

PRODUCTION OF MICROBIAL ENZYMES FOR CELLULOSE HYDROLYSIS

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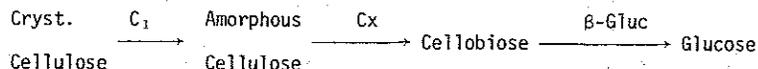
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SUMMARY

The conversion of cellulose to glucose by microbial enzymes on a commercial scale requires (a) an adequate supply of a readily hydrolyzable cellulosic material, and (b) an active enzyme source at low cost. A highly active source (*Trichoderma reesei*) was obtained by screening thousands of cultures. Three successive mutations resulted in an 8-fold increase in enzyme production. pH control in fermentors permitted the use of much higher cellulose concentrations (e.g., 6%) than could be used successfully in shake flasks. As a result, extracellular enzyme yields have again been increased by a factor of 8-10. These filtrates are of such a potency that they can be diluted at least 5 times for use in the digestion phase, thus greatly reducing enzyme costs.

INTRODUCTION

Our program on the biological decomposition of cellulose (4) began 33 years ago - as a long term basic study (which it has turned out to be). This program is a good example of how basic research, which began with one objective in view, can end up supporting exactly the opposite objective. The initial goal was to minimize the biological decomposition of cellulose. The present objective is to maximize it. We now want to hydrolyze cellulose to glucose as rapidly and as cheaply as possible.



There are two basic problems to be solved if an economic process is to be developed for converting cellulose to glucose. First is the difficulty which an enzyme has when its substrate is a highly organized impermeable solid. In these systems surface area measures solute concentration, and efforts must be made to increase the available surface. This involves pretreatment of the cellulosic material - a subject not to be discussed today. The second basic problem, the development of an inexpensive source of the catalyst (cellulase), is the area which I wish to consider (10).

ENZYME PRODUCTION

Before going into production of an enzyme, certain questions should be asked. Why do we want this enzyme? What requirements do we impose on it? Usually the requirements are quite simple. An amylase may be needed for desizing cotton fabric. It must be free of enzymes attacking the fabric (e.g., of cellulase). A protease may be needed to dehair hides, but it must not weaken the leather. In the case of cellulase, the preparation must be free of enzymes which inactivate the cellulase (proteases, etc.), and of enzymes which modify the products (glucose oxidase, etc.). Fortunately, these contaminants are rarely observed.

What are the goals we set for cellulase production? Obviously we want the most

enzyme at the least cost, a high concentration of extra-cellular enzyme, so high that it can be diluted rather than concentrated for its subsequent use in the hydrolysis reaction. Such a preparation minimizes both equipment and handling costs.

Goals in Enzyme Production (3)

1. Yields
2. Cost
3. Stability
4. Purity

Stability is important in commercial enzymes. For the cellulose hydrolysis process, the cellulases must withstand temperatures of 50°C for at least 3-4 days at the optimum pH for activity. Fortunately most microbial polysaccharases meet this requirement. Until recently we considered the cellulase of *Trichoderma reesei* to be remarkably stable to heat and resistant to chemical inhibitors. However, these early studies were based on endo-glucanase (Cx) activity, since CMC was used as substrate. Now we find (manuscript in press) that the cellobiohydrolases (CBH 2 C₁) are somewhat less stable, and are, under the cellulose hydrolysis conditions, inactivated by merthiolate, heat and shaking. Some attention must be paid to this type of inactivation if an enzyme system is to be developed that is suitable for commercial exploitation.

There is a related problem of some concern. Cellulase is adsorbed on the substrates and, to date, no good means of recovering it from the unhydrolyzed residues have been developed. With pure cellulose, much of the enzyme is liberated as the cellulose is digested. With more complex cellulosic materials, much less of the cellulase can be recovered. Re-use of enzyme would greatly reduce enzyme cost.

Purity is not an important requirement of the cellulase preparations to be used in the cellulose digestion process. The presence of most other enzymes (amylase, xylanase, etc.) does not interfere. The only limitation is that the microbe producing the enzyme does not simultaneously produce a compound toxic to the organism that is to use the glucose product. When present such toxic agents would have to be removed, thus imposing another cost on the process.

Purity can be defined as the ratio of the specific activity of a crude enzyme to that of the purified enzyme. From the purification data, the enzyme as percent of the total protein can be determined (Table I).

TABLE I

"PURITY" OF EXTRACELLULAR DEPOLYMERASES (3)

Enzyme	Source	*E/P x 100	Reference
exo-β 1,3 glucanase	Basidiomycete QM 806	14.	Huotari, 1968
exo-β 1,3 glucanase	Sclerotinia	12.	Ebata, 1963
endo-β 1,3 glucanase	Rhizopus	0.6	Marshall, 1974
α 1,3 glucanase	Cladosporium	7.	Walker, 1976
β-xylosidase	Penicillium	4.	Claeyssens, 1976
α 1,6 glucanase	Pseudomonas	2.	Richards, 1972
endo-β 1,6 glucanase	Rhizopus	0.2	Yama moto, 1974
β-glucosidase	<i>Asp. phoenicis</i>	14.	Reese, 1975
alkaline protease	<i>B. subtilis</i>	80.+	Ikeda, 1974

*E/P x 100 = enzyme as percent of total protein.

Generally these values are quite low, but they can be increased by improving the conditions for enzyme production. Thus, the extracellular protein of *B. subtilis* closely approaches "pure" protease (5); and the protein of our high yielding cultures of *T. reesei* nearly pure "cellulase". (Actually there is no such thing as a "pure" enzyme; there are only enzymes purified to a certain degree.) An enzyme source which requires a 2,000-fold purification is certainly a poor choice of starting material. The time spent in finding a better source of enzyme; i.e., in finding a preparation of high initial specific activity, is time well spent. In Table I are several examples of enzymes which make up 12-14% of the extracellular protein. Nearly all of these are systems that have been thoroughly investigated. The high initial concentration of enzyme greatly simplifies subsequent purification. Enzyme yields may be enhanced either by increasing the total protein secreted, or by increasing the percent of enzyme in the extracellular protein.

The best sources of cellulases are fungi. Because the substrate is insoluble, and cannot enter the cell, the fungus must secrete the solubilizing enzymes into the medium. Fortunately for us, this simplifies the recovery of the enzyme system. Only a simple filtration is required. The enzyme is also in a much more "purified" condition than enzymes extracted from whole cells.

When an organism approaches an insoluble substrate - and soluble substrates are absent - the hyphal tips (Figure 1) comes into contact with the substrate. The cell wall at these tips is very thin and permits the secretion of enzyme. The cellulase diffuses and attaches itself to the substrate in such a way as to catalyze its hydrolysis. (This is quite different from hydrolysis of soluble substrates, where the substrate does the diffusing and adjusting to the enzyme.) Diffusion of the enzyme is impeded by its large size relative to the pores of the substrate, so that much of the action takes place in close proximity to the hyphal tip. The soluble products resulting from the hydrolysis are taken up by the fungus, and growth continues into the space liberated, thus maintaining close contact between hypha and cellulose.

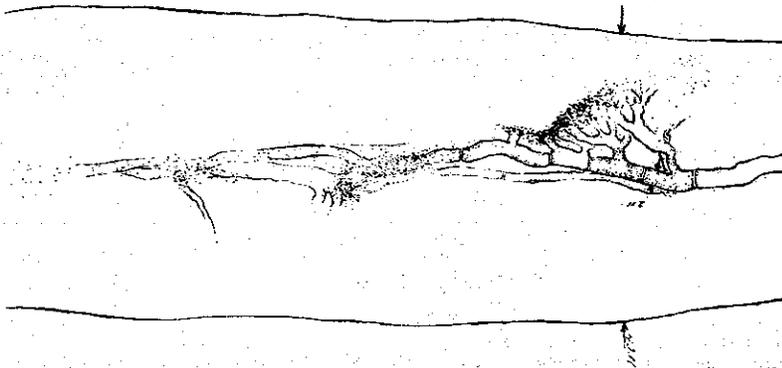


FIGURE 1. Growth of *Memnoniella echinata* in cotton fiber. (W.L. White)

Increasing Enzyme Yields

1. Selection of organism.
2. Mutation to a more productive form.

3. Selection of conditions favoring high yields.

- (a) Solid vs liquid media.
- (b) Salts, pH, aeration, temperature.
- (c) Inducing compounds.
- (d) Use of surfactants.

How does one go about obtaining the high yields of cellulase required for this process? Work! The steps to success are well known. The short-cuts few. In our laboratories, we have isolated thousands of fungi from deteriorating materials. These have been tested on tens of thousands of cotton fabric strips, and the organisms most active then compared in shake flasks for their ability to produce and secrete the enzymes responsible for the hydrolysis. During this work, we observed that the organisms which degrade cellulose the most rapidly are not necessarily those which secrete the most enzyme.

Laboratories in Japan and in Russia have done similar screening experiments. In all cases, *Trichoderma* species have been among the best for cellulase production. Many of the other good organisms; e.g., *Fusarium*, *Penicillium*, *Pestalotiopsis*, are related to *Trichoderma* in that their perfect states are perithecial ascomycetes. Phycomycetes and bacteria are poor producers of extra-cellular cellulase, even though many can rapidly decompose cellulose.

All microorganisms which grow well on cellulose must produce active cellulases (2, 4, 18). Why then are active enzyme preparations obtained from so few? It may be because cellulase is a complex of enzymes which act synergistically, and that only a few organisms secrete adequate levels of all members of the complex. Cellulase preparations which hydrolyze insoluble cellulose can be separated physically into 3 types of components: endo- β 1,4 glucanases (C_x); exo- β 1,4 glucanases (cellobiohydrolase, C_1 ?); and β -glucosidase (cellobiase). Of these, it is the CBH component which is deficient in the filtrates of most organisms, perhaps because of its high affinity for cellulose.

TABLE II
INCREASE IN ENZYME PRODUCTION BY MUTATION (3)

Enzyme	Organism	Enzyme Yield $\frac{\text{(Mutant)}}{\text{(Control)}}$
aspartate transcarbamylase	<i>E. coli</i>	500
cellulase	<i>Trichoderma viride</i>	2
dihydrofolate reductase	<i>Diplococcus pneumoniae</i>	200
β -galactosidase	<i>E. coli</i>	4
glucoamylase	<i>Aspergillus foetidus</i>	1.6
protease	<i>Bacillus cereus</i>	10
α -amylase	<i>Aspergillus oryzae</i>	10
protein	<i>Candida 25</i>	4

Having selected the best organism, one tries to produce a mutant that will far surpass the parent in yields of the desired product. Examples of 100-fold increases of some enzymes have been reported (Table II). Usually, however, one is satisfied with a doubling effect, and hopes to achieve 3 or 4 of these steps. To a great extent, the enhancement is a function of the initial level of production. When this is low, the enhancement is great; when it is high (as after a selection process), the enhancement is much less.

Several cellulase mutants of *Trichoderma reesei* have been produced at Natick (7) and at Rutgers (8) that produce higher levels of endo- and exo- β 1,4 glucanases (up to 15 international filter paper cellulase units/ml of broth), and higher productivity (up to 100 units/liter/hr., Figure 2). Some of these strains are partially derepressed. However, all strains presently available produce unsatisfactorily low levels of cellobiase (β -glucosidase), so that it is necessary to add supplemental cellobiase (from *Aspergillus phoenicis*) to achieve optimum saccharification rates (15). The ideal ratio of cellobiase to filter paper cellulase units is about 1.5. Current strains have ratios of about 0.5 at best. So we are looking for mutants which not only give increases in cellulase productivity, but also give higher levels of cellobiase. Other desirable properties of the mutants would be (a) resistance to catabolite repression, (b) removal of inducer requirement, (c) increased growth rate, (d) enzymes of structure modified in such a way as to increase stability, and to decrease product inhibition (8).

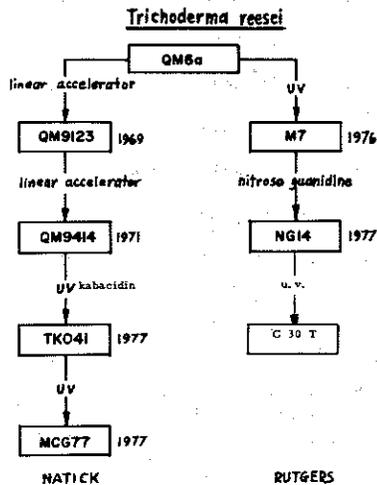
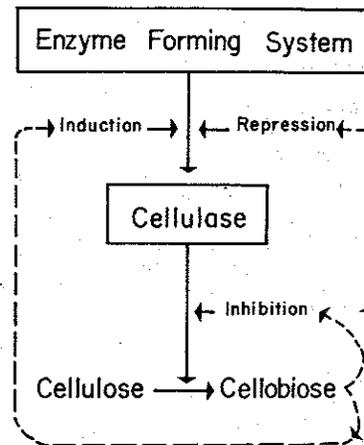
FIGURE 2. Mutants of *T. reesei*

FIGURE 3. Cellulase formation

CULTURAL CONDITIONS

The amount of enzyme produced is strongly influenced by the cultural conditions. Of these, we consider only two; e.g., induction and surfactants.

INDUCERS

The hydrolyzing enzymes of interest to us are polysaccharases secreted into the culture medium. These enzymes are of two kinds, the one (constitutive) is produced by the organism under nearly all conditions of growth; the second (induced) is produced only when an "inducer" is present in the medium (Table III). While this classification is generally good, there is some dependence upon the organism. α 1,3 glucanase, for example, is usually an induced enzyme, but in the exceptional case (*Cladosporium resinae*) it is constitutive. However, even this fungus produces its best yields of enzyme in the presence of the α 1,3 glucan. Cellulase always appears to be induced.

What is the nature of the inducer? In general, the inducer is considered to be the

substrate of the enzyme, or a modification of the substrate. But when the substrate is a molecule which, because of its large size, cannot enter the fungus, another inducer is required - one that is small enough to enter the cell. For polysaccharases, this turns out to be the soluble dimer (and related) products obtained by enzymatic hydrolysis of the polymer.

TABLE III
POLYSACCHARASE PRODUCTION BY FUNGI

Constitutive	Induced
α 1,4 Glucanase	α 1,6 Glucanase (Dextranase)
	α 1,3 Glucanase
β 1,3 Glucanase	β 1,4 Glucanase
β 1,6 Glucanase	β 1,2 Glucanase
Xylanase (?)	α 1,4 Polygalacturonase
	β 1,4 Mannanase
	β 1,4 Chitinase
	β 2,1 Fructanase
	β 2,6 Fructanase

According to this theory we assume that the organism is producing cellulase (for example) in very small amounts (usually undetectable) at all times. When the organism approaches cellulose, the secreted enzyme catalyzes the hydrolysis of a small amount of cellulose, the cellobiose produced enters the cell and triggers off the cellulase-synthesizing system. More enzyme leads to more cellobiose which promotes more enzyme production (Figure 3).

Several other enzymes have also been shown to be induced by the dimer product (Table IV).

TABLE IV
PRODUCT INDUCERS OF POLYSACCHARASES (10)

Enzyme	Product	Condition	Organism
Cellulase	Cellobiose & Oligomers	liberation from ester	Trichoderma
	Cellobiose & Oligomers	low concentration	Pseudomonas
β -amylase	Maltose		Bacillus
α -amylase	Maltose		Bacillus
			Pseudomonas
Dextranase	Isomaltose	liberation from ester	Penicillium
			Spicaria
Pullulanase	Maltose		Aerobacter

Induction by soluble products may easily be overlooked, because of product and/or catabolite repression. That is, the inducer at high concentrations acts as a repressor.

Thus at 0.5% cellobiose, very little cellulase is produced, and the same is true of other dimers inducing other polysaccharases (Table V). In cellulose cultures, cellulase is produced. When, however, glucose or cellobiose (1%) is added to these, enzyme production not only ceases, but the pre-formed enzymes disappears from the extra-cellular fluid. When the added sugar has been consumed, the organism again resumes its synthesis of cellulase.

Proof that the dimer is the inducer in these systems has been supplied in two ways. First, by maintaining the supply of dimer at a low level by constant feeding, Suzuki and co-workers (19) obtained yields of cellulase comparable to those obtained on cellulose. We had tried this but failed. Second, by supplying a precursor of the dimer, which slowly undergoes hydrolysis to maintain the dimer inducer at low concentrations. Our approach was to form esters of the particular dimer. In the presence of esterases produced by the fungus, the dimer was released slowly over a long period, and excellent yields of enzymes resulted (Table V). It is obvious that the method works, not only for polysaccharases, but for glycosidases as well. Thus, the nucleosidase is not induced by its substrate, adenosine (at 0.5% level). But when the same substrate is liberated slowly from RNA (by a nuclease), or from adenosine-5- PO_4 (by a phosphatase) the yields are quite good. It should be clear that in the latter examples, modified substrate, rather than modified product, is involved.

TABLE V
EFFECT OF MODIFIED INDUCERS ON ENZYME YIELD (10)

Enzyme	Organism	Inducer	Yield
Cellulase EC3.2.1.4	Trichoderma viride	Cellulose	22.5 ^a
		cellobiose	0.2 ^a
		cellobiose dipalmitate	4.8 ^a
	Pestalotiopsis westerdijkii	cellulose	35.9 ^a
		cellobiose	0.2 ^a
		cellobiose octaacetate	20.1 ^a
Pseudomonas fluorescens	cellulose	514.0	
	cellobiose (slow feeding)	430.0	
	sophorose	397.0	
Dextranase EC3.2.1.11	Penicillium funiculosum	dextran	1080.0 ^a
		isomaltose	2.0 ^a
		isomaltose dipalmitate	1098.0 ^a
Invertase EC3.2.1.26	Pullularia pullulans	sucrose	1.3 ^a
		sucrose monopalmitate	108.0 ^a
Purine nucleo- sidase a EC3.2.2.1	Aspergillus ambiguus	adenosine	0
		yeast RNA	57.0
		adenosine 5' PO_4	90.0

^aUnit values in International units. Others as defined by authors.

In apparent contradiction to the above, it is sometimes possible to obtain good enzyme yields at concentrations of inducer that normally repress. This is done by decreasing the rate of consumption of inducer by one of the following means:

- (a) lower-than-optimal temperature
- (b) addition of compounds at concentrations which are slightly toxic
- (c) restriction of one or more factors required for growth

Experiments of this nature have led us to conclude that it is not the concentration of cellobiose per se that represses the cellulase synthesizing system, but the rate at which the cellobiose is being consumed. This suggests that where there is a family of inducers, the ones most slowly consumed will induce the most, other factors being equal. For the cellulase system, cellulose is still the best C-source for obtaining high yields of cellulase, apparently because the organism consumes the cellobiose product as rapidly as it is being produced.

What, then, of the inductive effect of sophorose, and of lactose? These, and other analogues of cellobiose, often induce, and indeed in washed mycelium experiments yield much larger amounts of endo- β 1,4 glucanase than does cellobiose (but these yields are very much lower than the organism normally produces in a cellulose culture). Why this should be is difficult to say. It has been observed that sophorose is much more slowly absorbed than cellobiose by *T. reesei* under the assay conditions used (Sternberg, D. and G. Mandels, in press), a factor known to favor induction. Certainly if sophorose were the "true" inducer, it would have to arise in cellulose cultures from the transferase action of β -glucosidase on β 1,4 oligomers. The production of transfer products requires high concentrations (5-10%) of cellobiose. In nature, and in active shake flask culture, on cellulose, cellobiose is almost non-detectable. Hence, the likelihood of sophorose being produced is nil.

SURFACTANT EFFECTS

The work on inducers led to studies of surfactants. We thought we knew how to get good yields of enzymes by using esters of the substrate. So to make α -galactosidase, we prepared an ester of an α -galactoside, namely melibiose octaacetate. It didn't work! In the same experiment, we had a sucrose monopalmitate control, and on this the fungus (*Aspergillus fumigatus*) produced α -galactosidase (though there was no α -galactoside in the medium). This was entirely unexpected and forced us to recognize that the remarkable effects obtained previously with modified inducers were, to some extent, due to the surfactant properties of the esters used.

As Tween 80 (polyoxyethylene sorbitan oleate) a non-ionic surfactant has long been known to improve growth of some bacteria, it was compared with the sucrose monopalmitate (previously used) for its effect on enzyme production. For some organisms, one surfactant was superior, for other organisms, the other. Since Tween 80 is the more readily available product (Hercules Powder Co.), it was used as the standard. It gave very appreciable increases in yields in many enzyme systems where it was tried (Table VI). These were the maximum increases observed in the screening operation. Emphasis was then shifted to production of a single enzyme, and more variations were made in types and concentrations of surfactant. Even greater enhancement in yields were obtained.

TABLE VI
EFFECT OF ADDITION OF SURFACTANT (TWEEN 80) TO CULTURE MEDIUM ON ENZYME YIELDS

Enzyme	Source	R ^a $\frac{(\text{yield} + \text{surfactant})}{(\text{yield} - \text{surfactant})}$
Cellulase	Many fungi	20
Invertase	"	16
β 1,3 glucanase	"	10
β -glucosidase	"	8
Xylanase	"	4
Amylase	"	4
Nucleosidase	"	5
Esterase	"	6
Dextranase	<i>Penicillium funiculosum</i>	2
Pullulanase	<i>Aerobacter aerogenes</i>	1.5

R^a = Ratio of yield in shaken flasks (29°C) containing appropriate culture media plus Tween 80 (0.1%), to yield under identical conditions lacking Tween.

One surfactant may be best for enzyme production under one set of conditions, and a second surfactant best under other. Tween 80 is most generally stimulatory to *Trichoderma reesei* growing on cellulose. But Na oleate is much better than Tween when the same fungus is growing on cellobiose. In the absence of surfactant, no CMCase (C_x) was produced on cellobiose (Figure 4). In the presence of Na oleate (0.1%) 85 units/ml was produced (roughly half as much enzyme as appears in a cellulose grown culture).

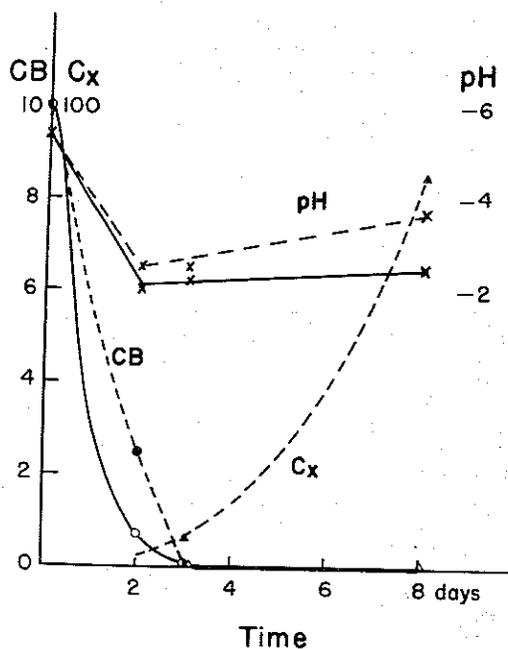


FIGURE 4. Effect of surfactant (0.1% oleate) on cellulase production on cellobiose (1%). Solid lines = control; dashed lines = + surfactant, *T. reesei* QM6a. (11)

The basis for the surfactant effect is not known (11). It is likely that more than one mechanism is involved. Fundamentally, however, surfactants are substances which accumulate at interfaces. Their incorporation into the cell membrane alters permeability, and this change is reflected in the metabolism of the organism. The change in permeability may be slight in the examples cited - facilitating access of substrate to the membrane-bound enzyme. More surfactant may assist in stripping away enzyme from the membrane. And still more, may lead to complete loss of selectivity by the membrane, in which case it is toxic and the organism dies.

The inherent "leakiness" of cell membranes varies from organism to organism, thus accounting for variability in enzyme-production (or in secretion of any compound). Surfactants further increase (or modify) the leakiness. As the amount of cell-bound enzyme appears to be constant (feed-back control), increased secretion leads to increased production, as the cell attempts to maintain the cell-bound level.

PRODUCTION IN FERMENTORS (1, 6)

The work described above was done in shake flasks, using small volumes. The next

step for increasing enzyme yields is through the use of fermentors, by which various of the cultural factors can be controlled. A major stumbling block in the shake flask work was the inability to control pH.

The fermentation profile of the Rutgers mutant C30 growing on 6% two roll milled cotton in a batch ten-liter fermentation shows three distinct phases (Figure 5). Growth dominates for the first 50 hours as pH falls to the control point of 3.0 and half the cellulose is consumed. Mycelial protein is synthesized rapidly reaching a peak of 5 mg/ml. Assuming 40% protein, this represents about 12 g/l of cells, a 40% yield from the cellulose consumed. Very little extracellular protein or cellulase has been synthesized. Enzyme production dominates for the next 200 hours. The pH holds at 3.0 as the cellulose is more slowly consumed, falling to 5 g per liter. Mycelial protein falls to 2 g per liter representing about 7 g per liter of older cells containing about 30% protein. Cellulase has risen to 12 units/ml and extracellular protein to 17 g/liter, 3.5 times the maximum level of mycelial protein. Not much happens in the final phase. Cellulose is slowly consumed but little new enzyme is produced. Eventually when cellulose is depleted pH will rise as cells autolyze (1, 14).

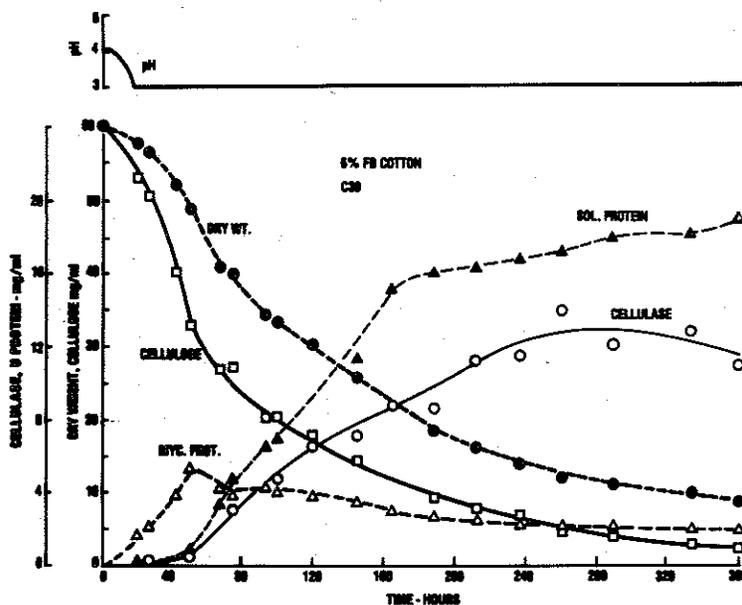


FIGURE 5. Fermentation profile of *T. reesei* C30 growing on 6% cotton. (Unpublished data of M. Mandels)

Enzyme production is not directly related to growth rate. Growth of *Trichoderma* is most rapid at about pH 4.0 (Figure 6). Cellulase production is at a maximum near pH 3.0 where growth is less rapid. The amount of cellulase is a function of amount of mycelium-produced in the presence of an inducer (i.e., cellulose). Mycelium grown on glucose or on other soluble C-sources is not induced, does not produce significant levels of cellulase, and secretes very little soluble protein into the medium. Under optimal pH and

temperature, *T. reesei* growing on glucose has a doubling time of about 3 hours (Sternberg unpublished).

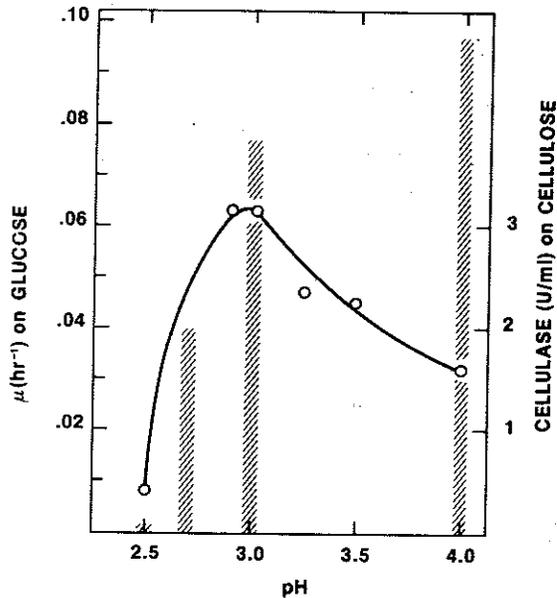


FIGURE 6. Effect of pH on growth (bars) and on cellulase production (o - o) of *T. reesei* QM 9414 (in fermentors). Growth data from Brown, D.E. (4); enzyme activity data from Sternberg, D.

CONTINUOUS CULTURE FOR ENZYME PRODUCTION

In the continuous culture (enzyme production stage) yields of up to 2 cellulase units/ml were attained, a low value compared to present yields on cellulose. But specific enzyme productivities were equal to those of cellulose grown batch cultures; e.g., up to 8 units/g biomass/hr., or 100 units/liter/hr. Growth was equal to or slightly less than zero, under these second stage conditions.

Batch culture has shown that cell growth and cellulase production require two distinctly different sets of conditions. Now Dr. Dewey Ryu is studying the two phases in continuous culture, the first stage being optimized for growth, the second for enzyme production (12). This allows independent evaluation of each process and permits conditions for zero or negative growth in the second stage. In his initial studies he has used mutant strain MCG77 which grows rapidly and shows high cellulase productivity. Lactose was used as a soluble substrate for both stages to simplify control of feed, measurement of biomass and substrate consumption, and to eliminate carbon source as a variable between the two stages. For strain MCG77 in batch culture, lactose is about 50% as effective as cellulose as a cellulase inducer, although for some other strains such as QM 9414 it is only about 20% as effective. In this experiment, the first stage was optimized for growth with pH 4.5, temperature 32°, and a rapid dilution rate. The

second stage was optimized for enzyme production with lower pH (3.5) and temperature (28°) and a slower dilution rate. Data was collected only from steady state conditions. Uptake rates (Q) as mmoles or mg of carbon, oxygen, and nitrogen were determined for cells in the first and second stages and plotted against μ , the specific growth rate. In the first stage μ is equal to the dilution rate (D) and ranged from 0.01-0.06 per hour. In the second state μ_2 ranged from -0.01 to +0.02. From these results the yields and the maintenance coefficients (Table VII) were determined

TABLE VII (12)
METABOLIC CONSTANTS FOR T. REESEI⁽⁴⁾

OXYGEN ⁽⁵⁾	CARBON ⁽⁶⁾	NITROGEN
$M_O^{(1)} = 0.85(\text{mmole } O_2/\text{g biomass/hr})$	$M_C = 0.14(\frac{\text{mmole hexose}}{\text{g biomass/hr}})$	
$M_O = 27.2(\text{mg } O_2/\text{g biomass/hr})$	$M_C = 10. (\frac{\text{mg C}}{\text{g biomass/hr}})$	
$Y_{x/O}^{(2)} = 32.3(\text{mg biomass/mmmole } O_2)$	$Y_{x/C} = 80. (\frac{\text{mg biomass}}{\text{mmole hexose}})$	$Y_{x/N} = 12.5(\text{mg x/mg N})$
$Y_{x/O} = 1.01(\text{mg biomass/mg } O_2)$	$Y_{x/C} = 1.11(\frac{\text{mg biomass}}{\text{mg C}})$	$Y_{x/N}^{(3)} = 16.6(\text{mg x/mg N})$

(1) M = Specific uptake rates of O_2 and C for energy metabolism that is equivalent to maintenance coefficient.

(2) Y = Yield constant for biomass (x) with respect to O_2 , C, and N sources.

(3) $Y_{x/N}$ = Value in the enzyme production stage differs from that in the growth stage.

(4) Strain MCG77 on lactose.

(5) and (6) The values for the first and second stages are the same.
First stage at 32°C and pH 4.5, second stage at 28°C and pH 3.5.

CURRENT STATUS OF THE PROCESS

We summarize the developments in the cellulose conversion process in Table VIII. The screening operation gave us T. reesei QM 6a. Modification of medium improved the cellulase yields; the extent of improvement being at least 2-fold. Two successive mutants gave additional doubling effects. These steps were based on laboratory experiments in shake flasks. The next improvements were based on pH control which permitted the use of much higher cellulose concentrations, and on the use of pretreated (2-roll milled) cotton as a growth substrate. This mill disrupts the "ordered" structure of cellulose by a shearing effect, forming a product which is very reactive (16).

The sum total of the step improvements shows roughly an 8-fold increase in enzyme production to 1970, and a further 8-fold increase to the present time. The result of all these improvements is a fungal filtrate containing two per cent protein, most of which is enzyme. This is, indeed, an accomplishment, surpassing even B. subtilis (5) which produced 1.4% solution of extracellular protein (mostly alkaline protease).

TABLE VIII

DEVELOPMENTS IN CELLULASE PRODUCTION BY T. REESEI

1. Screening of 1,000± organisms + QM 6a		1951
2. Modification of medium (2x)		1962
3. Mutant QM 9123 (2x)		1970
4. Mutant QM 9414 (2x) Protein 1.5 mg/ml		
5. pH control in fermentors		1974
6. Increase in cellulose concentration (2%) 4 mg/ml		1976
7. Supplementation with β -glucosidase (2x)		1976
8. Increase in cellulose conc. (6%)		
	Ball-milled 9 mg/ml	
	2-roll milled 16 mg/ml	
9. Mutant, Rutger's	20 mg/ml	1977

A process improvement, not related to cellulase production, is the addition of β -glucosidase to the cellulase at the hydrolysis step (15). *Trichoderma* has an excellent balance of enzymes for its growth on cellulose and is a very successful organism in nature. The sugars produced are consumed by the fungus and do not accumulate. However, when the enzymes are removed from the fungus and used to saccharify cellulose, high levels of sugar accumulate. The cellobiose component inhibits cellulase action; the glucose inhibits the β -glucosidase. Supplementation with β -glucosidase can double the cellulose hydrolysis rate.

Time does not permit discussion of the hydrolysis step. However, with 2 filter paper unit/ml enzyme (0.2% soluble protein) we can achieve 50% saccharification of a 10-15% slurry of cellulose pulp in 24 hours yielding 6-9% sugar syrups. We are now developing a pilot plant process. The flow scheme for this process (Figure 7) shows the two important steps, enzyme production, and enzymatic hydrolysis. The product is glucose.

The matter of estimating costs of producing glucose from cellulose is difficult and uncertain. All of the factors to be considered (Table VIII) are continually fluctuating. Four years ago, the lowest estimated price was \$.33 per kg. Subsequent improvements further reduce the cost of enzyme. Increasing the final sugar concentration in the digest will reduce the cost of water removal. Addition of β -glucosidase will reduce the hydrolysis time. At Natick we are optimistic that commercial utilization of enzymatic saccharification will soon contribute to the utilization of waste cellulose.

During the past year, our engineers (13) have made another attempt to estimate the cost of producing ethanol from urban waste (Table IX). The various assumptions are realistic based on the available laboratory data. Enzyme cost remains the largest single item. As yet, we foresee no satisfactory method for enzyme recovery and re-use. A breakthrough in this area would be most helpful. The analysis shown is based on the partial hydrolysis of the 30% charge to the digester, yielding a 10% glucose syrup, which is then subjected to fermentation to yield alcohol. The cost (\$1.42/gal.) is still non-competitive in today's market; but no credits have been taken for any by-products.

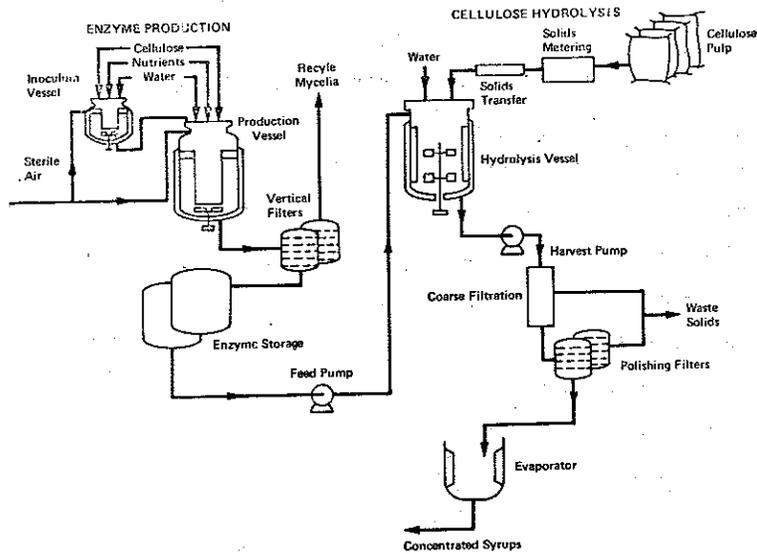


FIGURE 7. Flow sheet for enzymatic hydrolysis. (9)

TABLE IX

COST ANALYSIS - ETHANOL FROM CELLULOSE (13)
 BASED ON 25×10^6 GALLONS/YEAR FROM 5×10^8 KG URBAN WASTE

Cost Factor	Assumption	\$/gallon 95% Ethanol
Substrate	30% Charge to Reactor	0.11
Pretreatment	Two Roll Mill	0.30
Enzyme	10 I.U./g Substrate	0.57
Hydrolysis	45% Yield (10% syrup)	0.13
Ethanol Production	40% Yield from Sugar	0.31
Total Factory Cost	No Credit for By-Products	1.42

Enzyme Productivity = 100 International Units/Liter/Hour
 Enzyme Utilization Efficiency (24 Hours) = 39%

The Natick method for enzymatic conversion involves the use of highly developed techniques, and expensive equipment. The Japanese methods seem to be moving in the opposite direction; i.e., toward a simplified process, the development of a "cottage" industry. Dr. Toyama (17) envisages the production of enzyme by the Koji method; i.e., growth of *Trichoderma* on pasteurized bran in trays, followed by an aqueous extraction of enzyme. Major advances in enzyme production on Koji have been reported. Substrate preparation is also being simplified in such a way that it can fit into farm operation requiring a minimum of equipment. The digestion of the cellulosic charge is carried out in crude vessels ("Sho Chu" jars already used in the Orient for fermentation of sweet potatoes) over a period of several days to yield rich sugar solutions, equivalent in concentration to sugar cane juice. This can then be used to produce single cell protein or alcohol. Perhaps the ultimate process will combine elements from both the American and the Japanese developments.

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