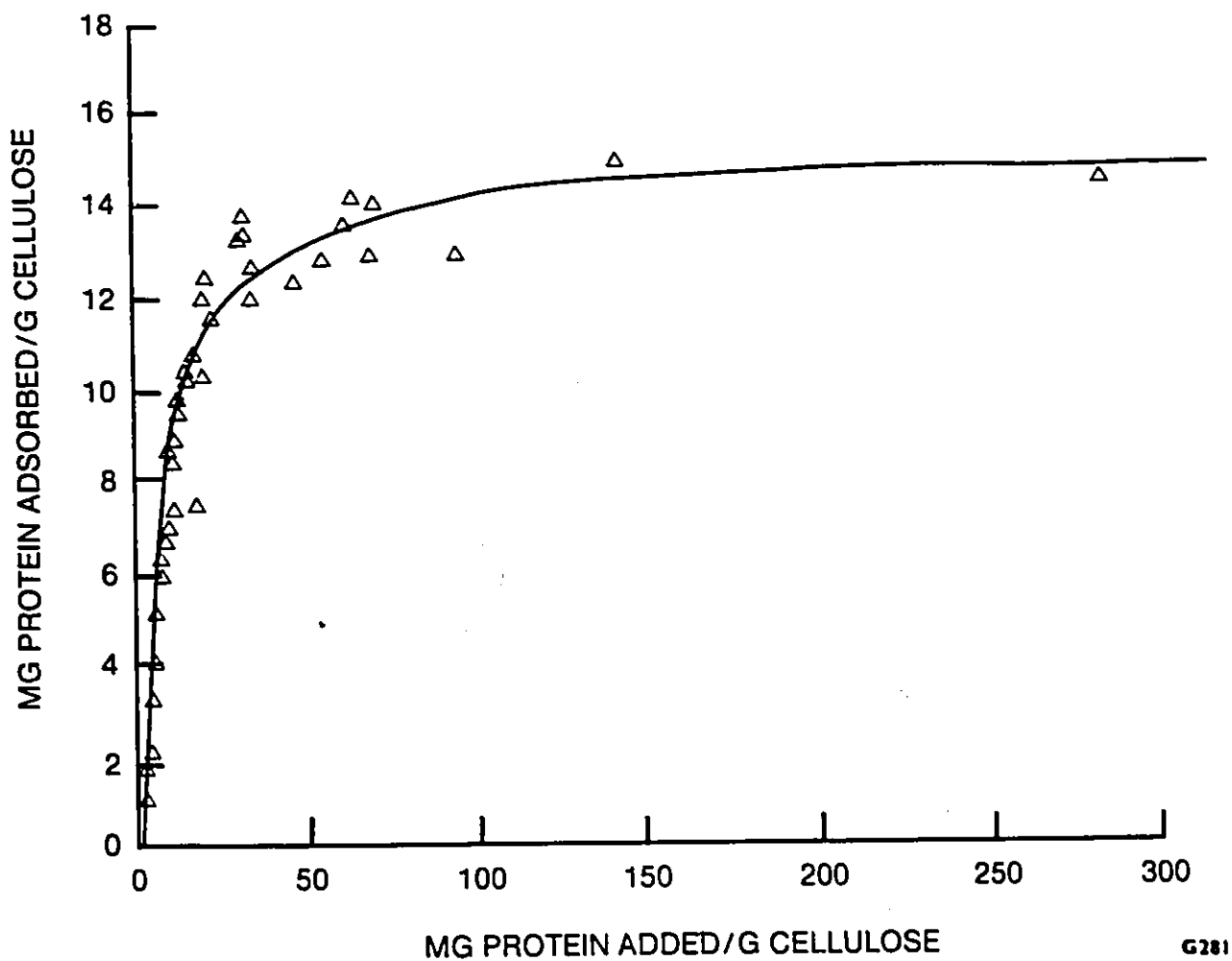
Incubation time for the adsorption study was 10 minutes.

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The relationship between the amount of purified exoglucanase adsorbed to the amount of protein adsorbed in the culture filtrate was examined. In Figure 14 varying concentrations of culture filtrate were added to the Solka Floc BW300, and the amounts of adsorbed and unadsorbed proteins determined. These values were plotted using the Langmuir Isotherm Equation, and it was determined that the maximum amount of protein able to be adsorbed was 15.1 mg/g substrate. This value is comparable to that obtained with the purified exoglucanase, suggesting that the maximum amount of enzyme able to adsorb to the substrate could be determined by examining protein adsorption from the culture filtrate.

To determine the minimum amount of enzyme needed for efficient hydrolysis the saturation level of the adsorbed protein (15.1 mg/g substrate) must be correlated with the total amount of protein added. The amount of enzyme which bound at saturation corresponded to an enzyme loading of 23 filter paper units per gram cellulose.

Using T. harzianum E58 cellulases in combination with the standard cellulose substrate, Solka Floc BW300 it has been established that: a) a substrate has a limited number of binding sites for the cellulases to adsorb to in order to effect hydrolysis; b) the cellulases initially adsorbed to the substrate have a major role in governing hydrolysis; c) the enzymes that are not adsorbed in the initial phase of hydrolysis have only a minor role in the hydrolysis of the substrate.

The enzyme loading where the substrate is saturated with

enzyme is evidently the most economical enzyme concentration to use for hydrolysis. With Solka Floc BW300 the optimal enzyme loading was determined to be 20-25 FPU/gram.

II. ENZYME RELATED FACTORS LIMITING HYDROLYSIS

A. End-product Inhibition

End-product inhibition is a serious limitation in the hydrolytic process and adequate β-glucosidase levels are essential to good hydrolysis. The cellulases and β-glucosidase in most Trichoderma spp. are strongly inhibited by cellobiose and glucose, respectively (Ladisch et al., 1983). There are a number of ways to circumvent the problem of end-product inhibition. Supplemental β-glucosidase derived from other sources have been shown to alleviate glucose inhibition and increase the efficiency of the process (Bissett and Sternberg, 1978; Sternberg et al., 1977). Most commercial enzyme preparations require the addition of β-glucosidase for effective glucose production. The use of commercial enzymes would add to the expense of the process. T. harzianum E58 is a high producer of β-glucosidase and may be able to achieve high hydrolysis without being handicapped by end-product inhibition.

Using the previously determined "optimal" filter-paper loading of 25 FPU/gram substrate, the effect of supplemental β-glucosidase on hydrolysis was studied. β-Glucosidase, purified from the culture filtrate of T. harzianum E58 (Tan et al., 1987) was used as the source of supplemental β-glucosidase. Varying concentrations of the purified β-glucosidase were combined with a T. harzianum E58 enzyme preparation and incubated with Solka Floc BW300 for a 4 day period (Figure 17). After a 24 hour incubation, hydrolysis to glucose was enhanced 10% by the addition of 4 IU

Figure 17.

Effect of supplemental β-glucosidase purified from _T. harzianum_ E58 on the long-term hydrolysis of a 2% (w/v) concentration of Solka Floc BW300 to a) reducing sugars and b) glucose.
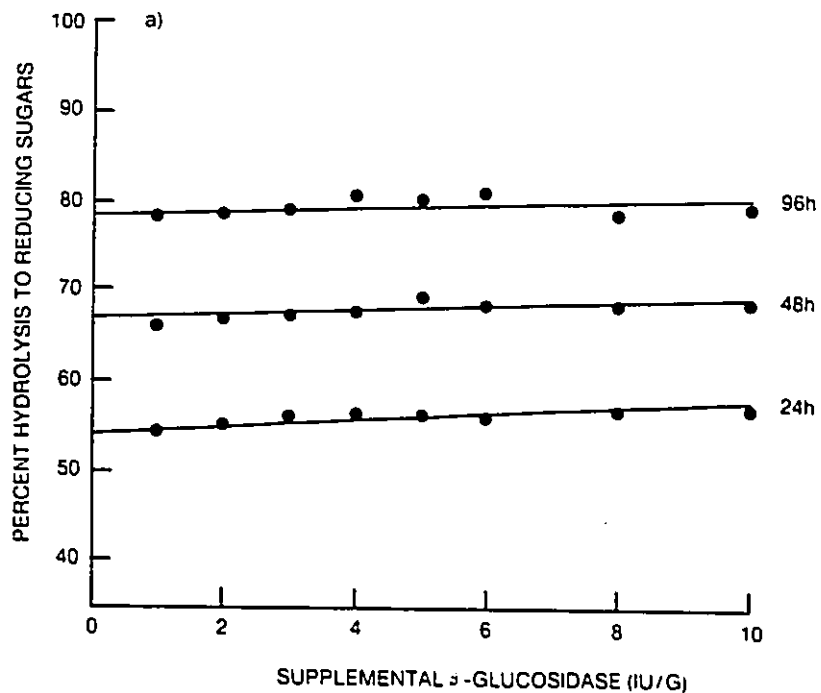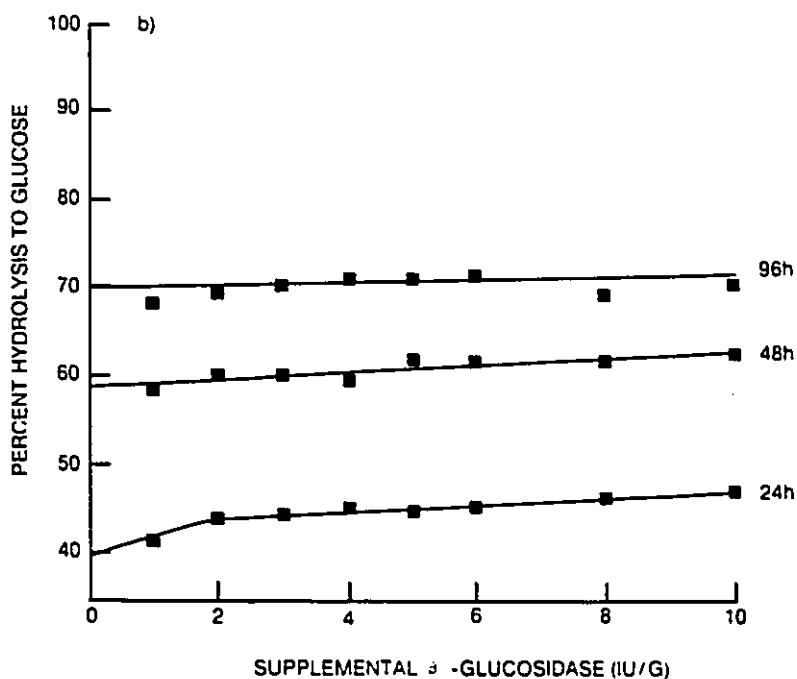
Enzyme activity (IU/mg protein) profile for the _T. harzianum_ E58 cellulase preparation was:
Endoglucanase, 19.07
β-glucosidase, 0.55
Filter paper activity, 0.85
Enzyme loading was 25.7 FPU/g substrate

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β-glucosidase/g substrate. Additional β-glucosidase had little effect on hydrolysis. After 48 hours the beneficial effects of supplemental β-glucosidase were slight. Evidently, the β-glucosidase of T. harzianum E58 is present in sufficiently high quantities to handle end-product inhibition for long-term hydrolysis. The enzyme activity of the control (no supplemental β-glucosidase) was 25.7 FPU and 16.5 IU β-glucosidase per gram substrate. For this enzyme system, acting on 2% Solka Floc, a filter paper activity to β-glucosidase ratio of 1.6 FPU: 1.0 IU β-glucosidase is sufficient to cope with end-product inhibition.

B. Combined Hydrolysis and Fermentation

β-Glucosidase, in converting the cellobiose to glucose, removes the more potent inhibitor of the exo- and endocellulases. Glucose is still present, in increasingly greater amounts, as hydrolysis proceeds. Glucose is also an inhibitor of the cellulases, although at a much reduced level (Sternberg et al., 1977; Bissett and Sternberg, 1978; Hsu et al., 1980).

To avoid the problem of glucose or cellobiose inhibition while examining hydrolysis a combined hydrolysis and fermentation (CHF) process can be used. The glucose is continuously removed from the reaction mixture by fermentation to ethanol, greatly stimulating the hydrolysis of the cellulose (Takagi et al., 1977; Ghosh et al., 1982; Saddler et al., 1982). Since end-product inhibition is virtually eliminated, the unhindered action of the endoglucanase and exoglucanase on the substrate can be assessed.

Enzyme loadings ranging from 0-30 FPU/gram cellulose were

examined. The filter paper to β-glucosidase ratio of the
cellulase preparation was 1.0 FPU:1.0 IU β-glucosidase. In one
system the yeast, Saccharomyces cerevisiae, was added
concurrently with the cellulases, and the fermentation vials
incubated at 37°C. A hydrolysis-only system was run in parallel
at 37°C to observe the extent to which glucose inhibition limits
the reaction (Figure 18).

In the hydrolysis-only systems, the amount of sugar released
increased with the enzyme loading for the entire range studied.
The amount of sugars released per enzyme unit was greater in the
CHF systems, especially at the lower enzyme loadings. As the
enzyme loading increased above 20 FPU the differences in the
sugar yield between the CHF and hydrolysis-only systems narrowed.
Twenty to twenty-five FPU has been estimated as the minimal value
to fulfill the endoglucanase and exoglucanase requirements for
efficient hydrolysis, so hydrolysis is not limited by
insufficient cellulases. In the hydrolysis-only system it
appears that there is insufficient β-glucosidase at an enzyme
loading of 20-30 FPU; enhanced hydrolysis at the higher enzyme
loadings is due mainly to the alleviation of inhibition by the
additional β-glucosidase. The β-glucosidase required to avoid
end-product inhibition is higher than estimated in the above
section, with the optimal ratio being 1 FPU:1 IU β-glucosidase.
In this case hydrolysis was run at 37°C, rather than 45°C,
lowering the activity of the β-glucosidase (Figure 7) and
increasing the amount of enzyme required.

In the CHF systems the amount of hydrolysis reached a
plateau at an enzyme loading of 15-20 FPU per gram of substrate

Figure 18.

Comparison of a combined hydrolysis and fermentation process (■) and hydrolysis process (●) on the degradation of a 5% (w/v) concentration of Solka Floc BW300 at day 2 (——) and day 4 (—·—) using a range of enzyme loadings.
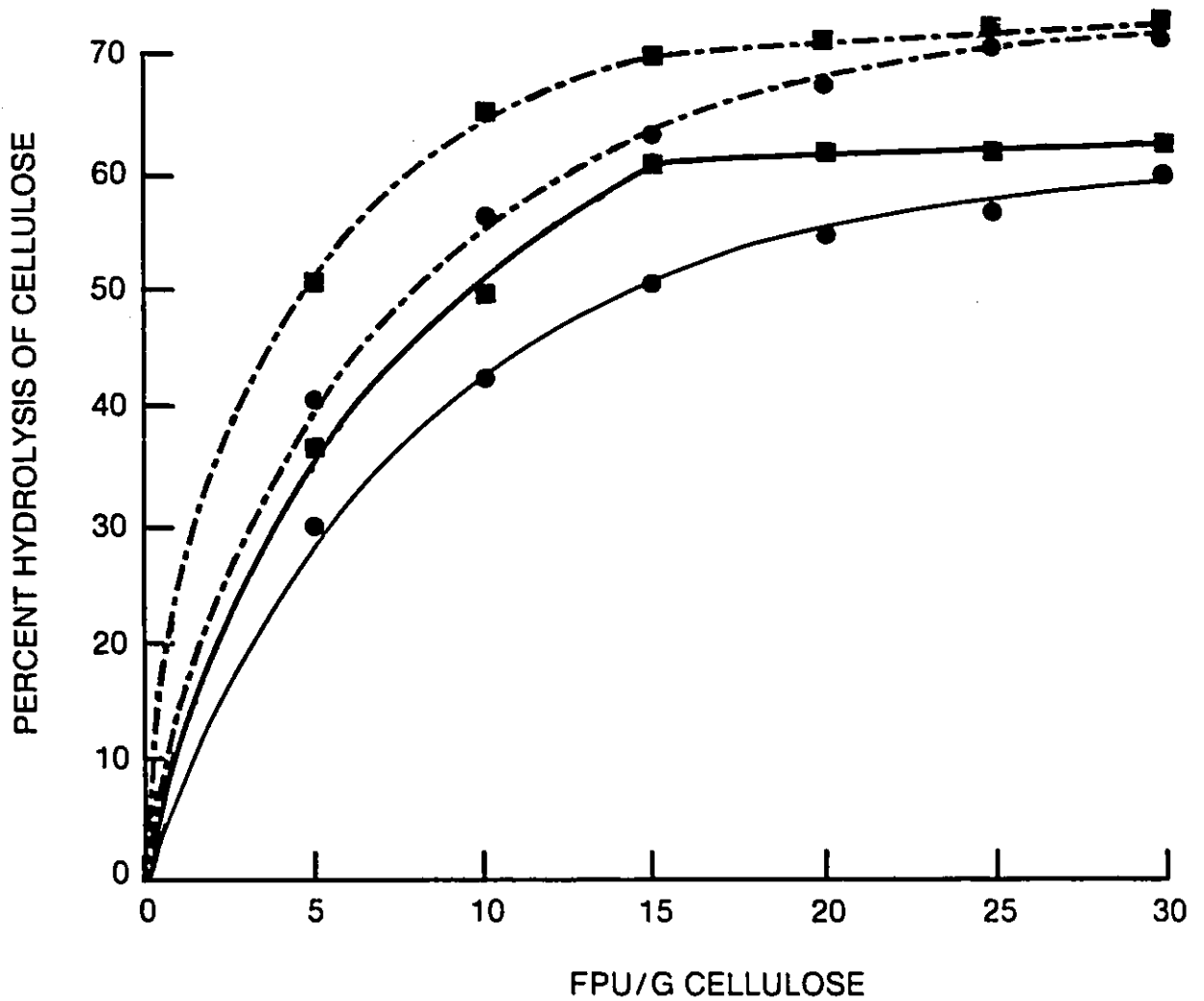
In the CHF systems percent hydrolysis was based on the ethanol yield; no glucose was detected on either day 2 or day 4.

Enzyme activity (IU/mg protein) profile was:
Endoglucanase, 19.87
β-glucosidase, 0.84
Filter paper activity, 0.87

at both day 2 and 4. Additional enzyme had no effect on hydrolysis, indicating that for efficient hydrolysis of this substrate the maximum amount of enzyme that should be used is 15-20 FPU per gram substrate. The enzyme loading corresponding to the plateau point in the CHF process provides an alternate method to initial adsorption to determine the most efficient enzyme loading for hydrolysis of the cellulose. Once the enzyme requirements are met, it is the substrate that imposes a limit on hydrolysis.

C. Thermal Inactivation

The extent to which thermal inactivation contributes to the drastic slow-down in hydrolysis is unknown. When enzymes are applied to long-term reactions, such as hydrolysis of crystalline cellulose, thermal inactivation becomes an important concern.

An investigation of enzyme stability was carried out under the conditions considered optimal for the enzymatic conversion of cellulose to glucose (45°C, pH 4.8, four days incubation). The enzyme system was buffered and agitated to duplicate the hydrolysis conditions, but in the absence of substrate. The endoglucanase component appeared to be the most thermal-labile, losing 20% of its activity over a 4 day period (Figure 19). The preparation lost only 10-15% of the filter paper and β-glucosidase activities, and 5% of the purified exoglucanase activity after 4 days.

In order to evaluate the effect of thermal inactivation on the hydrolytic efficiency of the cellulases, the ability of the

Figure 19.

Thermal stability of the endoglucanase ($\Diamond$), β-glucosidase ($\blacktriangle$), and filter paper (o) activities of T. harzianum E58 cellulases and purified exoglucanase ($\triangle$) activity after incubation at 45°C, pH 4.8.
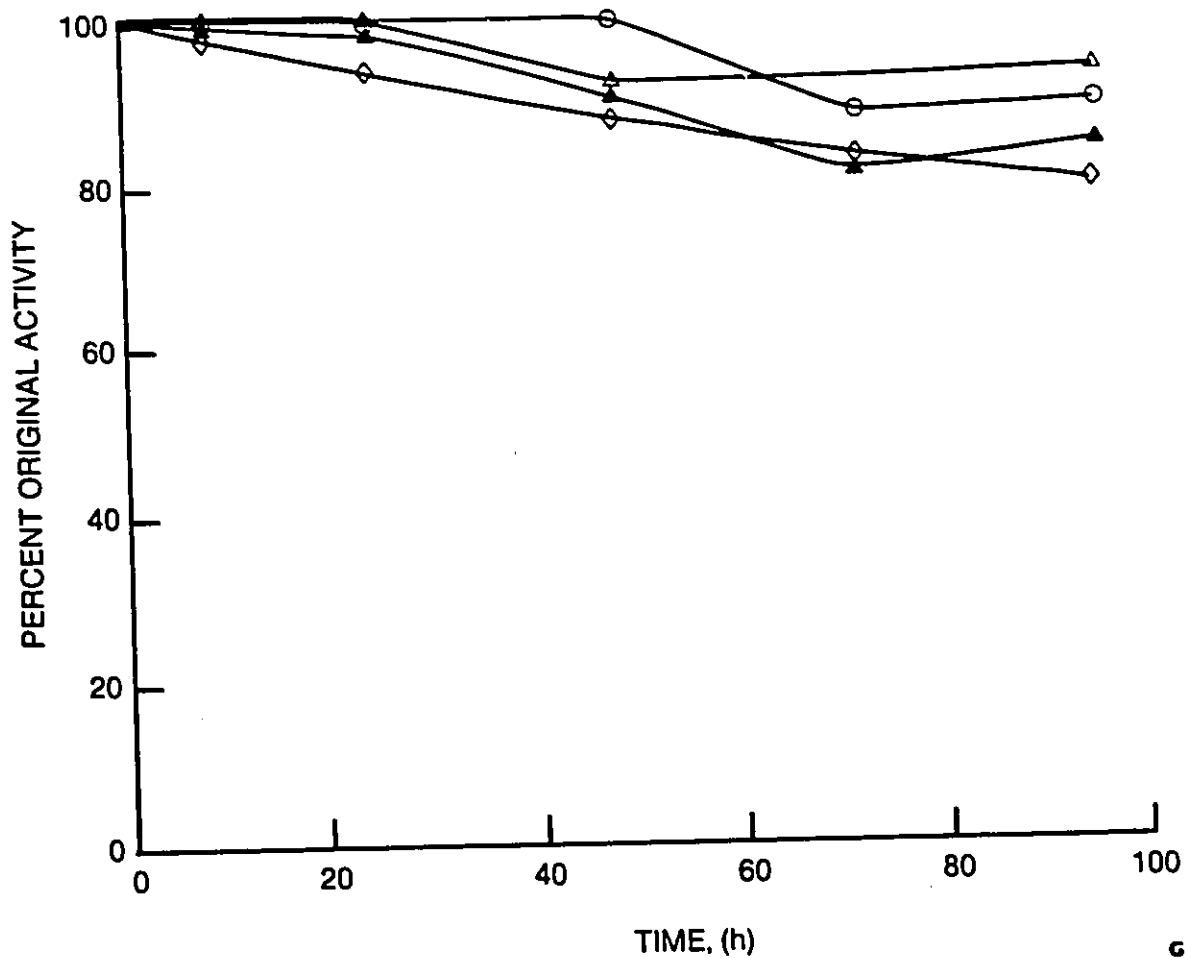
Enzyme activity (1U/mg protein) profile for the cellulase preparation was:
Endoglucanase, 13.30
β-glucosidase, 0.45
Filter paper activity, 0.67

Protein concentration of the incubated cellulases was 1.12 mg/mL.

Protein concentration of the incubated purified exoglucanase was 1.04 mg/mL.

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preincubated cellulases to hydrolyze cellulose was tested. Enzyme
samples were recovered at various times during incubation at 45°C
(Figure 19), then incubated with fresh crystalline cellulose for
24 hours at 45°C (Figure 20). Enzymes that had been preincubated
for 4 days at 45°C had lost only 20% of its ability to degrade
Solka Floc BW300 to glucose. The results indicate enzyme
stability is a contributing factor to the slow down in long term
hydrolysis.

D. Site Blockage

The effect of enzyme loading and incubation time on the rate
and extent of substrate conversion was examined (Figure 21).
After 5 days of incubation at the lower enzyme loadings the
substrate had a slower rate of hydrolysis, and a lower extent of
conversion. Some of the factors partially responsible for this
lower conversion have been examined, e.g. end-product inhibition,
thermal inactivation. These factors limit the reaction at low
enzyme loading (<25 FPU/g), but at higher enzyme loadings their
effect is negligible. Complete hydrolysis of the substrate was
obtained, but only at very high enzyme loadings. It is curious
that the substrate is not completely hydrolyzed after 5 days at
enzyme loadings over the predetermined minimal amount (>25
FPU/g).

Site blockage might explain why complete hydrolysis is
prevented. During long-term incubation, inactivation of the
cellulases occurs, the extent being dependent on the incubation
conditions. If these "inactivated" enzyme were irreversibly

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Figure 20.

Effect of thermal inactivation on the hydrolytic efficiency of the
preincubated cellulases to degrade a 2% (w/v) concentration of Solka Floc
BW300 to reducing sugars (●) and glucose (■).
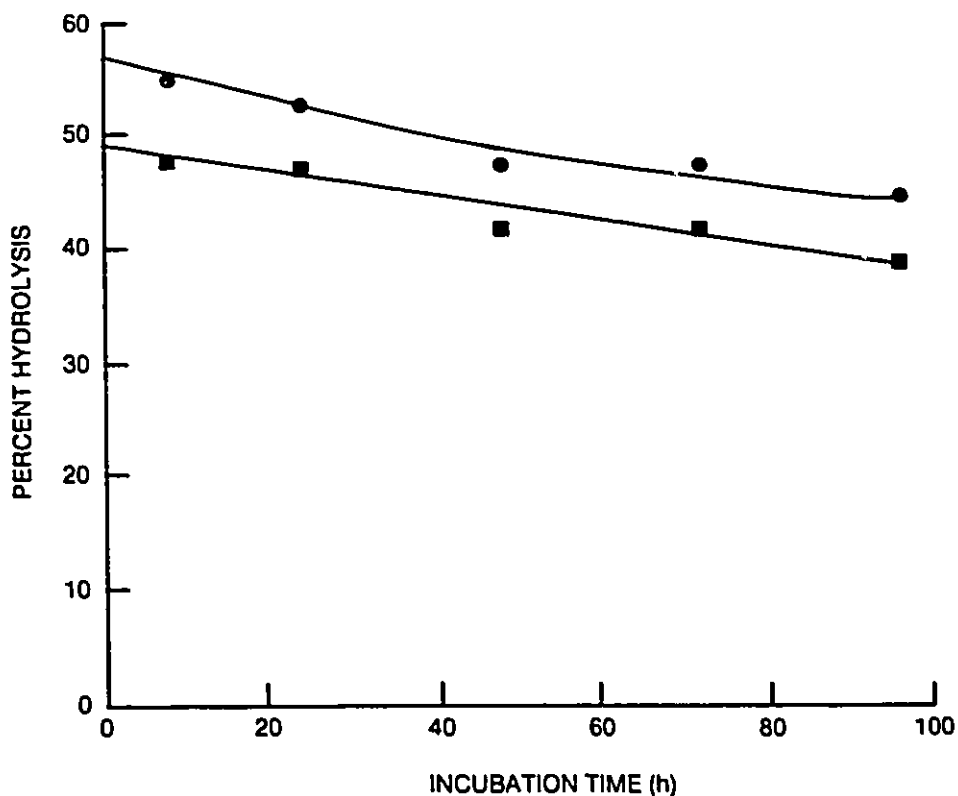
Enzyme fractions were collected at various times during incubation at 45°C (Figure 19). The enzyme
solutions were filter-sterilized and added, without dilution, to the hydrolysis systems. After 24 h
incubation at 45°C the amount of sugars released by the various systems were compared.

Initial enzyme loading, at t=0 h, was 30 FPU/g substrate.

Figure 21.

Long-term hydrolysis of a 2% (w/v) concentration of Solka Floc BW300 incubated with a range of enzyme loadings.
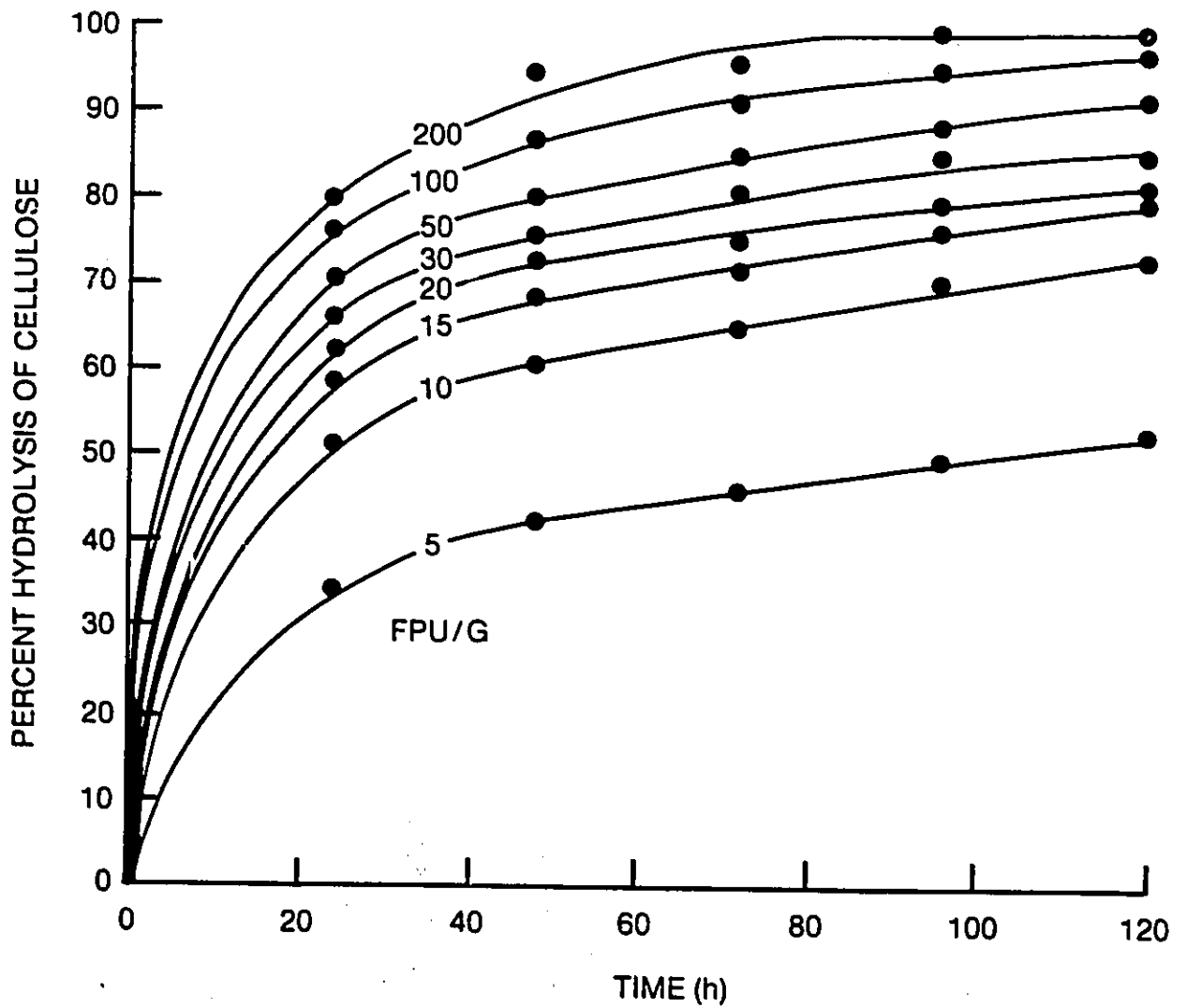
G281

bound to the substrate, they might block binding or catalytic sites from attack by the active cellulases. This would result in a slow down, or total prevention of catalysis on different sites on the substrate. Site blockage would be more of a problem at low enzyme loadings, as the slower rates of hydrolysis demand longer incubation times, and increase the amount of inactivation.

If adsorption of inactivated enzymes to the substrate is irreversible, then complete hydrolysis of the substrate is not possible, even if fresh additional enzyme is added to the system. To test the existence of site blockage, Solka Floc BW300 was incubated with various levels of enzyme; those encompassing the saturation point, as well as sufficient levels to ensure complete enzymatic hydrolysis (Figure 22). The substrate was incubated for two days, after which time a portion of the enzymes had been inactivated (Figure 19), and should presumably have been in place to block further attack by active enzymes. Complete hydrolysis of the substrate had been achieved at enzyme loadings of 100 FPU/g, so fresh enzyme was added to bring the final concentration of the systems up to this concentration. After 5 days incubation all the systems were 95-98% hydrolyzed, regardless of their original enzyme loadings (Figure 22). If enzyme blockage did occur, the lower enzyme loadings were expected to have lower conversion levels than if all the enzyme had been added directly. These results demonstrate that site blockage by inactivated enzyme is not limiting the extent of hydrolysis. Inactivated enzymes either do not bind to the substrate, or if bound, they are displaced by the active enzyme.

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Figure 22.

Measurement of the role of site blockage in hydrolysis. A 2% (w/v) concentration of Solka Floc BW300 was incubated for 2 days with a range of enzyme loadings (Figure 21), then supplemented with enzyme to produce a final enzyme loading of 100 FPU/g. The arrow marks the addition of fresh enzyme.
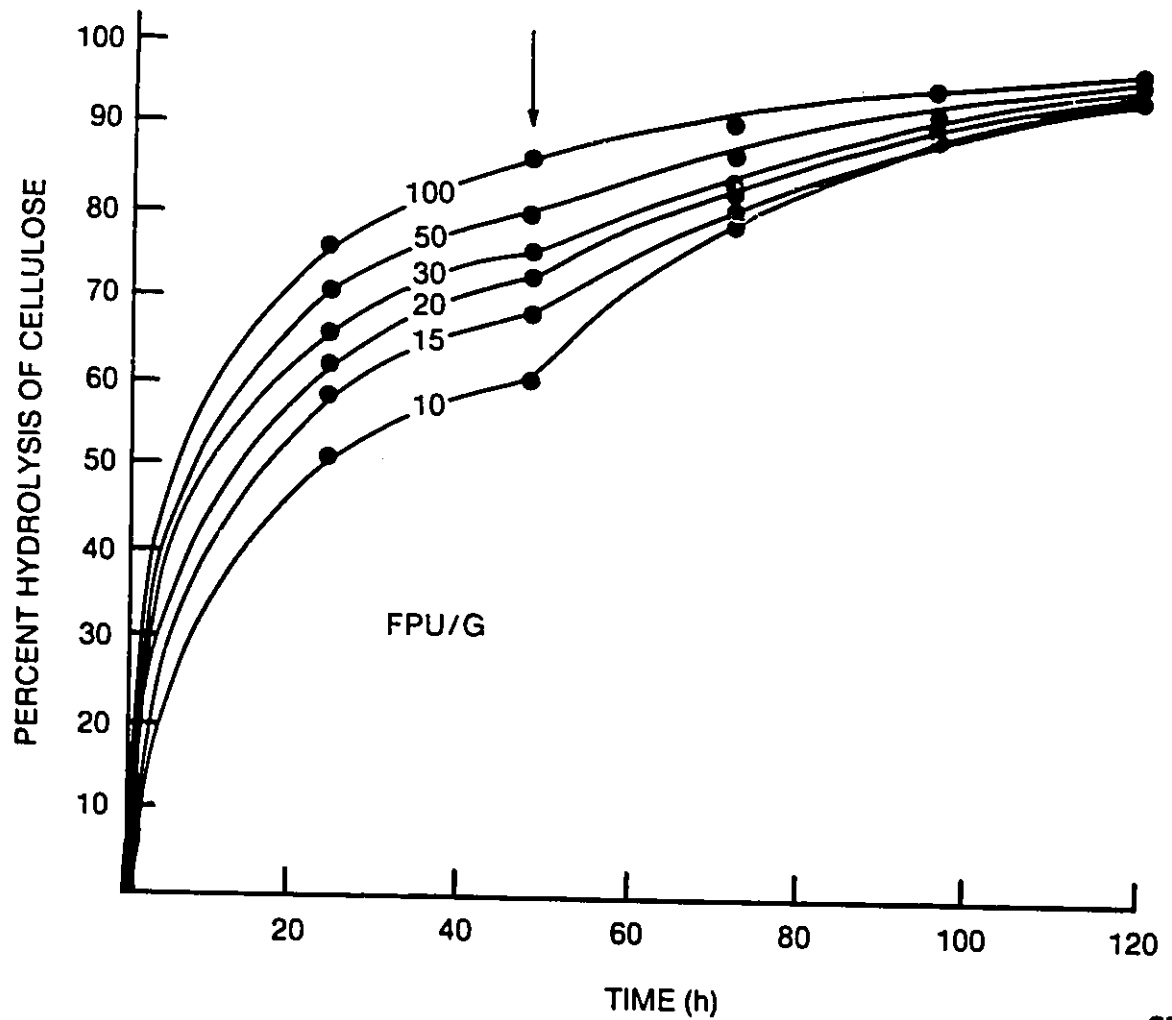
G281

III. STRUCTURAL FEATURES LIMITING HYDROLYSIS

Once the enzyme requirements are fulfilled it is the substrate that imposes a limit on hydrolysis. The main structural features determining the susceptibility of the substrate to hydrolysis include crystallinity and surface area accessible to the enzyme (Cowling, 1975; Klesov and Sinitsyn, 1981; Fan et al., 1980).

Water has a profound effect on the structure of cellulose. The specific surface area of cellulose increases drastically upon wetting (Browning, 1963), and partial recrystallization of fairly amorphous, dry preparations of cellulose under the action of water occurs (Caulfield and Steffes, 1969). As hydrolysis takes place in an aqueous environment, the changes hydrolysis causes to the substrate should be duplicated prior to any structural analysis.

The initial crystallinity of the substrate has been shown to be inversely related to its rate of hydrolysis. Strong correlation between initial crystallinity and hydrolysis has been reported using both substrate treated in various ways to lower its crystallinity, and substrates derived from different sources. The strong correlation is highly dependent on using water-swollen, dried cellulose rather than unwetted samples, for determination of the crystallinity index (Fan et al., 1980; Klesov and Sinitsyn, 1981).

Klesov and Sinitsyn standardized their cellulose preparations by determining the crystallinity after moistening and air-drying the sample. When cellulose is air-dried from a

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water-swollen state the capillary structure collapses and the physical parameters are changed drastically. Alternately, Fan et al. (1980) maintained the water-swollen nature of the cellulose by drying using a solvent-drying technique. The structural features of the water-swollen cellulose are better reflected in the solvent-dried cellulose than in the air-dried cellulose. Accordingly, for these experiments the cellulose sample was water-swollen and solvent extracted prior to determination of its crystallinity index, surface area, or other structural features.

The relationship between structural features and hydrolysis was defined using a range of pure cellulose substrates. All the commercially available cellulose substrates were highly crystalline. Since amorphous cellulose is a useful reference material for studying enzyme-substrate interactions, it was prepared by a number of techniques; ball-milling, solvent-swelling, and phosphoric-acid swelling.

Solka Floc BW300 was ball-milled in several jars for up to two weeks. Based on the literature (Fan et al., 1980) it was predicted that a one week milling was necessary to completely break down the crystalline structure, rendering the substrate amorphous. Five milling jars were charged with the substrate, and milled for 1, 2, 4, 7 or 14 days. As discussed earlier, the milled materials were then water-swollen for 24 hours, then solvent-dried prior to determination of their crystallinity index. Contrary to our predictions, the substrates were found to be completely amorphous after only one day milling, as determined by x-ray diffractometry.

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A number of cellulose-dissolving solvents have been developed (Schroeder et al., 1986). Cellulose dissolves in the solvent, losing its crystalline structure. Upon addition of excess water the cellulose is reprecipitated out in an amorphous form, and can be easily hydrolyzed to glucose.

Inorganic acids can be used as solvation agents for cellulose (Walseth, 1952; Tsao et al., 1979). As high acid concentrations are needed for complete solvation, degradation losses are high. Phosphoric-acid swollen cellulose was prepared following the technique of Walseth (1952), producing amorphous cellulose as determined by x-ray diffractometry.

Chelating Metal Caustic Swelling Solvent, CMCS, (Hamilton et al., 1984) was employed as the dissolving agent on a number of crystalline substrates in order to produce amorphous cellulose. The substrates were water-swollen and solvent dried prior to determination of their crystallinity index. X-ray diffractometry of the substrates indicated the substrates had partially recrystallized into the Cellulose II form.

Cellulose has a polymorphic crystallinity. The native cellulose, Cellulose I, exhibits a diffraction pattern different from those of the three "man-made" cellulose crystal forms known as celluloses II, III or IV. Cellulose I occurs as a natural product, a result of biosynthesis. Cellulose II results from mercerization, or solubilization and regeneration of Cellulose I, and once Cellulose II has been generated the transformation remains irreversible. Cellulose III can be made from Cellulose I or II by treatment with liquid NH_3 or some amines, and can be

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converted to Cellulose IV by heating in glycerol to 260°C (Sarko, 1986). The x-ray diffractogram of Cellulose II differs from Cellulose I (native cellulose) in having its crystalline peak shifted from 22° to 20° and its amorphous component shifted from 18° to 15°. As this cellulose is radically different from cellulose I, it would likely deviate from the norm in its accessibility to hydrolysis. When these substrates were hydrolyzed concurrently with the other celluloses their conversion values were substantially lower. These substrates were therefore not included in the study of the relationship between crystallinity and hydrolysis.

A. Crystallinity

Once a series of substrates of varying crystallinities had been purchased or prepared, the relationship between hydrolysis and crystallinity could be studied. The substrates were digested with T. harzianum E58 cellulases using an enzyme loading of 30 FPU/gram. Hydrolysis was studied over a four day period to observe the influence of time on the relationship (Figure 23). As expected, the more amorphous substrates were more readily hydrolyzed at all times studied. Surprisingly, a bimodal pattern of hydrolysis was observed; the more crystalline substrate demonstrated a dramatic increase in susceptibility with a small decrease in crystallinity, while the susceptibility of the more amorphous substrates (CrI=65-80) was increased to a lesser extent with a drop in crystallinity. The transition point occured at approximately CrI=80, and remained constant throughout the 4 days of hydrolysis. These results imply that the relationship between

Figure 23.

Relationship between the initial crystallinity of a substrate and its
susceptibility to enzymatic hydrolysis after 8(●), 24(■) and 96(▲) h.
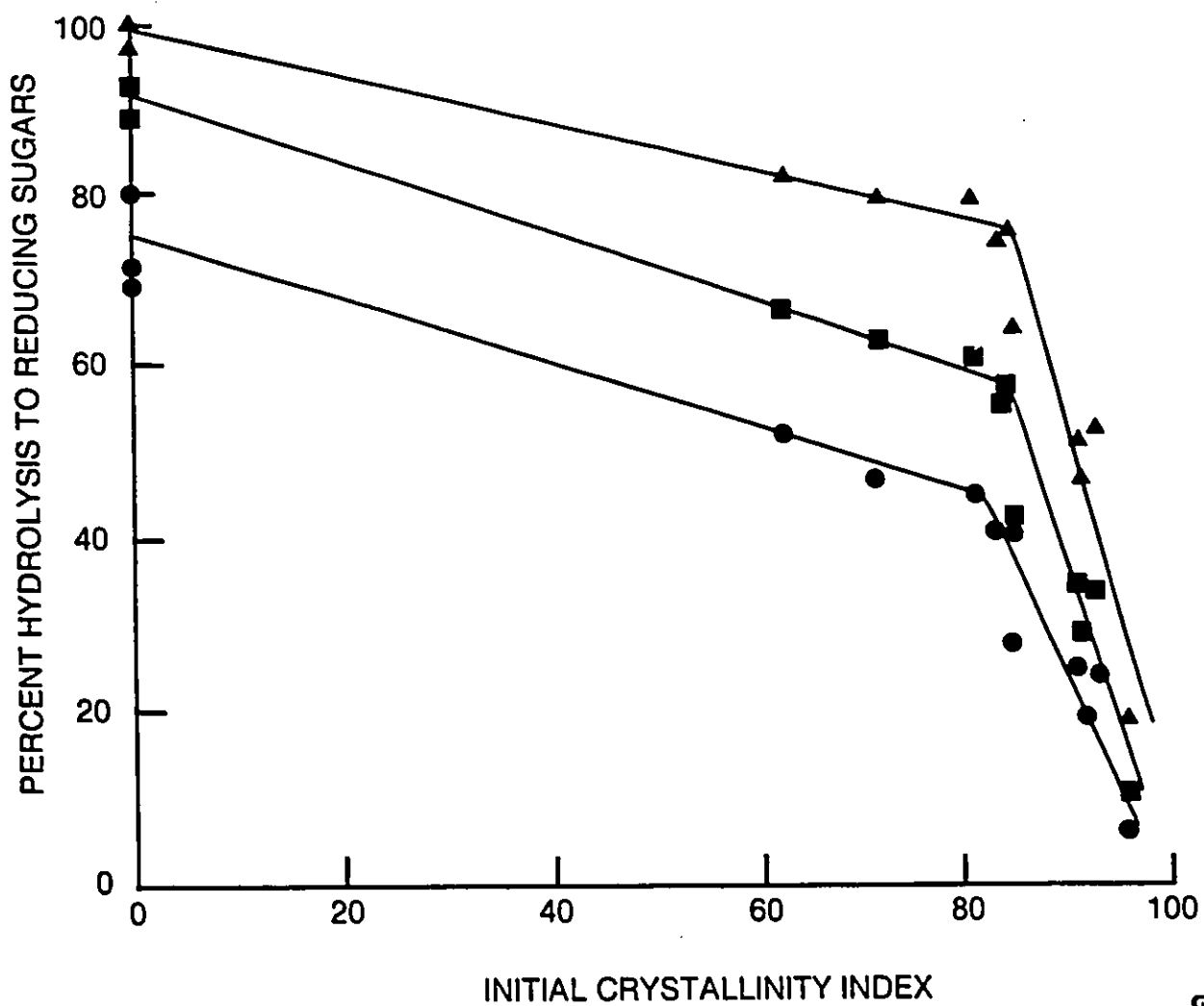
Enzyme loading was 30 FPU/g substrate.

initial crystallinity and hydrolysis may be a reflection of some

other factor. When a substrate is pretreated a number of factors

are changed simultaneously, i.e. crystallinity, particle size,

surface area, degree of polymerization. One, or several, of

these structural features may be limiting accessibility in the

more crystalline materials, so that a small change in

crystallinity may result in a dramatic increase in accessibility.

Caulfield and Moore (1974) also found ball-milling increases

the digestibility of both the amorphous and crystalline

components of cellulose, especially the latter. This led them to

propose that the increase in digestibility is a result of

decreased particle size and increased surface area, rather than a

result of reduced crystallinity. They suggested coupling

crystallinity measurements with surface measurements in order to

study more effectively the influence of each structural feature.

The amorphous substrates underwent about 75% conversion to

reducing sugars in 8 h, although fairly low enzyme loadings (30

FPU/g substrate) were used. In comparison, the more crystalline

substrates were only 5-50% hydrolyzed, depending on their initial

crystallinity. It appears that it is the structure of the

substrate, not the activity of the enzyme which primarily limits

hydrolysis.

B. Surface Area

Cowling (1975), Stone and co-workers (1969), and Greithlein

(1985) state the most important structural feature governing

enzymatic hydrolysis is the surface area available to the

enzyme. Many effective pretreatments increase surface area either by reduction in particle size (milling), by alkali-swelling, or dissolving and re-precipitating, producing a more susceptible substrate (Fan et al., 1981; Klesov and Sinitsyn, 1981).

There are conflicting reports on the relationship between surface area and susceptibility of the substrate to enzymatic attack. One must consider the technique used to measure accessible surface area when interpreting these results. Nitrogen gas adsorption has been used by several groups (Fan et al., 1980; 1981). As the nitrogen molecules can enter into pores that are inaccessible to the enzyme, surface area available to the enzyme can not be accurately determined.

Direct physical contact between the enzyme and the substrate is necessary for hydrolysis. The amount of cellulase adsorbed is proportional to the accessibility of the substrate to the enzyme (Lee et al., 1982); a substrate able to adsorb more enzyme apparently is more readily hydrolyzable. Adsorption of cellulase, or a protein possessing the same dimensions, provides the most direct measure of surface area available (Mandels, 1985). Berezin and Klesov (1981) using this procedure found the rate of hydrolysis proportional to the surface area.

Direct adsorption of cellulase to the substrate appeared to be both an accurate and quick way to measure the surface accesible to the enzyme. Cellulase adsorption was used to examine the relationship between accessible surface area and hydrolysis of a substrate.

The enzyme adsorption profiles for a number of pure

Figure 24.

Adsorption of varying concentrations of a) endoglucanase and b) filter paper
activities and c) protein onto a 5% (w/v) concentration of different
cellulosic substrates.
Substrates; Avicel (♦), CF11 (o), Solka Floc AS1040 (■), Solka Floc BW300 (●)
and Solka Floc SW40 (▲).
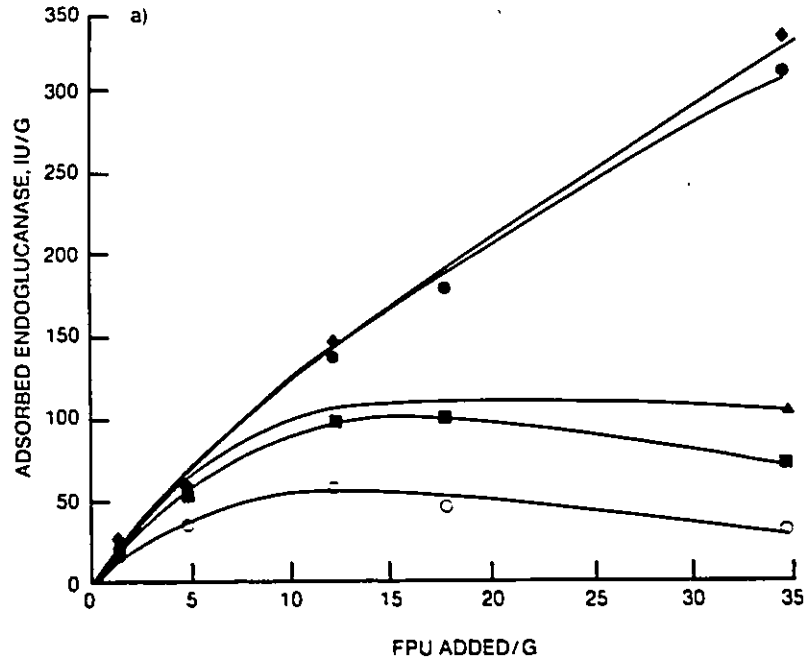
Figure 24.

Adsorption of varying concentrations of a) endoglucanase and b) filter paper
activities and c) protein onto a 5% (w/v) concentration of different
cellulosic substrates.
Substrates; Avicel (♦), CF11 (o), Solka Floc AS1040 (■), Solka Floc BW300 (●)
and Solka Floc SW40 (▲).

Incubation times for the adsorption study was 10 minutes.

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cellulose substrates, incubated with varying concentrations of cellulases, were determined (Figure 24). Avicel and Solka Floc BW300 ads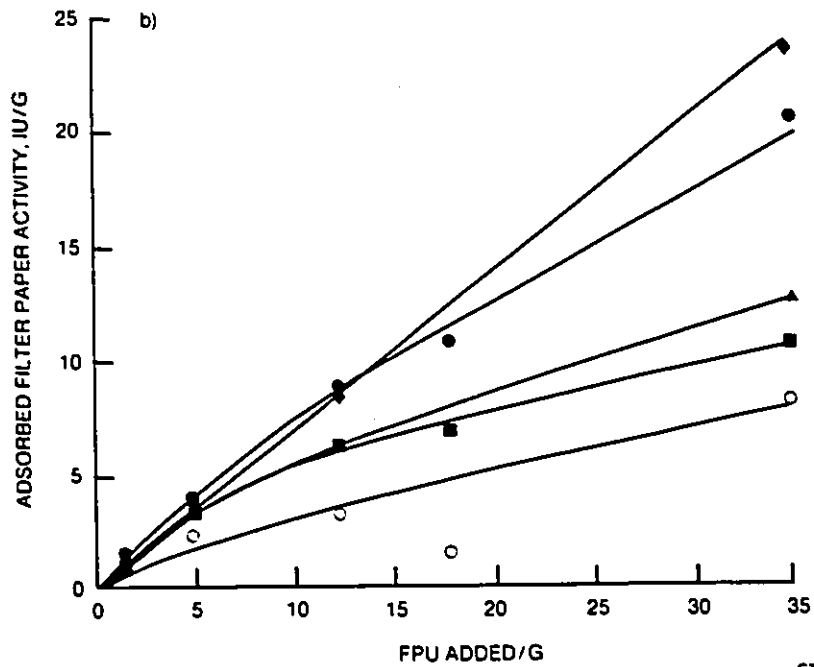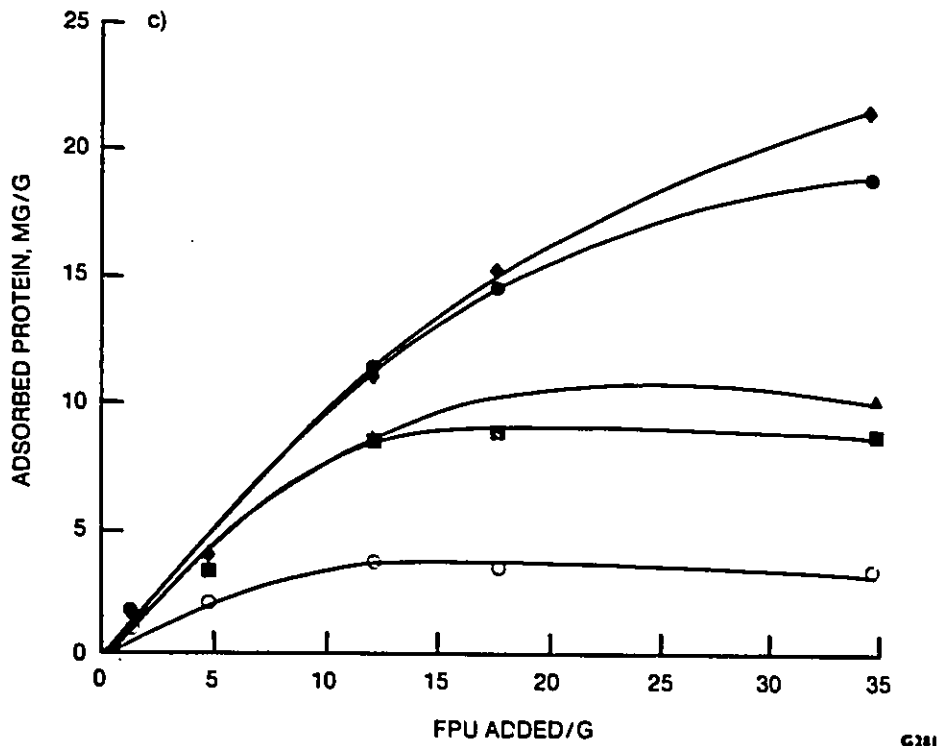orbed the greatest amount of enzyme, Solka Floc AS1040 and Solka Floc SW40 had intermediate levels of adsorbed enzymes, while CF11 had low levels of adsorption. The pattern of enzyme adsorption onto the substrates was identical for endoglucanase, filter paper activities, as well as for protein. From these results it was predicted that Avicel and Solka Floc BW300, with the highest accessible surface area (i.e. highest amounts of enzyme adsorbed) would have the fastest rates of hydrolysis, and CF11, with the lowest accessible surface area, the slowest.

The digestibility of the substrates was tested using a high and a low enzyme loading. At low enzyme loading equivalent amounts of enzyme were adsorbed to the different substrates, so comparable hydrolyses were expected. At high enzyme loading the benefits of the higher amounts of adsorbed enzyme should come into play, so Avicel and Solka Floc BW300 should be more readily hydrolyzed.

The various substrates were incubated for 4 days, and assayed at various times for reducing sugars and glucose released (Figure 25). Solka Floc BW300 was found to release the highest levels of sugars, at both enzyme loadings, with more sugars produced at the higher enzyme loading. The order of susceptibility of the substrates was the same at both enzyme loadings. Surprisingly, Avicel released only moderate levels of sugars, despite the fact that it had adsorbed the greatest amount of enzyme.

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Figure 25.

Hydrolysis of a 5% (w/v) concentration of different cellulosic substrates to reducing sugars and glucose at enzyme loadings of a,c) 5 and b,d) 30 FPU/substrate.
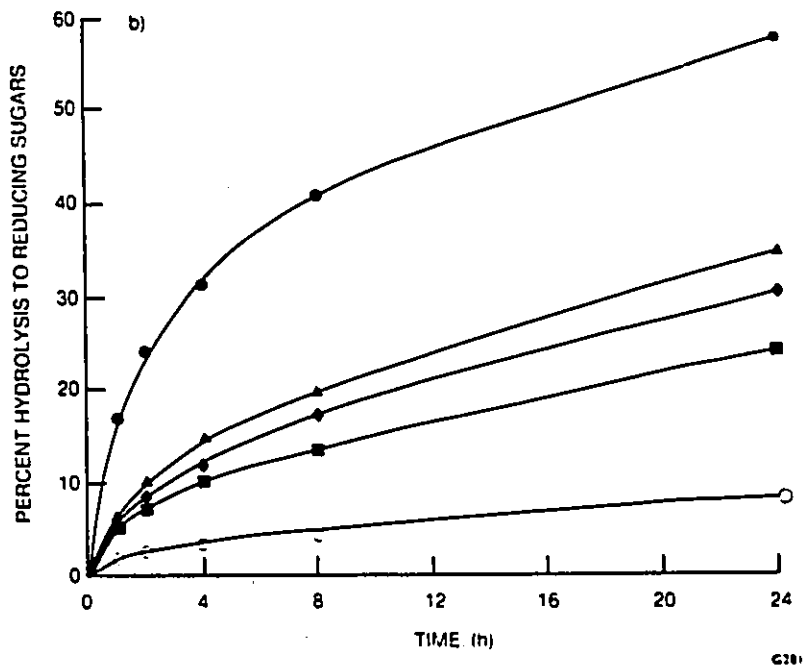Substrates; Avicel (◆), CF11 (O), Solka Floc AS1040 (■), Solka Floc BW300 (●) and Solka Floc SW40 (▲).
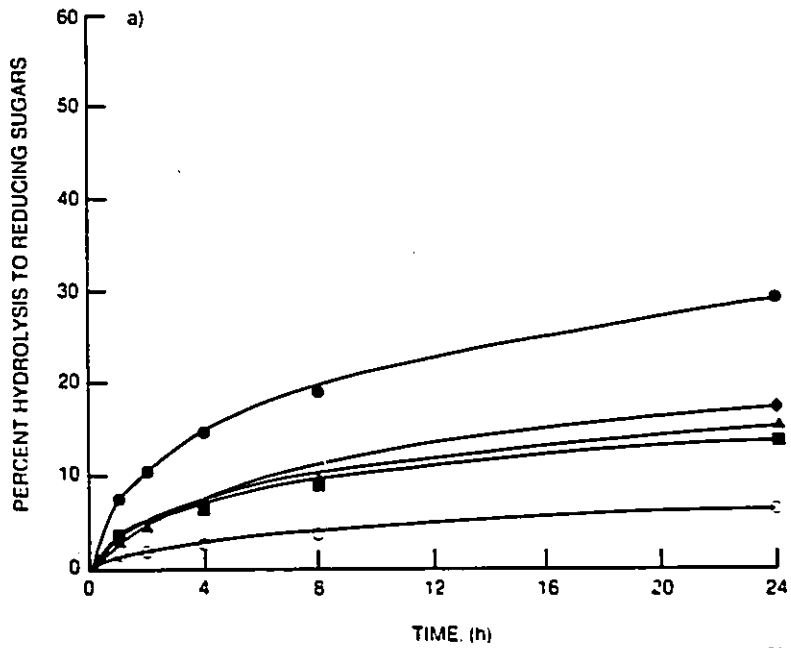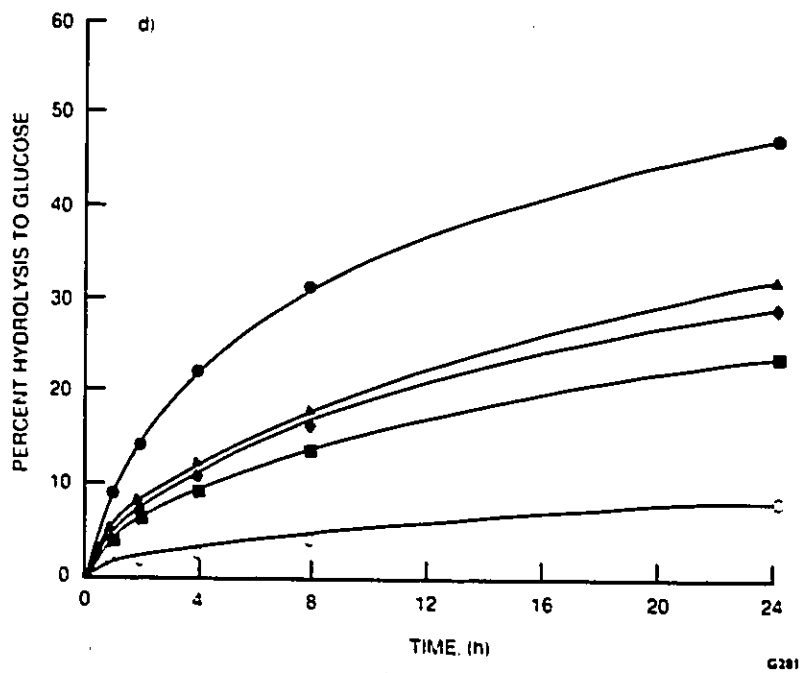
Figure 25.

Hydrolysis of a 5% (w/v) concentration of different cellulosic substrates to reducing sugars and glucose at enzyme loadings of a,c) 5 and b,d) 30 FPU/substrate.
Substrates; Avicel (◆), CF11 (o), Solka Floc AS1040 (■), Solka Floc BW300 (●) and Solka Floc SW40 (▲).
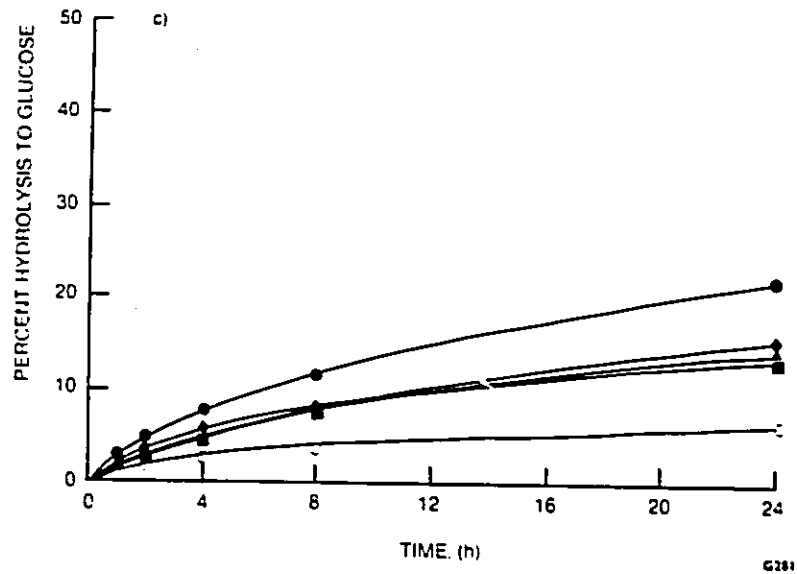
Two facts emerged. The amount of enzyme adsorbed onto a given substrate is related to hydrolysis of that substrate, with more adsorbed enzymes giving higher sugar yields. The effectiveness of the adsorbed enzyme to promote hydrolysis depends on the substrate. Effectiveness can be related to the structural features of the substrate, and the affinity of the enzyme towards the substate.

IV.   STRUCTURAL CHANGES DURING LONG-TERM HYDROLYSIS

Decrease in the hydrolysis rate can be attributed to several factors, including transformation of the structure into a more recalcitrant form, a decrease in the extent of adsorbed enzyme and depletion of the substrate.

There are numerous papers debating the relative importance of the structural features contributing to the increased recalcitrancy of the substrate. Changes in crystallinity (Caulfield and Moore, 1974) and specific surface area (Lee et al., 1980) due to cellulase action have been reported, but the results do not provide a clear picture of the overall hydrolytic mechanism.

A problem in measuring structural changes in the substrate during hydrolysis is the protein remaining adsorbed onto the recovered substrate. These proteins have been inactivated by boiling the substrate to terminate the enzymatic reaction, but the cellulases remain tightly bound to the cellulose, and are not removed by extensive washing. The proteins may interfere in the determination of structural characteristics. While most researchers, investigating structural changes of the "spent"

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cellulose, maintain the structural integrity of the water-swollen substrate by solvent extraction, the problem of adsorbed protein is not addressed. To eliminate possible protein interference a method of desorbing the protein from the residual substrate, without altering its structural integrity, had to be devised.

Otter et al. (1984) reported a mild alkali washing removes the bound cellulases from cellulose. A preliminary experiment was done to determine the optimal concentration for enzyme removal (Table 3). A 30 minute incubation in 0.1% NaOH was found to remove 90% of the protein.

Desorption of the adsorbed proteins by alkali, followed by solvent-drying the recovered cellulose, will ensure that a) the structural characteristics of the water-swollen cellulose are retained and b) the sites on the substrate accessible to the enzyme are not blocked by denatured enzymes. These conditions were employed in further studies requiring protein desorption of the spent cellulose (e.g. to study structural changes in the substrate during its hydrolysis).

A. Change in Crystallinity

Conflicting reports are prevalant on the change in crystallinity during hydrolysis. Norkrans (1950), Lee et al. (1983) and Ohmine et al. (1983) found that as hydrolysis proceeds the crystallinity increases, causing the substrate to become increasingly resistant to enzymatic attack. These results suggest that the amorphous portion of the cellulose is preferentially attacked; with the rapid removal of the amorphous

Table 3.   Removal of protein adsorbed onto 5% Solka Floc BW300
            after 30 minutes incubation in alkali

NaOH %	Protein recovered in filtrate, % original	Protein removed by alkali, % original	Total protein recovered, % original
0	47	3	50
0.02	46	15	61
0.05	47	30	77
0.10	48	42	90
0.20	47	45	92

Protein concentration=0.79 mg/mg powder

P_L=29.5 mg/g substrate

portion resulting in a marked increase in the crystallinity during hydrolysis.

In contrast, Lee and Fan (1983) noted only a slight increase in the crystallinity during 72 h hydrolysis of Solka Floc SW40 (From 77.8 to 83.7), with 50% of the substrate hydrolyzed. Other researchers report only a slight (Fan et al., 1980) or no change (Chang et al., 1981) in crystallinity of the substrate throughout hydrolysis.

In light of the conflicting data, we decided to examine the crystallinity index of Solka Floc BW300 at various times during hydrolysis (Figure 26). Crystallinity changed slightly during the initial 24 hours of hydrolysis, increasing from 81.6 to 82.0. During this time, the substrate underwent its greatest period of hydrolysis, releasing 40.6% of its weight in sugars. Although there was a slight increase in crystallinity it is difficult to reconcile this change with the drastic decrease in substrate hydrolyzability.

As the CrI increased only slightly throughout hydrolysis this indicates that a simultaneous hydrolysis of the amorphous and crystalline regions was occuring, rather than a selective attack of the amorphous region. Although the crystalline and amorphous regions are attacked simultaneously the amorphous regions may be more readily hydrolyzed, accounting for a slight increase in crystallinity throughout hydrolysis. These results support an "all-or-nothing" mechanism for cellulose degradation; when attack is initiated on a cellulose chain it goes to completion.

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Figure 26.

Change in the crystallinity index of a 5% (w/v) concentration of Solka Floc
BW300 during its enzymatic hydrolysis.
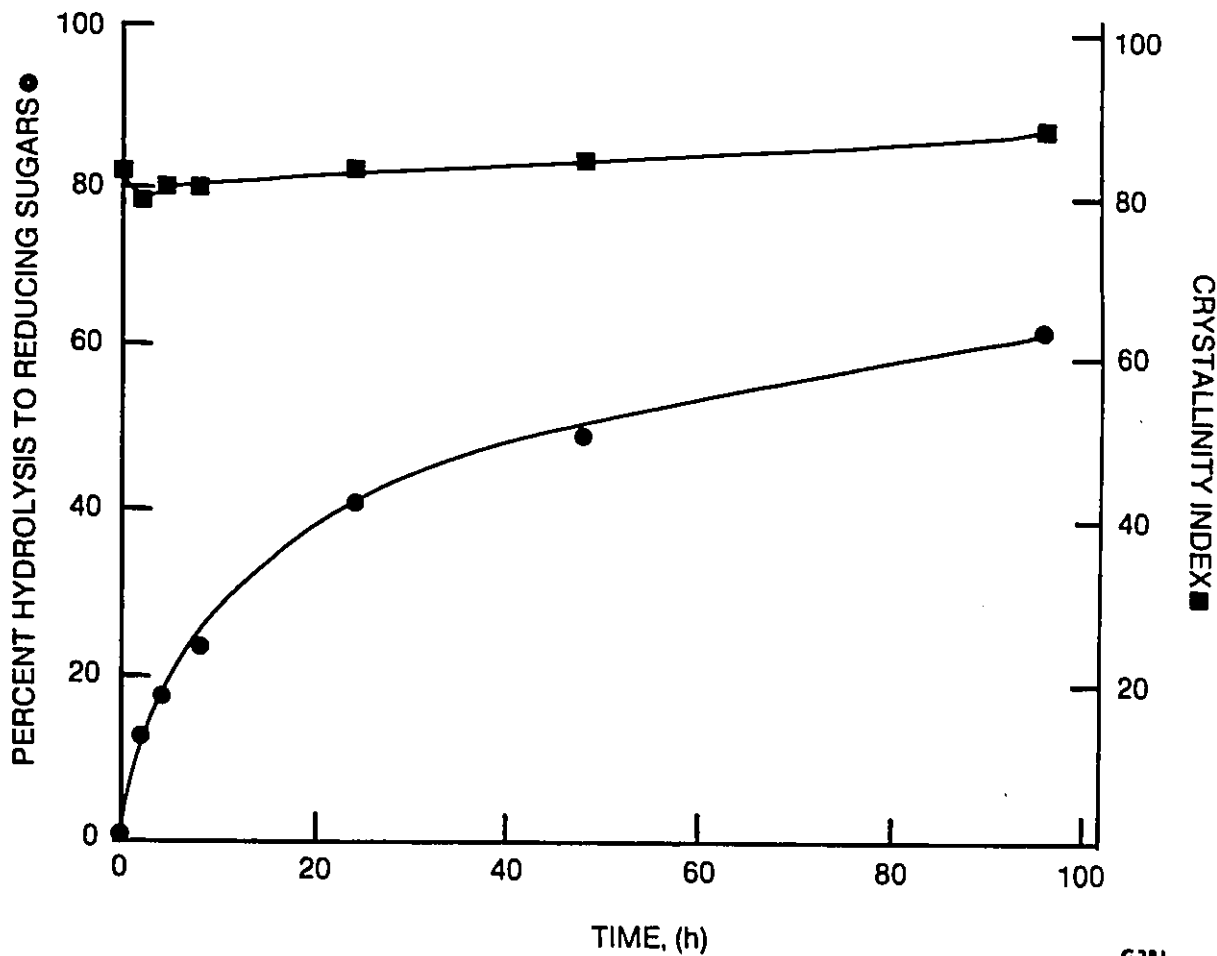
B. Change in Surface Area Accessible to the Enzyme

Increased recalcitrance of the substrate during hydrolysis may be due to a drop in the amount of adsorbed enzyme. If the amount of enzyme able to bind to the substrate declined during hydrolysis, the rate of hydrolysis would be expected to drop. The accessibility of the substrate, Solka Floc BW300, to the enzyme was followed during the course of the hydrolysis (See Methods and Materials; Spent Cellulose). Cellulose recovered at various times during hydrolysis was treated with mild alkali to desorb the bound protens, and solvent extracted. This cellulose was termed "spent cellulose". Adsorption of a fixed concentration of enzyme onto the spent cellulose was measured (Figure 27). During the initial stage of hydrolysis there was a rapid increase in the amount of enzyme adsorbed, signifying a drastic change in the structural features of the cellulose. The amount of enzyme adsorbed almost doubled in the initial 2 hrs of hydrolysis, and remained constant up to 12 hrs, after which the amount of enzyme adsorbed declined gradually. Even after 48 hours of hydrolysis the amount of enzyme adsorbed was greater than for the initial substrate (t=0) even though 50% of the substrate had been hydrolyzed. Lee and Fan (1983) have also observed the protein bound per weight of substrate remained relatively unchanged during hydrolysis. This indicates that the adsorption sites per unit weight of cellulose do not significantly change during hydrolysis, so the amount of enzyme adsorbed onto the surface is not a limiting factor.

The initial rapid increase in the amount of enzyme adsorbed

Figure 27.

Adsorption of T. harzianum E58 cellulases onto a 5% (w/v) concentration of
Solka Floc BW300 prehydrolyzed for different lengths of time.
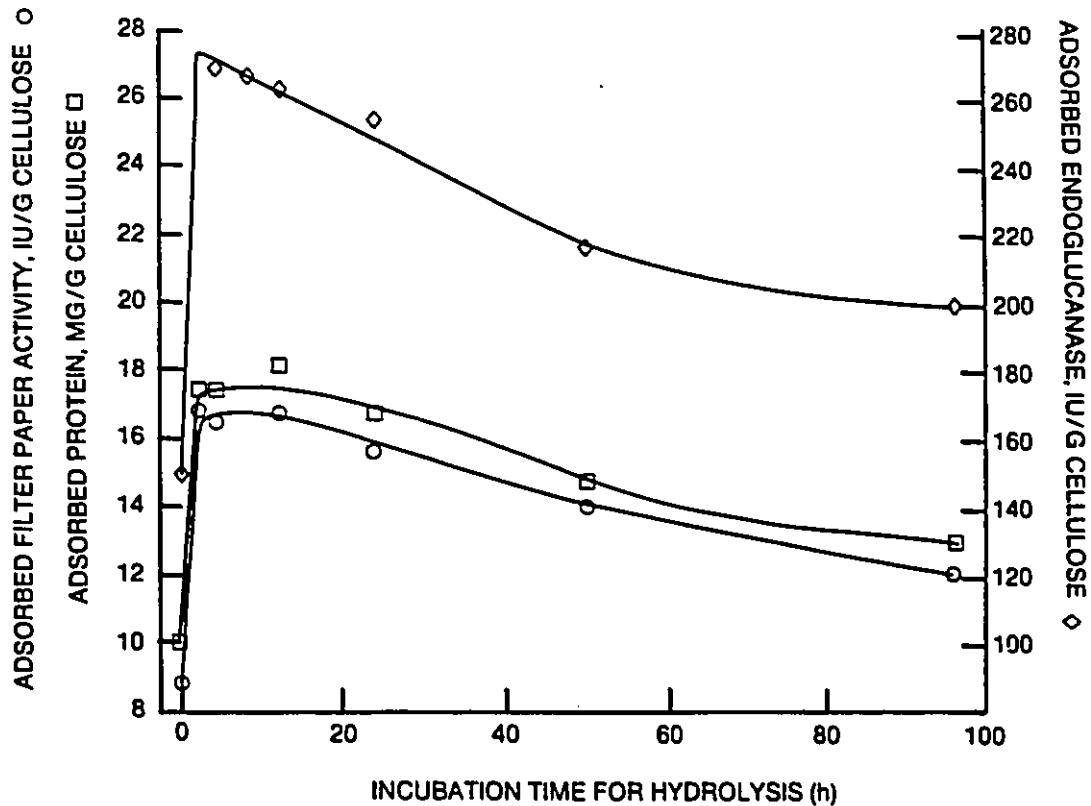
Substrate was recovered at various times during its hydrolysis at 45°C. The hydrolyzate was analyzed for
sugars, while the residue was deproteinized then solvent-dried. Cellulase adsorption studies were performed
on the dried residue.

Enzyme activity (IU/mg protein) profile of the cellulase preparation used for the adsorption study was:
Endoglucanase, 11.53
β-glucosidase, 0.63
Filter paper activity, 0.73

Enzyme loading for the adsorption study was 24.7 FPU/g substrate

was not surprising. During the early stage of hydrolysis the endoglucanase attacks the substrate, generating a large number of reducing ends for the exoglucanase to adsorb to and cleave. This occurrence was examined further by studying the adsorption of purified exoglucanase onto the spent cellulose (Figure 28). Exoglucanase adsorption onto the spent Solka Floc parallelled the pattern observed using the enzyme mixture. The substrate was drastically altered in the first hour of enzymatic attack, with its accessibility to the enzyme doubling. Apparently, the substrate had been altered to expose more binding sites for the exoglucanase.

Changes are occurring to the substrate that can not be related to either increased crystallinity or lack of surface area accessible to the enzyme. Although the increased crystallinity and changes in the surface area of the substrate during hydrolysis do not appear to limit hydrolysis, they reflect other structural changes.

C. Increased Recalcitrancy of the Substrate

Although there is plenty of substrate, and the amount of enzyme adsorbed to the substrate is high, the rate of hydrolysis drastically drops. The structure of the cellulose is transformed into a more resistant, or less accessible form during hydrolysis.

The extent to which changes in the structural features limit hydrolysis can be determined. Recovery of the unhydrolyzed cellulose throughout the hydrolysis, and testing it for hydrolysis with fresh enzyme will give a measure of the increased

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Figure 28.

Adsorption of purified exoglucanase, derived from T. harzianum E58, onto a
5% (w/v) concentration of Solka Floc BW300 prehydrolyzed for different lengths
of time.

INCUBATION TIME FOR HYDROLYSIS (h)

G28i
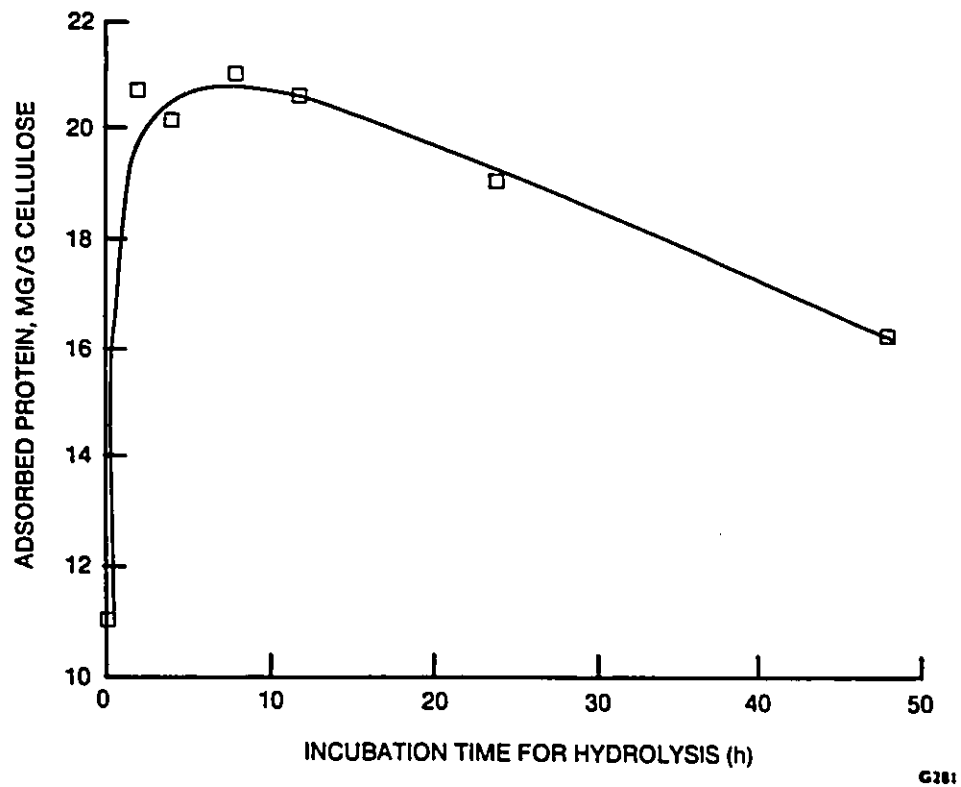
See Figure 27 for description of substrate.

Enzyme loading for adsorption study was 30.65 mg purified exoglucanase/g substrate

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recalcitrancy of the substrate. The use of spent cellulose
separates changes occurring to the substrate from the enzymatic
factors that inhibit hydrolysis e.g. end-product inhibition,
thermal inactivation.

Spent cellulose was recovered at various times during
hydrolysis (See Materials and Methods; Spent Cellulose). After
alkali desorption of the bound enzymes the residue was solvent
extracted. A 5% concentration of the spent celluloses were
incubated with fresh enzyme for 24 hours (Figure 29). The
results indicated the susceptibility of the cellulose to
hydrolysis decreased with prolonged incubation or with its extent
of hydrolysis. A 70% loss in susceptibility was noted, due only
to changes in the substrate.

As discussed earlier, neither an increase in crystallinity,
nor a lack of accessible surface area could be held accountable
for the reduction in the hydrolysis rate. There are a number of
hypotheses as to structural features that may be limiting
hydrolysis. Fan and Lee (1983) suggest the cellulose surface
area is composed of an active fraction and a less active
fraction; the former being easily digested and the latter being
the fraction resistant to hydrolysis.

Lee and Fan (1983) claim the transformation of cellulose
into the less digestible form cannot be understood by simply
examining the individual effects of several major structural
features. Several structural features are simultaneously
changing during hydrolysis, all contributing to its increased
recalcitrancy. It is difficult, if not impossible, to state
conclusively that only one factor is limiting hydrolysis. The

Figure 29.

The relationship between hydrolysis of a 5% (w/v) concentration of Solka Floc BW300 and the accessibility of the spent cellulose to further enzymatic hydrolysis.
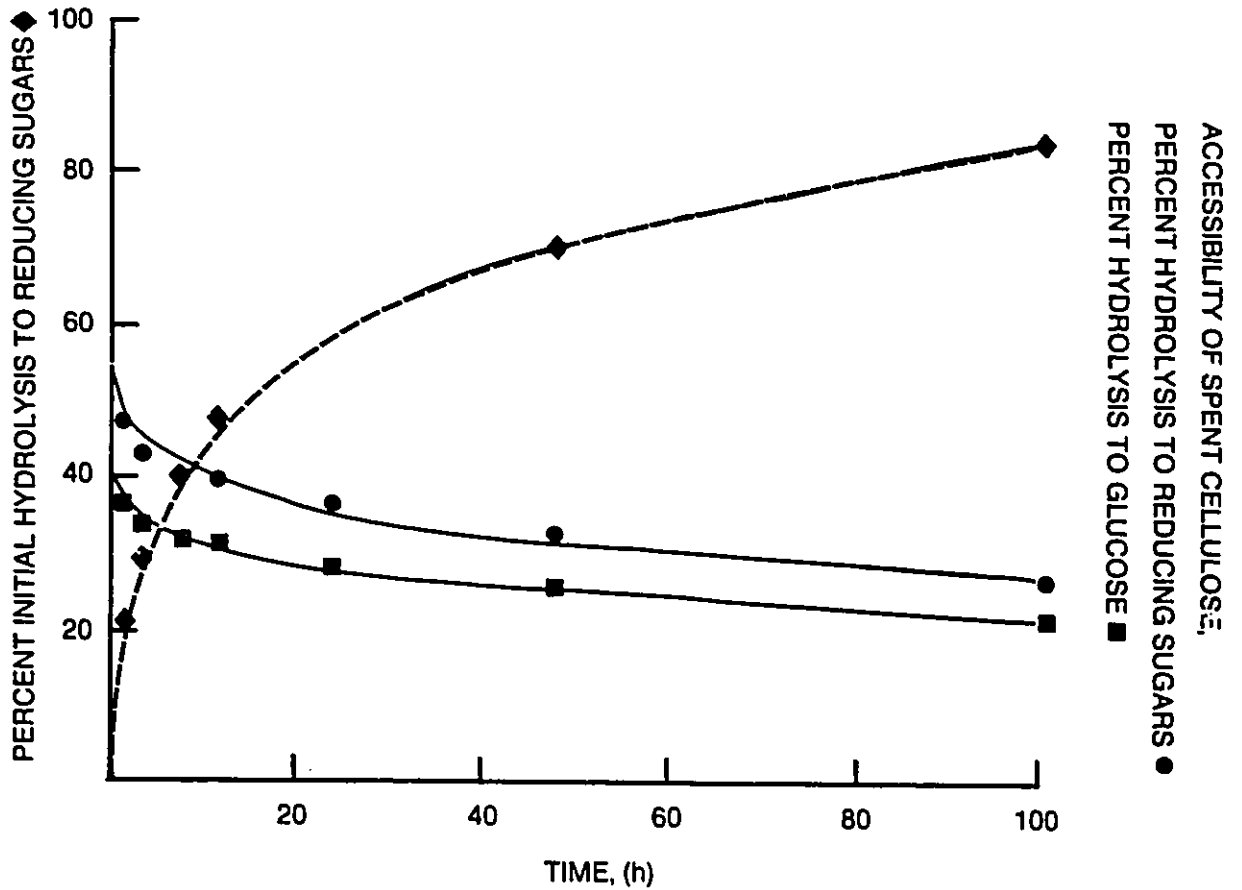
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Substrate was recovered at various times during its hydrolysis at 45°C (See Figure 27 for hydrolysis curve). The hydrolyzate was analyzed for sugars, while the residue was deproteinized then solvent-dried. A 5% (w/v) concentration of the spent cellulose was incubated with fresh enzyme (at an enzyme loading of 20 FPU/g substrate), for 24 hours, 45°C.

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synergistic influence of all the structural changes must be
assessed.

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CONCLUSION

A number of factors affecting and limiting enzymatic hydrolysis using T. harzianum E58 cellulases have been investigated. The conditions employed for hydrolysis are not conducive to maintenance of enzyme activity or stability. Factors such as high substrate concentration, high temperature, long incubation times, cellulase adsorption and end-product inhibition, all reduce the activity of the enzyme. The first step was to establish the optimal pH and thermal conditions for detecting enzyme activity. It was found that T. harzianum E58 cellulases had a temperature optimum of about 50°C and a pH optimum of 4.8. When applied to a long-term process the stability of the enzymes is a prime consideration. A temperature of 45°C was the best compromise between stability and a fast hydrolytic rate.

As enzyme adsorption is a prerequisite for hydrolysis, the relationship between the two processes was examined. Adsorption has much broader thermal and pH optima than hydrolysis. The optimal conditions for hydrolysis lie within the range for optimal adsorption onto the cellulases, so adsorption does not limit hydrolysis.

The relationship between amount of enzyme adsorbed to the substrate and its hydrolysis was studied. As the concentration of enzyme present in the system was increased the amount of enzyme adsorbed to the substrate increased, up to a saturation point. Hydrolysis of the substrate to reducing sugars reflected this pattern. It was established that the substrate has a

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limited number of binding sites for the enzyme to adsorb to. Additional cellulases (i.e. endoglucanase and exoglucanase) remained in solution and had only a minor role in hydrolysis. The enzymes that adsorb to the substrate in the initial stage of hydrolysis (i.e. within the first hour) have a strong role in governing the overall reaction. When working with an insoluble substrate it is the substrate, or the adsorptive process which controls the overall rate. The enzyme loading where the substrate is saturated with the enzyme is apparently the most economical enzyme concentration to use for hydrolysis. This enzyme loading can be determined by studying either adsorption of the cellulase preparation onto the substrate or by employing a combined hydrolysis and fermentation process.

The decline in the hydrolytic rate throughout the reaction can be attributed to changes occurring either to the enzyme or to the substrate.

Exoglucanase and β-glucosidase activity were resistant to thermal inactivation over a 4 day incubation period at 45°C. The endoglucanase component appeared to be the most thermolabile, losing 20% of its activity over the 4 days. Although the cellulases are partly inactivated at 45°C, this does not appear to be a major problem. Site blockage of adsorptive or catalytic sites on the substrate by inactivated enzymes also did not seem to be limiting hydrolysis.

End-product inhibition of the cellulases and β-glucosidase by cellobiose and glucose, respectively, is one of the major factors limiting hydrolysis. Inhibition can be alleviated by

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supplemental β-glucosidase or by removing the inhibitors by fermenting them to a less inhibitory product, e.g ethanol. Trichoderma harzianum E58 has high levels of β-glucosidase activity, and is able to achieve high conversion of the cellulose to glucose. Cellulase, added at an enzyme loading of 25 FPU and 17 IU β-glucosidase/g Solka Floc BW300, was able to cope with end-product inhibition of β-glucosidase by the accumulated glucose. Once the problem of end-product inhibition is removed, the major impediment to hydrolysis is the substrate. A faster rate of reaction could be obtained by increasing the susceptibility of the substrate to enzymatic attack.

Crystallinity and surface area accessible to the enzyme were investigated to observe the relationship between structural features of the substrate and its hydrolysis. The relationship between initial crystallinity and hydrolysis appeared to be bimodal. For crystallinities ranging from 80-100, a slight decrease in crystallinity was accompanied by a large increase in its hydrolyzability. No clear pattern between accessible surface area of the substrate and its hydrolysis emerged. The changes occurring to the crystallinity and surface area of the substrate, Solka Floc BW300 during hydrolysis were monitored. A slight increase in the crystallinity was found during the hydrolysis period of 4 days. The accessible surface area increased drastically in the initial phase of hydrolysis, then slowly declined through the remainder of hydrolysis. The structural changes could not be correlated to the rapid decline in the rate of hydrolysis during this time. The substrate becomes more resistant to enzymatic attack during hydrolysis due to structural

changes. The extent to which the substrate becomes inaccessible to enzymatic attack was examined. Cellulose was recovered at various times during hydrolysis, the bound cellulases desorbed, and the "spent" cellulose treated with fresh cellulase. A 70% drop in the accessibility of the substrate to hydrolysis was noted; this indicated that the substrate became more recalcitrant during hydrolysis.

The objective of a cellulose conversion process is to obtain high yields of glucose, using low enzyme loadings and short residence times. There are two main routes for further research into this area: to use a more "active" enzyme system or to increase the susceptibility of the substrate. The reaction rates of cellulase on amorphous cellulose are rapid, achieving 90% conversion to reducing sugars at low (30 FPU/g) enzyme loadings after 24 hours. So, it is the structure of the substrate, not the activity of the enzyme which primarily limits hydrolysis. In addition, the greatest problems in the course of hydrolysis e.g. adsorption of the cellulases to the substrate, mass transfer at high substrate concentration and long residence times, are due more to the insoluble and recalcitrant nature of the substrate. Treatment of the substrate prior to, or during, hydrolysis would probably be a promising area on which to concentrate further research.

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APPENDIX A

Determination of Maximum Enzyme Adsorbed and Enzyme Affinity

Many investigators have found that the extent of adsorption increases as enzyme concentration increases - the adsorption behaviour obeying the Langmuir Adsorption Isotherm (Mandels et al., 1977; Lee et al, 1982). Using the data generated in Figures 14 and 16 the maximum amount of enzyme and protein able to adsorb to the substrate, and the relative affinity of the cellulases to the substrate can be determined using the Langmuir isotherm equation. The Langmuir adsorption isotherm equation relates the adsorbed enzyme or protein concentration to the free enzyme, or protein concentration according to the equation:

$$\frac{X}{X_m} = \frac{K_1 E}{1 - K_1 E}$$

Where X = activity adsorbed/g substrate

   X_m = maximum activity adsorbed/g substrate

   K_1 = half-saturation constant for enzyme $(IU/mL)^{-1}$

   E = free enzyme activity in filtrate (IU/mL)

The isotherm equation can be rearranged to give:

$$\frac{1}{X} = \frac{1}{E K_1 X_m} + \frac{1}{X_m}$$

Where a plot of $1/E$ versus $1/X$ will give a straight line with the slope $1/K_1 X_m$ and the intercept $1/X_m$. Similar equations are obtained for the protein adsorption, by exchanging P (mg/mL) with E (IU/mL) and P_{ads} (mg/g substrate)

for X (IU/g substrate). The parameters, K_1 and X_m for Solka Floc BW300 were calculated by linear regression. Although the Langmuir isotherm is a useful equation, it must be kept in mind that other factors are present which the Langmuir equation does not take into account, e.g. formation of a cellulase complex on the substrate during adsorption, the heterogenuous nature of the substrate, the multiplicity of the enzyme system.
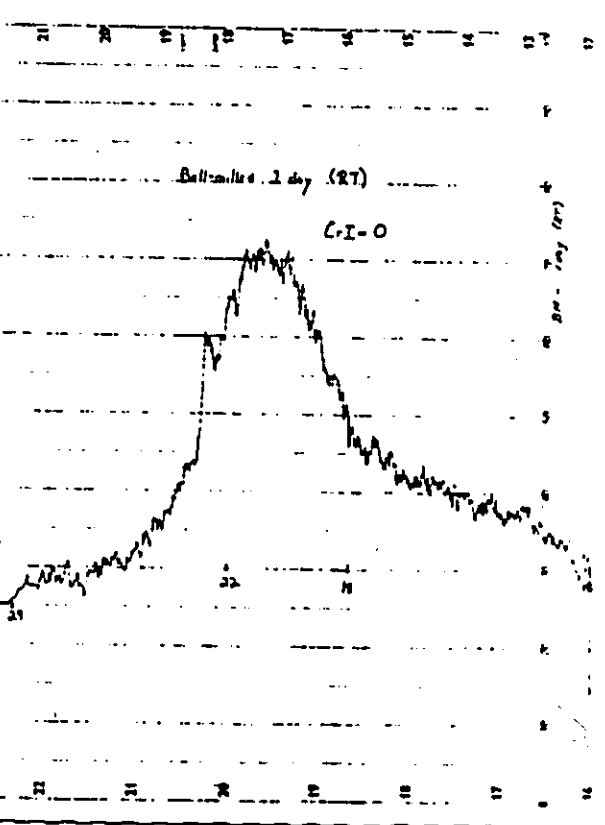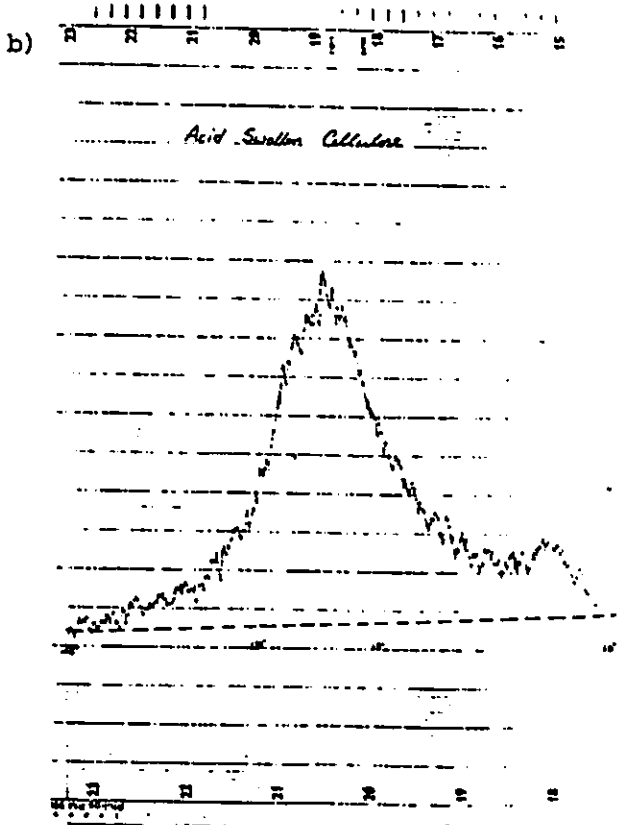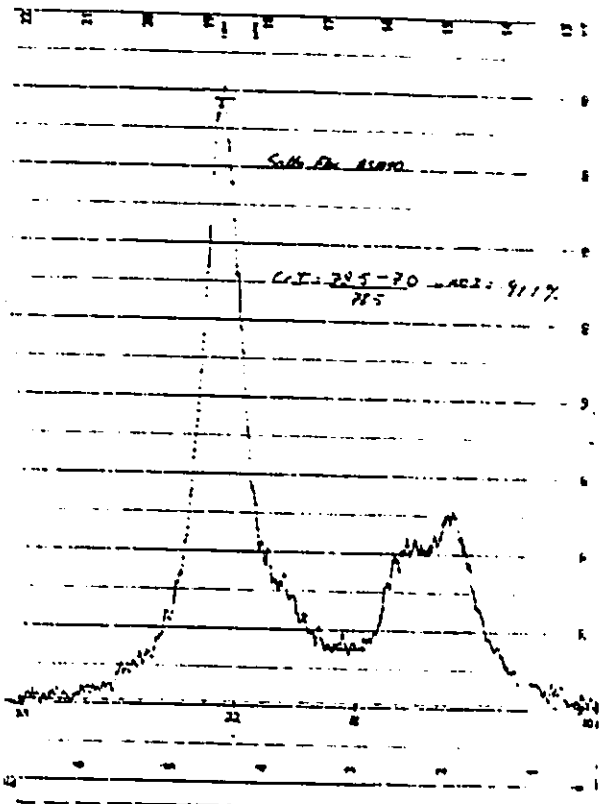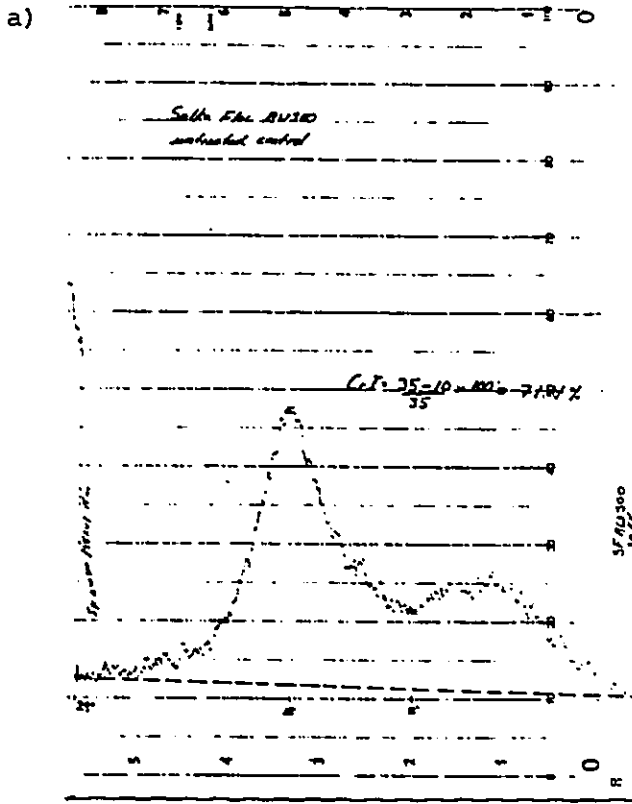
APPENDIX B

Crystallinity indices for commercial celluloses and
pretreated celluloses as determined by x-ray diffractometry.

Substrate	Crystallinity Index
Sigmacell 50	90.8
CF11	95.6
Avicel	92.8
Solka Floc AS1040	91.1
Solka Floc SW40	84.9
Solka Floc BW300 (Brown & Co.) air-dried	84.9
Solka Floc BW300 (Brown & Co.). treated with 0.4% NaOH then solvent-dried	83.3
Solka Floc BW300 (Brown & Co.) boiled for 5 minutes, then freeze-dried	80.7
Solka Floc BW300 (Brown & Co.)	71.4
Solka Floc BW300 (General Filtration)	62.5
Solka Floc BW300 (Brown & Co.) ball-milled 1 day	0.1
2 days	0.1
4 days	0.1
7 days	0.1
14 days	0.1
Whatman crystalline cellulose phosphoric-acid swollen	0.1

Unless otherwise stated the samples were water-swollen then
solvents dried prior to the crystallinity determination.

X-ray diffractograms of a) crystalline celluloses   b) amorphous celluloses and c) FeTNa- swollen cellulose.

a)

b)

c)

Fe T/Ho - Swollen - SF AU 300

$$C.I. = \frac{55.5-5}{55.5} = 91.0\%$$