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Review

Recent Advances in Cellulase Technology*

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Interest in studies of cellulase activities of micro-organisms has increased in recent years because it is hoped that such studies may contribute to the solution of some of our current pressing economic problems. Cellulose is the only organic material that is annually replenishable in very large quantities. The utilization of this resource is greatly simplified if cellulose is first hydrolyzed to its monomer, glucose. This conversion could be accomplished by either acid or enzymatic hydrolysis. When using acid, expensive corrosion proof equipment is required. Moreover, the crystalline structure of cellulose makes it very resistant to acid so that the temperature and acid concentration needed to achieve hydrolysis also cause decomposition of the resulting sugars so that yields of glucose are low and the syrups contain unwanted by-products and reversion compounds. The enzymes on the other hand are specific for cellulose and related polysaccharides and they do not react with impurities that may be present in waste cellulose. Moreover, the reaction takes place at moderate conditions so glucose is not degraded and enzymatically produced syrups are fairly pure and constant in composition. This paper is a review of the recent efforts at Natick to develop a practical process for enzymatic saccharification of waste cellulose and produce cheap technical glucose.

History of Cellulase Research at Natick

Until recently at Natick our research effort on cellulases was chiefly directed to prevention of microbial degradation of cellulosic materials such as tents, clothing, and sand bags. Early studies carried out by the taxonomists, Dr. William Weston and Dr. Lawrence White, involved collecting and identifying the organisms active in cellulose degradation. This resulted in the QM collection of over 14,000 fungi active in degradation of material such as wool, leather, cellulose, and other polysaccharides. This collection is now housed at the University of Massachusetts in Amherst. Its cultures are freely distributed to investigators all over the world. Recently we had the honor of adding Dr. Toyama's famous *Trichoderma* strains B1A (QM 9973) and LE (QM 9974) to this collection.

In the late 1940's Dr. Elwyn Reese began studying the mode of attack of fungi on cellulose and the cellulolytic enzymes elaborated by them. He noted that although many fungi degrade cellulose in nature, very few produce culture filtrates active against insoluble cellulose. This led him to the multiple enzyme C_1, C_x concept in 1950,[1] preceding by many years the isolation of the endo and exo glucanases recognized today. It also led him to appreciate the unique qualities of *Trichoderma* as a source of active cellulase containing

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all of the components required for hydrolysis of insoluble cellulose. In 1964[2] we showed that crude *Trichoderma* cellulase could totally hydrolyze cotton and succeeded in separating this cellulase on DEAE Sephadex into 2 fractions which we considered to be C_1 and C_x because they acted synergistically in hydrolyzing cotton. In 1968 Katz and Reese[3] reported the production of 30% glucose syrups from concentrated cellulase acting on ball milled cellulose pulp. Our interest shifted from prevention of deterioration to the serious development of a saccharification process.

Trichoderma Cellulase

Although many fungi and bacteria degrade cellulose, the products of growth on cellulose are microbial cells and metabolic products such as CO_2 and methane. Only a few fungi have been reported to produce high levels of enzyme capable of extensively degrading insoluble cellulose to soluble sugars *in vitro*. Rapid growth on and decomposition of cellulose and/or production of high levels of enzyme degrading soluble cellulose derivatives are not adequate criteria for selecting organisms to be used as a source of a stable cell-free enzyme preparation for use in practical saccharification of waste cellulose. For example, the cellulase of *Pestalotiopsis westerdijkii* (PW) is incomplete (Fig. 1). It contains high levels of *endo* β glucanases and cellobiase, but is lacking in C_1 activity. When insoluble cellulose was hydrolyzed by this enzyme, the reactive portion was rapidly hydrolyzed, but the rate then slowed down as the residue was increasingly resistant. The available or reactive portion ranged from less than 2% of cotton to about 24% of Sweco 270. The cellulase of *Trichoderma viride* (TV) is a complete cellulase (Fig. 2). When insoluble cellulose was hydrolyzed by this enzyme the reactive cellulose was very rapidly hydrolyzed, followed by a slower hydrolysis of the more resistant portions which continued until all available cellulose had been hydrolyzed. The extent of hydrolysis in 48 hours ranged from 6% for cotton to over 90% for Sweco 270. With long incubation even cotton would be totally hydrolyzed.

The difference between the two enzymes was most clearly evident when culture

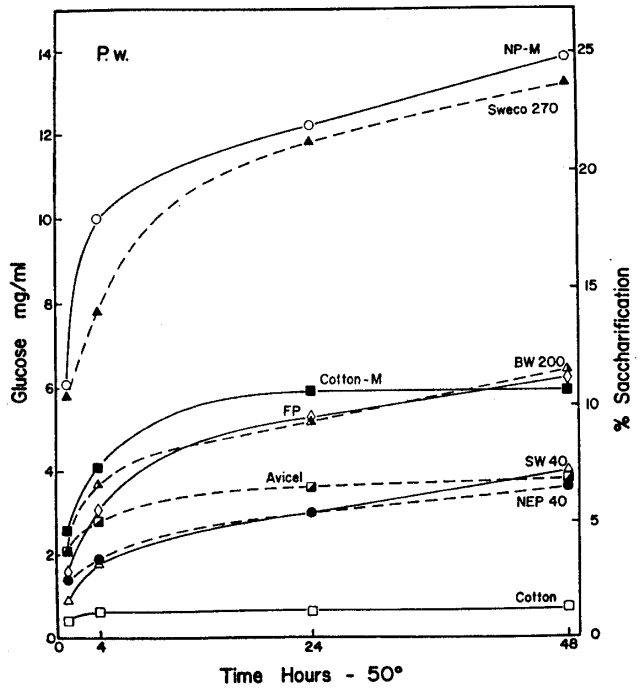

Fig. 1. Hydrolysis of insoluble cellulose by a C_x cellulase from *Pestalotiopsis westerdijkii*.
(Reproduced with permission from reference 6.)
5% Cellulose incubated at pH 4.8, 50°C with a filtrate of strain QM381 grown on cellulose medium. The enzyme preparation had 0.6 mg protein, 19 C_x units, 0.1 FP cellulase units per ml and a C_1 activity of 0.22 mg of glucose per 24 hours.

○—○ Newspaper, Sweco ball milled
▲—▲ Pure cellulose pulp, Sweco ball milled, 270 mesh
◭—◭ BW 200, pure cellulose pulp, ball milled, Brown Co., Berlin, NH
◇—◇ Whatman no. 1 filter paper
■—■ Ball milled absorbent cotton
◪—◪ Avicel pH 105, microcrystalline cellulose
△—△ Pure cellulose pulp SW 40, Brown Co.
●—● Hammer milled newsprint NEP 40, Brown Co.
□—□ Absorbent cotton, fibrous

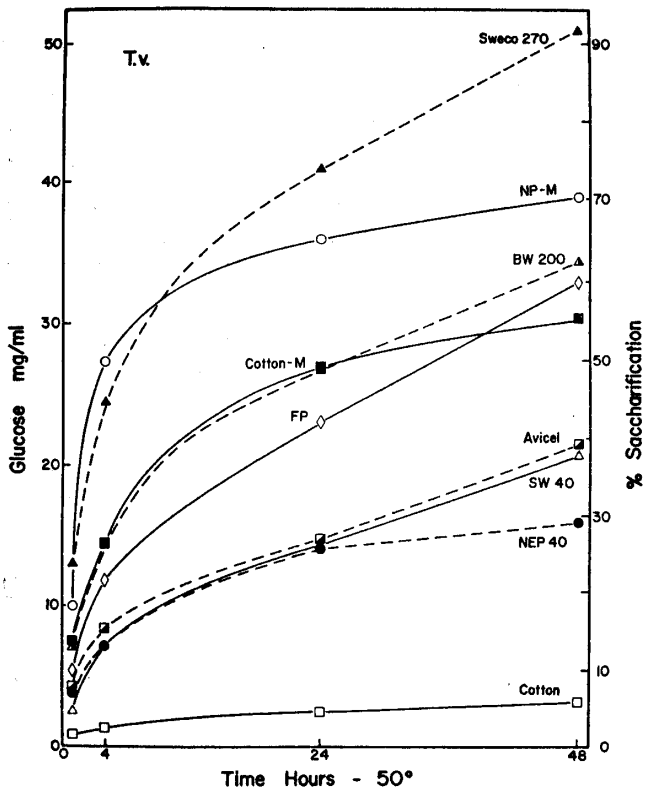

Fig. 2. Hydrolysis of insoluble cellulose by a complete cellulase from *Trichoderma viride*.
(Reproduced with permission from reference 6.)
5% Cellulose incubated at pH 4.8, 50°C with a filtrate of strain QM9414 grown on cellulose medium. The enzyme preparation had 0.8 mg protein, 25 C_x units and 0.6 FP cellulase units per ml and a C_1 activity of 2.8 mg of glucose per 24 hours.
○—○ Newspaper, Sweco ball milled
▲—▲ Pure cellulose pulp, Sweco ball milled, 270 mesh
◭—◭ BW 200, Pure cellulose pulp, ball milled, Brown Co., Berlin, NH
◇—◇ Whatman no. 1 filter paper
■—■ Ball milled absorbent cotton
◪—◪ Avicel pH 105, microcrystalline cellulose
△—△ Pure cellulose pulp, SW40, Brown Co.
●—● Hammer milled newsprint, NEP 40, Brown Co.
□—□ Absorbent cotton, fibrous

filtrates from the two organisms were diluted to equal activities on CMC or equal protein content and allowed to act on filter paper (Fig. 3). Initial hydrolysis by both preparations was rapid, but action by the incomplete cellulase (PW) leveled off after 30 minutes when the available substrate was all used up, while action by the complete cellulase (TV) continued on the more resistant portions of the substrate although at a slower rate. Measurement of cellulase activity therefore presents special problems that do not arise for enzymes hydro-

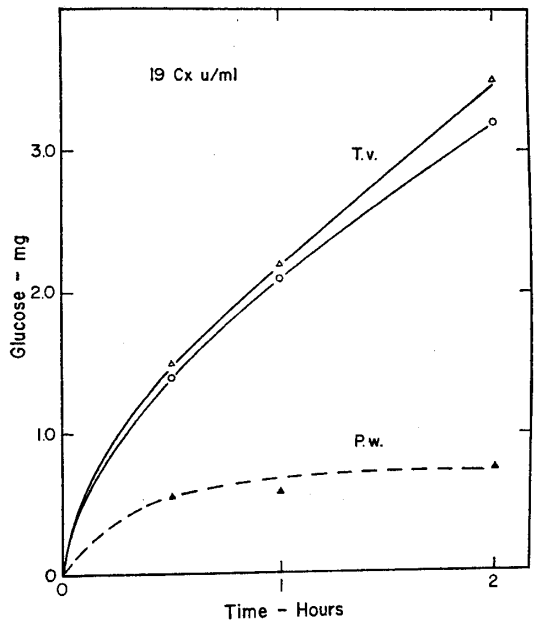

Fig. 3. Hydrolysis of filter paper by cellulase preparations from *Trichoderma viride* and *Pestalotiopsis westerdijkii* adjusted to equal activities on carboxymethyl cellulose.
(Reproduced with permission from reference 6.)
Culture filtrates diluted to 19 C_x units per ml. 0.5 ml enzyme+1 ml pH 4.8 buffer+50 mg paper. Incubated at 50°C.
△—△ Tv QM9123 Culture Filtrate
○—○ Tv QM9414 Culture Filtrate
▲—▲ Pw QM381 Culture Filtrate

lyzing soluble substrates. There is no absolute unit as can be measured for a single enzyme acting on a soluble substrate. The more resistant the substrate and the longer the time of exposure, the greater the difference between complete and incomplete cellulases. The unit value will depend on the substrate chosen, its concentration and the extent of conversion.

The filter paper assay[4] as developed at Natick has been used for a simple, easily reproducible measurement of saccharifying cellulase to predict the action of the enzyme in extensive conversion of concentrated cellulose slurries from a reasonably short assay based on limited conversion of a much smaller quantity of substrate (Fig. 4). Filter paper activity is the mg of glucose produced when 0.5 ml of enzyme solution acts on 50 mg of Whatman no. 1 filter paper at pH 4.8, 50°C, for 1 hour. Filter paper units (Fig. 5) are calculated as the micromoles of glucose produced per minute in the above assay based on the enzyme dilution to give 2.0 mg of glucose. The cutoff value of 2.0 mg was chosen because the hydrolysis curve is fairly linear to above this value and it represents 4% hydrolysis of the filter paper, well over the value that could be expected from an incomplete cellulase.

Most culture filtrates even from very active cellulose degraders including the thermophiles show low filter paper unit values and limited conversions of shredded or ball milled

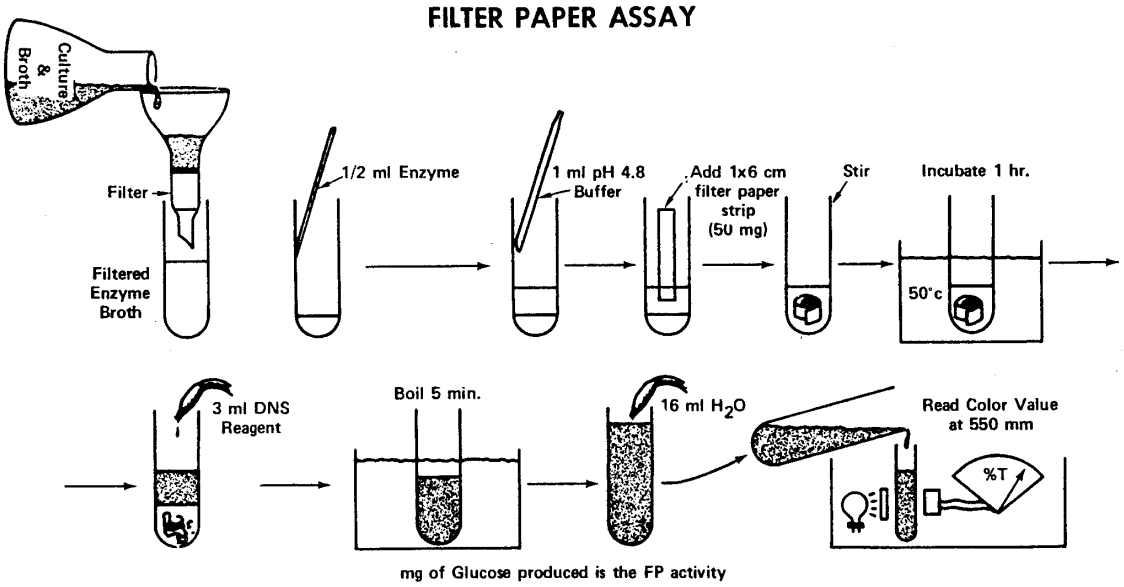

Fig. 4. Filter paper assay procedure. (Reproduced with permission from reference 4.)

1. Whatman no. 1 filter paper cut into 1×6 cm strips (50 mg)
   Buffer=0.05 M Na citrate pH 4.8
   Glucose standards in buffer
   Dinitrosalicylic Acid (DNS) Reagent for reducing sugar (27)
2. Filter or cent rifuge culture sample to remove solids. Dissolve enzyme powders at 1.0–5.0 mg/ml in buffer. Dilute enzyme solutions in buffer.
3. Place 0.5 ml enzyme solution and 1.0 ml buffer in 18 mm test tube. Add a filter paper strip and mix on Vortex mixer to coil the paper in the solution. Incubate 1 hour at 50°C. Add 3 ml DNS reagent to stop reaction. Place tubes in boiling water for 5 minutes and determine reducing sugar as glucose. Include a blank tube (without filter paper) to correct for any reducing sugar present in the enzyme preparation. The mg of glucose produced in this test is the filter paper (FP) activity.
4. The DNS reagent (27) measures reducing sugar nonspecifically. When glucose is used as a standard values for cellobiose will be about 15% low and values for xylose about 15% high on a weight basis.

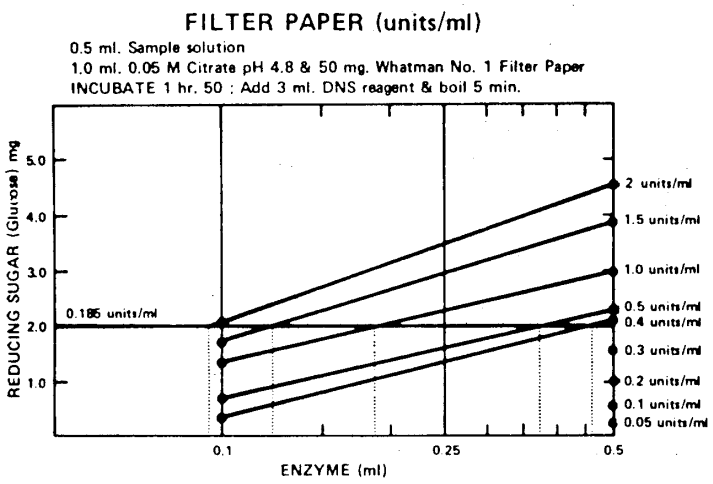

Fig. 5. Measurement of filter paper cellulase units per ml. (Reproduced with permission from reference 4.)

Follow procedure as outlined in Fig. 4. If filter paper activity for 0.5 ml of enzyme is equal to or less than 2.0, units per ml equal FP activity $\times 0.185$. If the FP activity is greater than 2.0, repeat using diluted enzyme and estimate the ml of enzyme required to give a FP activity of 2.0. Units per ml equals 0.185/ the ml of enzyme to give a FP activity of 2.0.

Table 1. *Trichoderma* strains in Natick collection.

QM No	ATCC No	Type	Cellulase FP units per ml
6a	13631	Wild strain	0.5–0.7
9123	24449	Enhanced cellulase mutant derived from QM6a	1.0–1.2
9414	26921	Enhanced cellulase mutant derived from QM9123	1.5–2.0
9136	26920	Cellulase negative mutant derived from QM6a	0

Cellulase activity in shake flasks grown on cellulose media.

These strains are available from Dr. Emory G. Simmons, U.S. Army Development Center Culture Collection of Fungi (QM), Dept. Botany, University of Massachusetts, Amherst, MA 01002 USA, or from The American-Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, MD 20852 USA.

newspaper or other complex cellulose materials as compared to enzyme preparations from the mesophilic fungus, *Trichoderma viride*. This is the reason we have focused our attention at Natick on *Trichoderma* as a cellulase producer. The strain has been treated by irradiation and chemical mutagens to induce mutation (Table 1). Two strains, QM9123[5] and QM9414[6] have been selected that yield higher levels of cellulase. The specific activity of the enzyme and the proportions of the components of the complex are similar to the wild strain.

Enzyme Production

Trichoderma grows readily on nutrient salts plus a carbon source (Table 2). The fungus is a vigorous acid producer when consuming carbohydrate above a minimum rate with NH_4^+ as the nitrogen source (Fig. 6). The rate of acid production is directly related to the rate of sugar consumption.[7] After the carbohydrate is consumed, the pH rises again. Cellulase in this fungus is an induced enzyme produced when the fungus is grown on cellulose, sophorose, lactose, or cellobiose.[8] Pure glucose is not an inducer and glucose strongly represses enzyme production in the presence of cellulose or other inducers.[9] The highest yields are obtained on pure or complex cellulose (Table 3). Although sophorose is a powerful inducer, active at extremely low concentrations, the levels of enzyme produced

Table 2. *Trichoderma viride* medium for cellulase production.

	g/l		mg/l
$(NH_4)_2SO_4$	1.4	$FeSO_4 \cdot 7H_2O$	5.0
KH_2PO_4	2.0	$MnSO_4 \cdot H_2O$	1.6
Urea	0.3	$ZnSO_4 \cdot 7H_2O$	1.4
$CaCl_2$	0.3	$CoCl_2$	2.0
$MgSO_4 \cdot 7H_2O$	0.3		

Cellulose 0.75%–1.0%, Proteose peptone 0.075–0.1%, Tween 80 0.1–0.2%, Initial pH 5.0–6.0

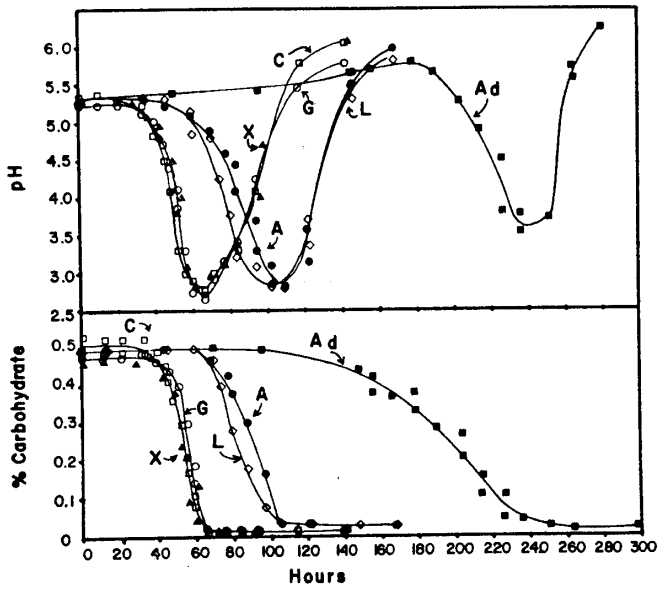

Fig. 6. Growth of *Trichoderma* on soluble sugars. (Reproduced with permission from reference 7.)
QM9414 on 0.5% sugars. Carbohydrate by phenol sulfuric method. Spore inoculum.
○—○ Glucose G
□—□ Cellobiose C
△—△ Xylose X
◇—◇ β Lactose L
○—○ L-Arabinose A
□—□ D-Arabinose Ad

Table 3. Effect of cellulose growth substrate on enzyme production by *Trichoderma viride* QM9414. (Reproduced with permission from reference 16.)

Substrate growth (1%)	Soluble protein (mg/ml)	C_x CMC (μ/ml)	Cellulase FP (μ/ml)	C_1 Cotton (mg glucose/ml)
SW40	1.84	152	1.48	7.6
BW200	1.40	89	1.11	2.4
Sweco 270	1.56	102	1.30	3.5
Avicel pH 105	1.68	144	2.04	3.5
Absorbent cotton	1.84	85	1.30	4.6
Jay bee newspaper	1.28	24	0.93	7.6
NEP40	1.44	48	1.48	7.7
Sweco newspaper	1.44	56	1.11	8.6
Milled computer paper	0.46	22	0.74	5.9

Grown 13 days on *T. viride* medium with 1% cellulose 0.1% proteose peptone, 0.2% Tween 80. Soluble protein and enzyme activities determined on the culture filtrates.

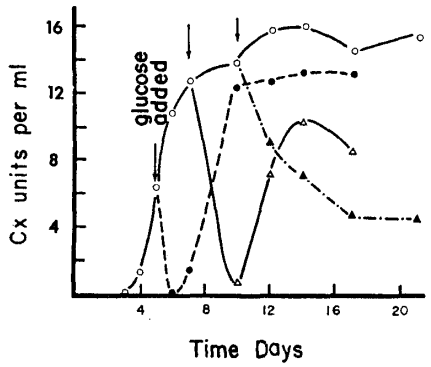

Fig. 7. Inactivation of cellulase by adding glucose to a culture of *Trichoderma* growing on cellulose. (Reproduced with permission from 13.)
○—○ QM6a on 1.0% SW 40 (Hammer milled cellulose pulp). Spore inoculum.
●—● 0.5% Glucose added at 5 days
△—△ 0.5% Glucose added at 7 days
▲—▲ 0.5% Glucose added at 10 days

are not equal to those on cellulose, and in any case the use of this rare sugar for producing enzyme in quantity would not be practical. Commercial glucose containing sophorose as a trace impurity has been used for fermentation production of cellulase,[10] but the yields of enzyme are only about 20% of the yields attained on cellulose as a substrate. Cellulase yields are increased by the addition of Tween 80 and peptone to the medium.[8,11]

If cellobiose, glucose or other rapidly metabolized carbohydrates are added to cultures growing on cellulose and producing cellulase, as the sugar is consumed, the pH falls rapidly and the cellulase enzymes also fall.[12] At pH values below 3.5 β glucosidase is inactivated, below 3.0 considerable filter paper cellulase is lost.[13] This acid inactivation is irreversible. If cellulose is still present in the culture medium, the enzymes will reappear after the glucose is consumed, but if all the cellulose has been consumed, the enzymes will not recover (Fig. 7). However if the pH is controlled at around 5.0 during the consumption of the sugar, the enzyme activities remain stable[7] (Table 4). Thus the apparent "glucose effect" is related to pH. Similar acid production and inactivation occurs when fresh cellulose is added to a cellulose culture[13] (Fig. 8). The cellulase enzymes present in the medium hydrolyze susceptible portions of the added cellulose so rapidly that a "glucose" or pH effect results.

The more rapidly cellulose (or any carbon source) is consumed, the greater the rate of acid production which in turn leads to a loss in cellulase activity. By this means the production and consumption of glucose is limited. When the rate of glucose consumption falls, pH will rise permitting further enzyme accumulation. This system may allow for a negative feedback control over extracellular enzymes. This control mechanism acting on already synthesized enzymes should not be confused with glucose repression.

Table 4. Effect of addition of glucose to a cellulase induced culture with and without pH control. (Reproduced with permission from reference 7.)

	Enzyme Activity				
	pH Uncontrolled		pH Controlled at 5.0		
	0	22	0	22	hrs after glucose addition
β-Glucosidase:	0.15	0	0.18	0.16	units per ml
Saccharifying Cellulase:	0.48	0.32	0.77	0.75	units per ml

QM9414 was grown in a 10 *l* fermenter on 0.5% BW 200 with pH not allowed to drop below 4.0. After pH rose (cellulose consumed), 0.5% glucose was added and pH controlled not to fall below 5.0. Glucose was rapidly consumed. After pH rose again, 0.5 % glucose was added, pH was not controlled and fell to 2.4 as glucose was more slowly consumed.

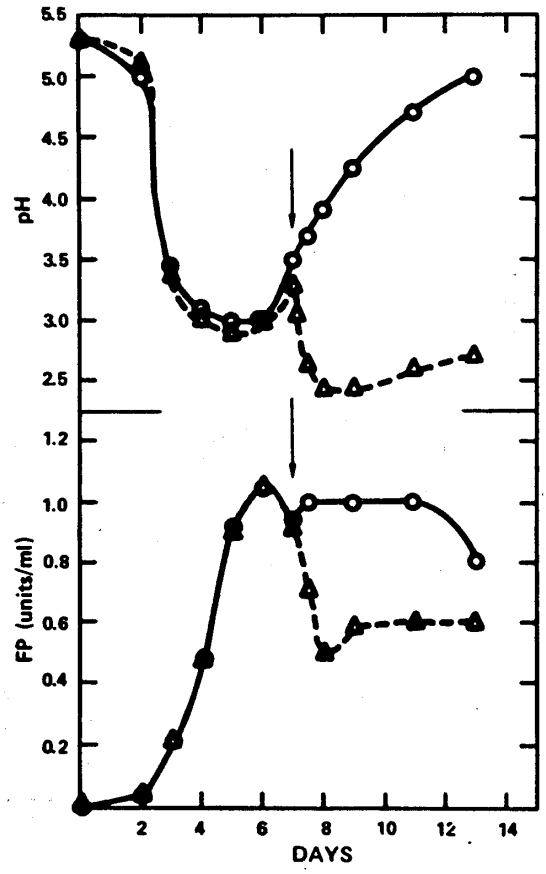

Fig. 8. Inactivation of cellulase by adding cellulose to a culture of *Trichoderma* growing on Cellulose. (Reproduced with permission from reference 13.)
○—○ QM9414 growing on 0.75% BW 200 (Ball milled pulp). Spore inoculum
▲—▲ 0.5% Fresh cellulose added at 7 days

It has been suggested that cellobiose is the true inducer in a cellulose culture, but that yields are low when *Trichoderma* is grown on cellobiose because the cellulase is repressed and/or inactivated by the rapid metabolism of the sugar.[12] In a culture growing on cellulose, cellobiose is slowly released so that repression and inactivation do not normally occur. However we have never achieved significant cellulase yields by slow addition of cellobiose to a culture, nor by maintaining continuous cultures on low levels of cellobiose. We have achieved good yields of cellulase on cellobiose by using a high cellobiose concentration (1% or more) and slowing metabolism by decreased aeration, suboptimal temperature, a marginal nutrient deficiency or a marginally toxic excess of trace metals.[12] Or excellent yields of cellulase on cellobiose can be attained by adding surfactants such as Tween 80 or sodium oleate to a 1% or more cellobiose culture.[11] If we use lower concentrations of cellobiose, around 0.5% we can not achieve good yields by these means. Thus the question remains open as to whether cellobiose is in fact the true inducer or whether an inducer is produced from cellobiose perhaps by a transferase. Soluble inducers which appear to be glucose trimers (predominantly β 1,6 β 1,4 linked) have been isolated from cellobiose media after growth of *Trichoderma*.[12]

When *Trichoderma* spores are inoculated into glucose medium, there is a lag of one or two days and then the glucose is rapidly consumed, yielding about 0.4 g of mycelium and 0.07 g of extracellular soluble protein per gram of glucose consumed. Maximum rate of glucose consumption is about 0.25 mg/ml per hour. Only traces of cellulase are produced on pure glucose.

In a cellulose batch culture inoculated with spores (Fig. 9) there is a lag of 30 or more hours before active growth begins as indicated by pH fall. The weight of insolubles increases

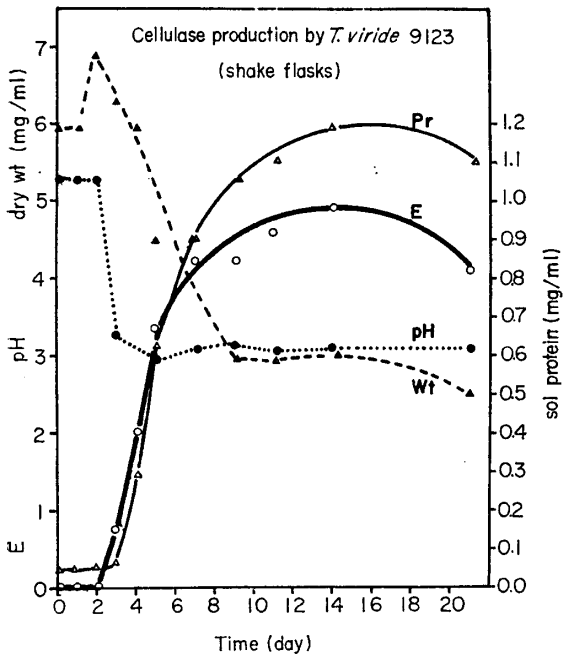

Fig. 9. Growth of *Trichoderma* on cellulose medium. QM9123 on 1% cellulose pulp, shake flask, spore inoculum.
●—● pH
▲—▲ Wt mg/ml includes residual cellulose
○—○ Enzyme-Filter paper activity (supernatent)
△—△ Soluble protein mg/ml (supernatent)

as peptone and Tween 80 are taken up and then falls as cellulose is consumed. Mycelial weight can not be estimated from the protein values. The yield of protein per gram of cellulose is about 0.12 g for QM6a and 0.24 g for QM9123, or 9414, both significantly higher than the yield from glucose. PH falls to about 2.8 during consumption of the cellulose and begins to rise a day or so before the cellulose is consumed. After the cellulose is consumed production of cellulase ceases.

We have made many attempts to improve cellulase yields in batch cultures.[7] Use of mycelial inoculum shortens the lag that occurs when spores are used, and mycelial inoculum grown on cellulose is superior to inoculum grown on glucose. Growth is more rapid at 30° but enzyme yields are higher at 25°–28°. The addition of peptone at one-tenth the cellulose level stimulates growth and enzyme production. With 1% cellulose the best yields have been attained in fermentations without pH control (Fig. 10). When pH is controlled, it is found that the less the pH is allowed to fall the less enzyme is produced although growth is markedly stimulated. A possible explanation for this effect is that at higher pH's closer to the optimum (4.8) for the enzyme the cellulase enzymes are more efficient releasing more soluble sugar per unit of enzyme and thus leading to catabolite repression of the cellulase. An interesting feature of growth on cellulose is the close relationship between secretion of extracellular protein and cellulase activity (Fig. 11). The specific activity of the enzymes acting on crystalline cellulose is very low, for example about one filter paper unit per mg of protein.[4]

Trichoderma can be grown continuously on cellulose but the cellulase yields are not equal to those from batch cultures. When *Trichoderma* is grown continuously on glucose the culture can be maintained at a rapid growth rate with a μ Max of about 0.2 hr^{-1}, but a continuous culture on cellulose could not be maintained at such a growth rate. Maximum cellulase productivity of about 0.2 units per ml per day was achieved at a dilution rate of 0.02 hr^{-1}.[6] When the dilution rate was raised above this level, soluble protein and cellulase in the effluent declined rapidly and dry weight (cellulose) rose. It is interesting that Brown also found a minimum residence time of about 50 hours required for cellulase production in continuous culture on glucose (plus sophorose) medium.[10]

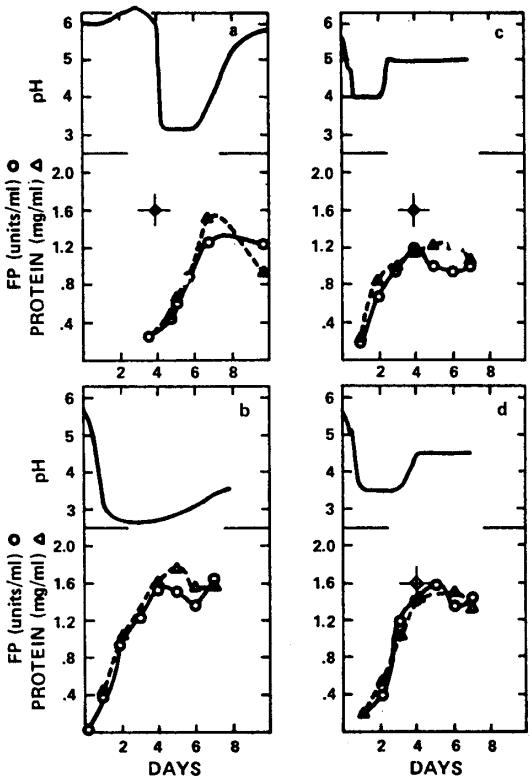

Fig. 10. Growth of *Trichoderma* in 10 *l* fermenter culture QM 9414 on 0.75% cellulose pulp.
(Reproduced with permission from reference 13.)
(a) Spore inoculum-no pH control
(b) 10% Mycelial inoculum-no pH control
(c) 10% Mycelial inoculum-pH controlled not to drop below 4.0 nor rise above 5.0
(d) 10% Mycelial inoculum-pH controlled not to drop below 3.5 nor rise above 4.5
—— pH (recorder tracing)
○—○ Filter paper units/ml (supernatent)
△—△ Soluble protein mg/ml (supernatent)
▲—▲ FP units in (b) at 4 days

Growth of a fungus on the insoluble substrate cellulose is more complicated than growth on a simple soluble carbohydrate. Cellulose is a multiple substrate which is broken down to soluble sugars and finally glucose by a series of reactions catalyzed by a complex of enzymes. Some of these reactions almost certainly are involved in changing the more crystalline and resistant forms of the cellulose to less crystalline and more susceptible forms. Only the final soluble products can be absorbed by the fungus and utilized for growth. The enzymes involved in cellulose breakdown are not constitutive; they are induced

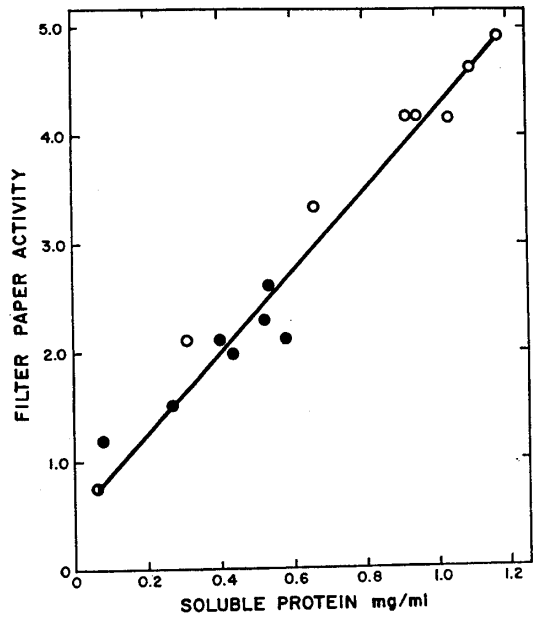

Fig. 11. Relations between soluble protein and cellulase activity.
(Reproduced with permission from reference 5.)
Cultures were grown on 0.5% cellulose plus 0.05% proteose peptone and 0.2% Tween 80. Filter paper activity and soluble protein in the supernatant were measured frequently over a 21-day growth period.
● QM6a ○ QM9123

directly or indirectly by products of their action; yet the fungus must grow and produce mycelium before it can secrete these enzymes and the synthesis of cellulolytic enzymes is strongly repressed by the soluble products of their action and levels controlled through a feedback mechanism related to rapid metabolism and acid production. There are advantages of this repression to the fungus since excess sugars are not available to competing organisms.

In nature the fungus grows initially on soluble materials in the environment. When these are exhausted, hemicelluloses and amorphous cellulose are attacked. Only when all other substrates are exhausted are the enzymes to attack crystalline cellulose produced. A similar process occurs in a batch culture. Most media incorporate low levels of a soluble substrate to initiate growth. When the soluble substrate is gone, the fungus is derepressed and low levels of cellulolytic enzymes are produced. Hydrolysis of cellulose releases inducers and cellulase levels rise rapidly until feedback controls intervene to slow down the rate of cellulose breakdown. A drop in enzyme level is frequently observed in cultures at this point.

The situation in a continuous culture does not favor rapid growth and enzyme production on cellulose. If the cellulase level is high, the amorphous portion of added cellulose is rapidly hydrolyzed producing sugars that inactivate the already formed enzyme and/or repress further enzyme production. The crystalline portion of the added cellulose then is broken down very slowly. but adsorbs enzyme and carries it off with the harvest insolubles. Controlling pH at higher levels prevents enzyme inactivation and leads to more rapid consumption of the cellulose but reduces enzyme yield.

Saccharification Technology

For simplicity and economy culture broths from cellulose fermentation can be used directly for saccharification. Optimum saccharification conditions in a stirred tank reactor are pH 4.8, 50°C. Cellulase is a very stable enzyme. Broths can be kept indefinitely under refrigeration and little activity is lost during prolonged digestion at 50°C. Culture filtrates can be rapidly concentrated by the use of Amicon or Abcor ultrafiltration membranes of 10,000–30,000 molecular weight cutoffs with little loss of activity and precipitated by 66% acetone with no loss of activity. The enzyme is remarkably stable to inhibitors.[14] A few heavy metals inhibit at about 10^{-3} M, but Merthiolate at 0.01% can be safely used as a preservative. Inhibitors such as the halogenators and reduced anthocyanins are unstable and would not be present in substrates.

There are severe constraints on the enzymatic conversion of cellulose due to its insolubility, its high degree of crystallinity, and its admixture with impurities, chiefly lignin, that restrict access of the enzyme to the glycosidic bonds.[15] Therefore in order to obtain glucose yields of 50% in 24 to 48 hours, it is usually necessary to pretreat the cellulose[16] (Table 5). Chemical pretreatments to remove lignin, swelling the cellulose with acid or alkali, or dissolving and regenerating cellulose increase the availability of cellulose but yield low bulk density products such that maximum solids content in a batch reactor will be about 7.5%. Physical pretreatments such as shredding or grinding do not remove lignin or swell the cellulose, but they reduce particle size and increase available surface. Cellulose is difficult to mill efficiently because of its fibrous and resilient nature. It gives without breaking, and the fibers tend to build up on the walls of the chamber or on top of the grinding media. Screens and small orifices become clogged. Hammer milling, fluid energy milling, and wet colloid milling gave good size reduction, but at high cost and with only moderate increases in enzyme susceptibility. The most successful milling treatment is ball milling

Table 5. Pretreatment of newspaper. (Reproduced with permission from reference 16).

Pretreatment 5% Newspaper	% Saccharification	
	4 hr	24 hr
Granulator-comminuter 0.12	15	20
Boiled-wet	9	21
Soaked 20°-wet	13	24
Hammer mill, Jay Bee	12	24
Jet pulverized-single	16	26
Colloid mill-0.001-wet	17	27
2% NaOH-wet	14	28
Varikinetic-wet	16	30
Mulched Mighty Mac	24	31
Viscose-wet	30	44
Cuprammonium-wet	35	52
Sweco mill	28	53
Pot mill	50	65

Jay bee hammer milled newspaper served as the feed for the other treatments (except the mulcher). Samples from wet treatments were evaluated without drying. Saccharification was at 5% with *Trichoderma viride* QM9123 cellulase culture filtrate 1.2 filter paper units per ml.

(Fig. 12, 13) which reduces crystallinity as well as particle size yielding products of maximum availability and high bulk density so that suspensions of 10–20% can be easily handled. For pure cellulose heating at 200°C during or before milling has been used to increase the efficiency of milling, but this has been avoided with complex cellulose for fear of cross reactions with impurities. Ball milling is energy intensive and the cost has been estimated at $0.10 per kg of cellulose or $0.20 per kg of glucose assuming a 50% conversion.

Saccharification of pure cellulose was studied in one liter stirred tank reactors by Ghose.[17~19] A continuous process was established using a feed of 10% milled pure cellulose in unconcentrated culture filtrate and with a 40-hour retention time yielding an effluent of 5% glucose (Fig. 14). A system was devised whereby the enzyme and undigested cellulose could be retained in the reactor and the glucose syrup continuously removed through an

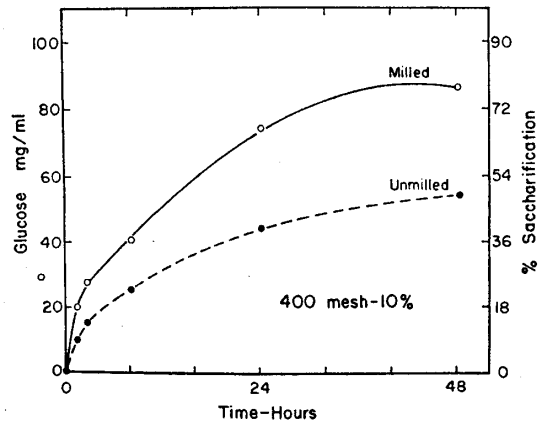

Fig. 12. Effect of milling on susceptibility of cellulose to cellulase.
(Reproduced with permission from reference 16.)
400 Mesh (38 μm) cellulose at 10% saccharified with Tv QM9123 cellulase, 1.76 mg protein, 1.4 FP cellulase units per ml.
○—○ 400 Mesh fraction from Sweco milled pure cellulose pulp
●—● 400 Mesh fraction from unmilled pure cellulose pulp

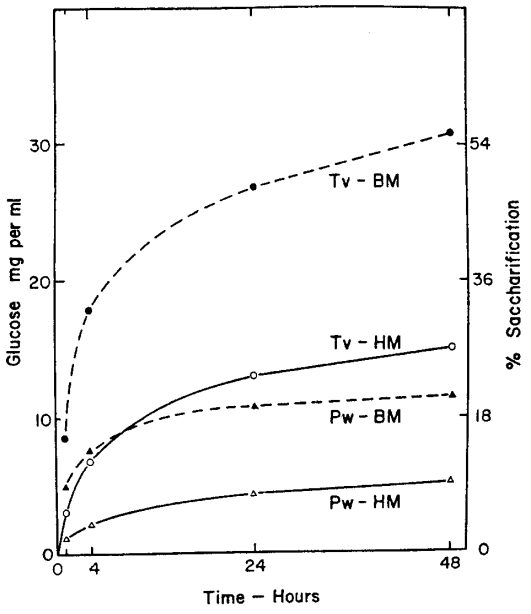

Fig. 13. Effect of enzyme pretreatment on hydrolysis of newspaper by cellulase.
Cultures grown on 1% cellulose for 14 days.
Tv = *Trichoderma viride* QM9414 1.85 filter paper units/ml
Pw = *Pestalotiopsis westerdijkii* QM381 0.14 filter paper units/ml
HM = Hammer milled newspaper 5%
BM = Sweco ball milled newspaper 5%

ultrafiltration membrane of 10,000–30,000 molecular weight cutoff. Water, makeup enzyme, and cellulose were added to the reaction vessel to maintain a continuous process. The advantages to this process were (a) the enzyme was retained in the reaction vessel for further use, (b) the undigested cellulose was also retained so that conversion was increased, (c) removal of the products increased the rate of the reaction, and (d) the glucose was removed as a clear aqueous syrup free of cellulose and protein.

The system was simplified and improved by taking advantage of the fact that milled cellulose strongly adsorbs cellulase at 25–50°C, pH 4.0–5.0, over a range of particle sizes from 7–50 μ in diameter, that is, under conditions optimum for enzyme action[20] (Fig. 15). The adsorbed enzyme was sufficient to digest the cellulose with no replenishment of enzyme even though the liquid phase was continuously removed (Fig. 16). As cellulose was digested the released enzyme was readsorbed on excess or freshly added cellulose with retention of activity. As long as the cellulose concentration in the reactor is kept high, the enzyme is adsorbed and the glucose syrups can be removed through a coarse filter retaining the undigested cellulose and adsorbed enzyme in the reactor, thus eliminating the need for high pressures and expensive membranes.

The above studies were carried out on pure cellulose. Continuous processes have not

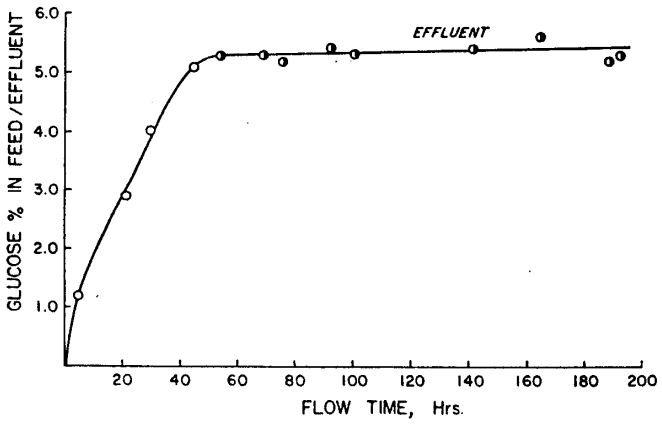

Fig. 14. Continuous saccharification of 10% cellulose. (Reproduced with permission from Cellulases and Application [18].)
Trichoderma QM6a cellulase, FP Activity 1.0+10% heated milled pure cellulose pulp at pH 4.8, 50° in stirred tank reactor 4.0 *l* volume. Operated 40 hours as batch ○—○ and then as a continuous slurry ◑—◑ D=0.025 hr^{-1}.

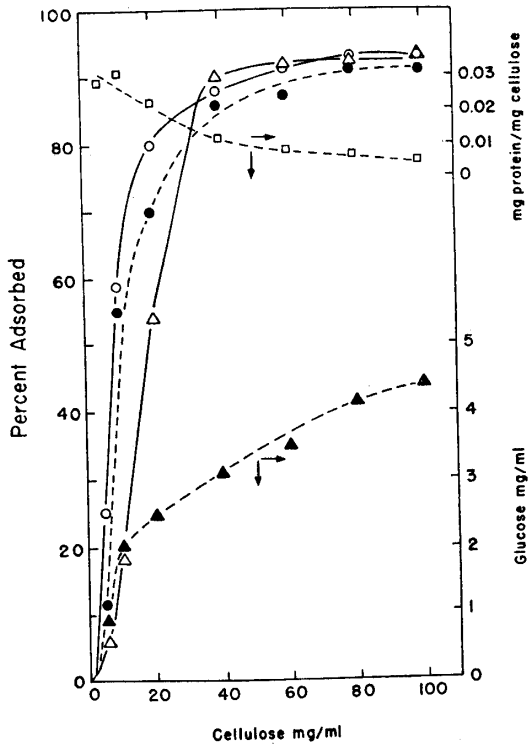

Fig. 15. Effect of cellulose concentration on adsorption of cellulase by cellulose.
(Reporduced with permission from reference 20.)
Unconcentrated cellulase (0.56 mg protein/ml, 0.7 filter paper units per ml, 18 C_x units per ml mixed with milled cellulose (400 mesh average particle size 30 microns) at pH 4.7 and incubated at 25°C for 30 minutes.
○—○ Protein adsorbed %
●—● Cellulase (F.P.) adsorbed %
△—△ Cellulase (C_x) adsorbed %
▲—▲ Glucose produced mg/ml
□—□ Mg protein adsorbed/mg cellulose

been developed for complex celluloses because of the rapid build-up of lignin and other unhydrolyzable materials. These residues also adsorb enzyme and satisfactory processes for recovering enzyme from them have not been developed. However we have studied the hydrolysis of complex cellulose in batch process. More than 100 examples of typical cellulosic waste materials have been sent to us by agricultural operators or companies who hope to find a profitable use for them.[16,21] These have been evaluated in 100 ml volume as 5% slurries in shake flasks using one to two filter paper cellulase units per ml. Samples were tested as received, after drying if the original condition was wet, and after ball milling. The last gives an estimate of the total hydrolyzable cellulose content (Table 6).

Total U.S. municipal wastes in 1973 were equal to 130 million tons, 40–60% of this is cellulose. A number of processes such as the Black Clawson (wet) or Bureau of Mines

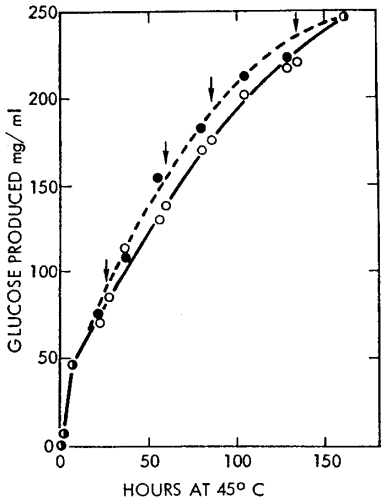

Fig. 16. Digestion of cellulose by adsorbed enzyme with replenishment of the cellulose.
(Reproduced with permission from reference 20.)
10% milled cellulose (400 mesh) was incubated with concentrated cellulase (2.08 mg protein/ml) in shaken flasks at pH 4.8, 45C. At ↓ cellulose (22 hr 59 mg/ml, 55 hr 37 mg/ml, 80 hr 29 mg/ml, 130 hr 33 mg/ml) was added to all flasks to restore the concentration to about 10%. One hour later the series B flasks were centrifuged, the supernatant removed, and replaced with 0.05 M citrate buffer.
●—● Series A. not centrifuged
○—○ Series B. centrifuged at ↓, supernatant removed and replaced with buffer.

Table 6. Hydrolysis of cellulose by *Trichoderma viride* cellulase.

Substrate	% Saccharification			
	1 hr	4 hr	24 hr	48 hr
Pure cellulose				
Cotton-fibrous	1	2	6	10
Cotton-ball milled	14	26	49	55
Cellulose pulp-SW40	5	13	26	37
Cellulose pulp-ball milled	23	44	74	92
Waste cellulose				
Bagasse	1	3	6	6
Bagasse-ball milled	14	29	42	48
Rumen fibers	7	12	16	—
Rumen fibers-ball milled	35	49	51	—
Newspaper-shredded	10	24	31	42
Newspaper-ball milled	18	49	65	70
Corrugated fibreboard-shredded	11	27	43	55
Corrugated fibreboard-ball milled	17	38	66	78
Black Clawson fiber	5	11	32	36
Black Clawson-ball milled	13	28	53	56
Bureau of mines-cellulose	7	16	25	30
Bureau of mines-ball milled	13	31	43	57
Paper mill sludge-wet	18	38	57	60
Paper mill sludge-dry	14	33	44	46
Paper mill sludge-ball milled	21	41	53	55
Glassine paper waste-shredded	21	30	48	53
Glassine paper waste-ball milled	18	36	49	65

QM 9414 cellulase 1.2 FP units per ml
Saccharification at 50°-pH 4.8

(dry)" now exist to shred and classify urban wastes after collection yielding among others a fibrous cellulosic fraction. As produced these fractions resemble newspaper (which is a major component) in reactivity with 30–50% saccharification in 24 hours. This yield can be increased to 50–60% by ball milling. Thus pretreatment is not absolutely required. Readily collectable agricultural residues and by-products, including feed lot wastes, in the United States have been estimated at 220 million tons per year and these average 40–50% cellulose. These materials are very resistant unless pretreated.

Large quantities of sludges, wood residues, and other cellulosic wastes are generated by the pulp and paper industry as by-products of pulp manufacture and paper making. Cellulose content averages about 50% and most have received some delignification or other pretreatment so that many are readily hydrolyzable as produced. Paper mill wastes also have the advantages of being produced in quantity at a central location so that costs of collection have already been paid. They are true wastes with many now disposed of as land fill. Furthermore some paper companies already ferment their waste sulfite liquors with yeast to produce alcohol or single cell protein so they would have a direct application for low concentration sugar syrups.

Syrups have been analyzed by high performance liquid chromatography.[21~23] The chief products are glucose, cellobiose, and xylose with traces of other sugars not yet identified

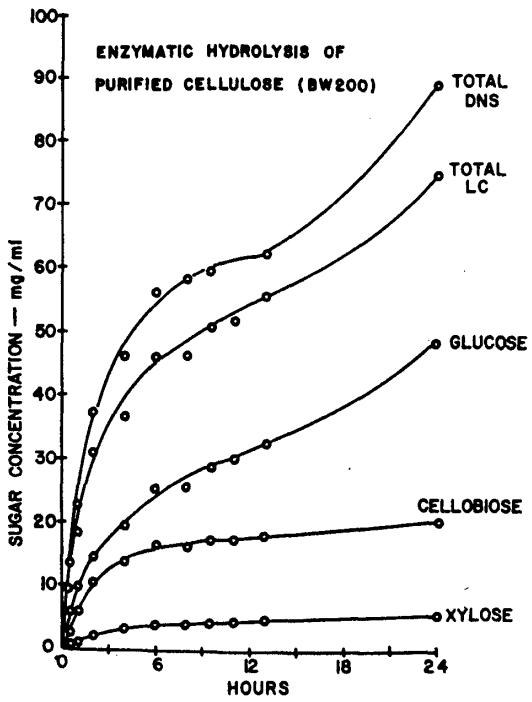

Fig. 17. Accumulation of sugars during enzymatic hydrolysis of pure cellulose.
(Reproduced with permission from reference 23.)
20% Ball milled pure cellulose pulp saccharified with *Trichoderma* QM9414 cellulase at 2 FP units per ml in a 1 *l* STR at 50°, pH 4.8. Reducing sugar by DNS, individual sugars by liquid chromatography.

Table 7. Effect of enzyme and substrate concentration on hydrolysis of hydropulped paper waste.

Substrate Conc. (wt %)	Enzyme conc. FP units/ml	Temperature (°C)	Reducing sugar at 24 hrs (mg/ml)	Saccharification at 24 hrs (%)
2.5	0.5	50	16	64
5.0	0.5	50	23	43
7.5	0.5	50	26	33
2.5	1.0	50	20	77
5.0	1.0	50	33	60
7.5	1.0	50	39	47
2.5	1.5	50	21	79
5.0	1.5	50	39	67
7.5	1.5	50	46	53
5.0	1.0	45	28	54
5.0	1.5	45	33	59
5.0	1.0	55	30	54
5.0	1.5	55	33	61

1 *l* stirred tank reactor pH 4.8
Enzyme *Trichoderma* QM9414 Culture Filtrate. Specific activity 0.62 FP units/mg Protein
Substrate hydropulped paper waste from the Pentagon

$$\% \text{ Saccharification} = \frac{\text{glucose mg/ml} \times 0.9}{\text{Substrate mg/ml}} \times 100$$

(Fig. 17, 18). The ratio of glucose to cellobiose varies depending on the enzyme preparation and hydrolysis conditions but glucose is almost always the principal product. In many cases cellobiose reaches a peak at about 4–6 hours when it may represent 40% of the total,

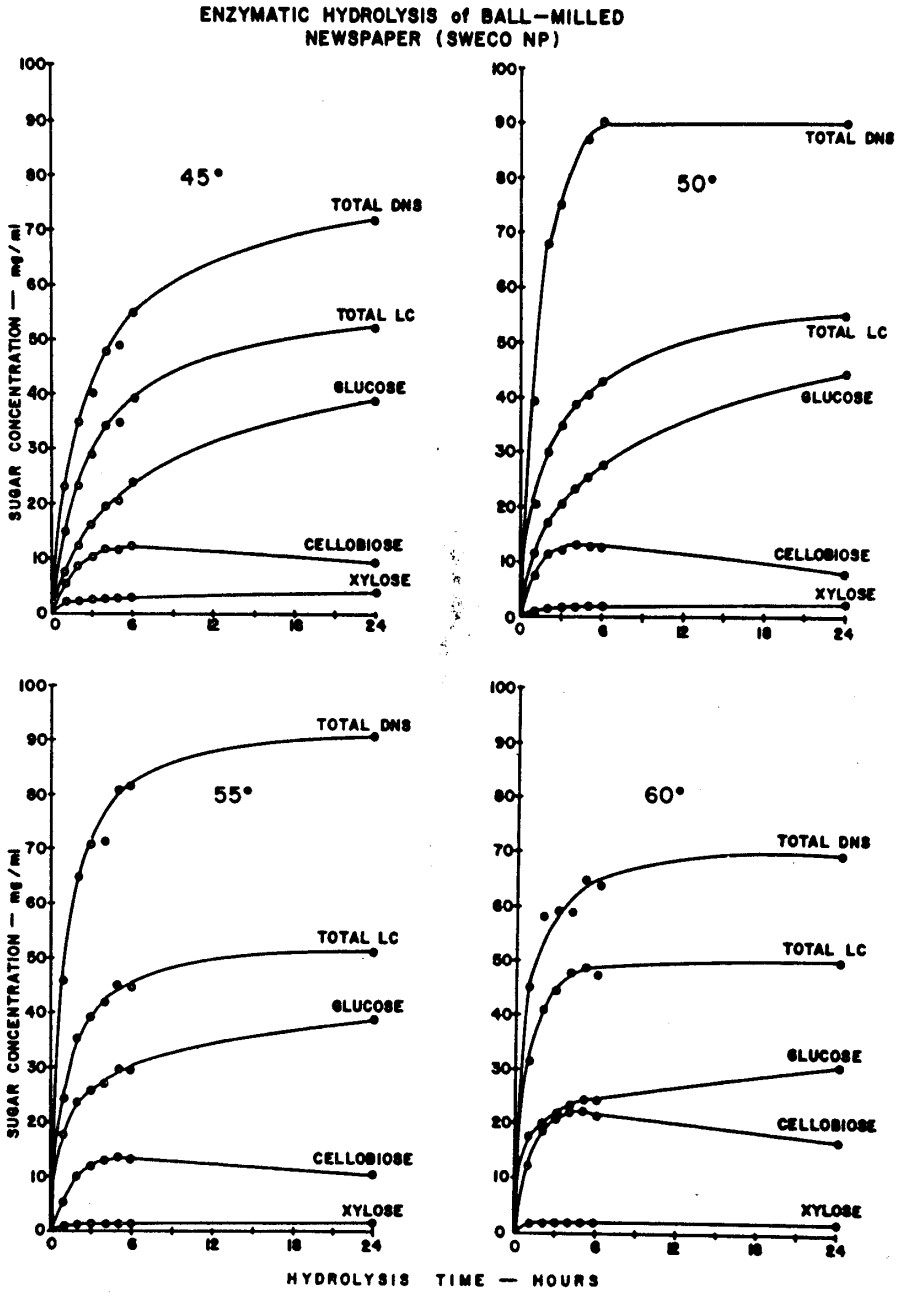

Fig. 18. Accumulation of sugars during enzymatic hydrolysis of newspaper. (Reproduced with permission from reference 23.)

17% Ball milled used newspaper saccharified with *Trichoderma* QM9414 cellulase at 2 FP units per ml in a 1 *l* STR at pH 4.8. Reducing sugar by DNS, individual sugars by liquid chromatography.

and then levels off or declines as its rate of hydrolysis equals or exceeds its rate of formation. Xylose ranges from traces when the substrate is pure cellulose to as much as 30% from some agricultural residues or wood wastes.

Some of the more promising substrates have been more extensively analyzed in a series of one liter stirred tank reactors varying enzyme and substrate concentration and saccharification temperatures (Table 7). Saccharification kinetics are complex and have a major impact on processing costs. There is a rapid initial rate lasting 4–6 hours as the

amorphous portions of the substrate are attacked, but as hydrolysis proceeds the rate decreases due to increasing crystallinity and product inhibition (Fig. 17). As substrate concentration is increased the amount of sugar is increased, but percent conversion is less. As enzyme concentration is increased the sugar increases, but the amount of sugar produced per unit of enzyme is less. Therefore the fastest rates are attained by using high substrate and enzyme concentrations for short hydrolysis times, but such conditions lead to only 20–30% conversion, yield dilute sugar syrups, and consume large quantities of enzyme. Longer hydrolysis times yield more concentrated syrups and conserve enzyme, but require long residence time which increases capital costs.

Similar problems arise in choosing the optimum temperature for saccharification (Fig. 18). Higher temperatures (55°–60°) lead to rapid initial hydrolysis rates, but by 6 hours the enzyme has been largely inactivated. The resulting syrups have low glucose to cellobiose ratios. Lower temperatures (45–50°) give slower rates but the enzyme is preserved and hydrolysis continues until all available cellulose is consumed.

Present Status and Future Outlook

Today probably the most important commercial application of enzymes is for saccharification of corn starch. Advantages favoring the enzymatic process over acid hydrolysis include less capital expense because acid resistant materials are not required, better yield, and a higher grade product with fewer impurities. The prospects seem good that a similar process can be developed for enzymatic saccharification of cellulose. The advantages of cellulose as a substrate are (a) there is so much of it, (b) it is annually renewed, (c) it is frequently a component of waste, and (d) it can be converted to glucose, a nearly universal growth substrate and a promising chemical feedstock. The disadvantages of cellulose include its recalcitrance as a substrate and its admixture with lignin which impedes enzyme action and dilutes the substrate, occupying valuable reactor space and adsorbing expensive enzyme. The process is technically feasible, but it remains to be proven that it is economically viable.

To answer this question and help solve some of the problems, a highly instrumented research prepilot plant containing fermenters, enzyme reactors, holding tanks and auxiliary vessels, instrumentation modules, substrate handling and preparation equipment, and enzyme recovery and concentration equipment has been set up at Natick. Batch, semi-continuous, and continuous processes can be studied. Data from this research pilot plant is now being collected.[24]

The chief problems are:

a. Availability of suitable substrate Despite the abundance of cellulose and its widespread occurrence in waste, much waste cellulose is of seasonal occurrence, and it is often thinly scattered over wide areas. In order to obtain glucose yields of 50% or more in 24 to 48 hours, it is necessary in most cases to pretreat the cellulose. Even if the original waste has no or negative value costs of collection and pretreatment are high. Furthermore clean ground cellulose may have value in its own right as a carrier or diluent for other chemicals, as a cleaning and polishing agent, or as a feed for ruminant animals.

b. Enzyme production Our current enzyme yields of about 0.5–2 units of cellulase per ml of fermentation broth are quite adequate for saccharification (Fig. 19), but because of the low bulk density of cellulose, its resistance, and its admixture with impurities, large quantities of enzyme are required. When impure cellulose is the substrate, it is not presently feasible to recover and reuse the enzyme. The best research approaches to improve enzyme yield are by strain mutation and selection and study of fermentation

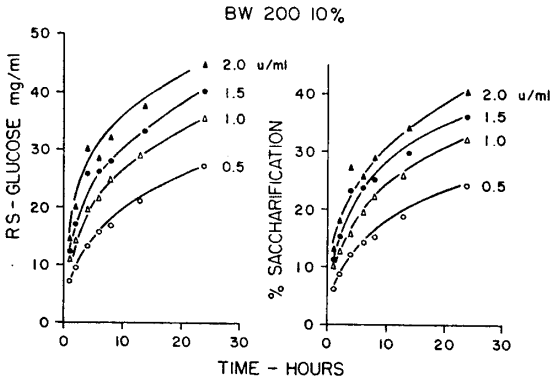

Fig. 19. Hydrolysis of milled cellulose pulp by *Trichoderma* cellu ase.
1 *l* stirred tank reactor at 50°C with 10% ball milled pure cellulose (BW 200), cellulase culture filtrate from QM9414 at 0.5–2.0 FP units/ml, Specific activity = 1.0 FP unit/mg protein. Percent saccharification = glucose mg/ml × 0.9.

processes to permit media enrichment to give higher growth and high r yields of enzyme. To achieve this goal means must be found to by-pass repression and enzyme inactivation. Our present maximum yields on 1% cellulose of 2 mg of protein per ml represent 20% of the cellulose as secreted protein, that is, at least 40% of the biomass produced. Cellulase appears to be a major component of this extracellular protein so higher yields will require richer media. When cellulose level is greater than 1% some pH control is required. Otherwise the severe acid conditions that develop reduce fungus metabolism and cause enzyme inactivation. We are now achieving enzyme yields of 3–4 units per ml on 2–3% cellulose by controlling pH at 3.0–3.25, and we anticipate further improvement.[13] Yields of 14 mg per ml of pure extracellular alkaline protease have been achieved from a *Bacillus*.[25]

c. The Saccharification process The process must be developed to minimize enzyme use and maximize sugar concentration in the digest. A serious consideration of the use of pure cellulose as a substrate must be made. This would have marked advantages if it could be produced cheaply from sources not suitable for fiber production and by processes that made no attempt to conserve fiber structure since all of the reactor space and enzyme would be utilized by substrate, greater substrate conversion would be possible, and enzyme could be conserved and recycled. Development of methods to desorb and recover enzyme from residues would be of great value.

d. Applications of the product Cost estimates have a notoriously short half-life and really should be made by industrial people who have access to realistic and current cost data. However it would appear that large-scale enzymatic conversion of waste cellulose to glucose may be economically competitive with the starch hydrolysis process.[24] The fermentation of glucose at such price could not compete economically with either alcohol production from hydrocarbons or soy protein.[25] Applications for by-products such as the cellobiose and xylose, the mycelial and lignin residues could improve the economic outlook.

Despite the above problems we believe that further development should continue. Political and economic instabilities in the world markets underscore the need for alternate sources of materials and energy. Enzymatic utilization of cellulose may become economically viable initially in a particular locale or industry due to feedstock availability and a direct application for low concentration glucose syrups. As material and energy resources are depleted, utilization of cellulose through enzymatic conversion offers a reasonable means of drawing on replenishable resources.

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