

Stability of the Cellulase of *Trichoderma reesei* under Use Conditions

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Summary

Enzyme stability studies have been reinvestigated under the conditions used for cellulose hydrolysis (pH 4.8, 50°C, 24 hr). The cellobiohydrolase (CBH) component as measured on Avicel is less stable than other enzymes of the cellulase complex, and is 60% inactivated by *merthiolate* (and other Hg compounds) under the above conditions. Endo- β -1,4-glucanase is much more stable, and more resistant to *merthiolate* and other compounds. Under unshaken conditions the Avicelase of the Rutgers strain C 30 shows greater stability to heat than that of other available strains. Biocides must be selected not only for their ability to prevent contamination, but also for their compatibility with cellulases. Tetracycline and chlortetracycline are inexpensive, effective in very low concentrations, have no harmful effect on the enzymes, and are compatible with the yeasts that subsequently grow on the sugar solutions to produce alcohol. Attempts have been made to stabilize the enzymes by chemical modification in such a way as to maintain their solubility. Glutaraldehyde treatment greatly increased the enzyme size, lowered the pI values, and gave a slight shift in the pH activity curve. There was, unfortunately, *no* increase in enzyme stability, and the activity of enzymes on solid celluloses was adversely affected. Shaking greatly reduced the hydrolysis of Avicel by *Trichoderma reesei* C 30 enzyme. The adverse effect was accompanied by a decrease in recoverable enzyme and protein.

INTRODUCTION

When an enzyme is applied to a practical process such as cellulose saccharification, stability becomes a factor of increasing importance. Whereas these enzymes may be sufficiently stable for the growth of the organism, they are less stable under the conditions we wish to employ; e.g., high substrate and product concentrations, high temperature, long incubation, nonsterile environment, the presence of assorted foreign materials, and shear resulting from vigorous agitation. Since low cost is often a critical determinant of whether a process is practical, many of the enzymes are not purified, and as a result contain materials that may contribute to inac-

tivation. These include proteases which are often involved in the degradation of other enzymes,¹ usually under conditions unfavorable to that enzyme. The best means of achieving a stable enzyme is to find one through screening organisms and by modifying growth conditions. It is much more difficult to correct later for instability. Additives that have been useful in stabilizing some enzymes fall into several categories: 1) Metals, especially Ca^{2+} . 2) Proteins, such as bovine serum albumin (apparently because protein reactants will act on it, and spare the enzyme). 3) Biocides, to prevent inactivation by contaminating microorganisms. 4) Substrates, or products of the desired enzyme.¹ 5) Enzyme modification; e.g., immobilization.

The cellulases of *Trichoderma* were reported to be remarkably stable and resistant to inhibitors and other toxic compounds.² However, these early studies were based on endoglucanase activity, since carboxymethylcellulose (CMC) was used as the substrate. More recent studies have shown that the cellobiohydrolases (CBH) are much less stable.³⁻⁵ Fortunately the maximum pH stability of CBH (ca. 5.0) coincides with its optimum pH activity.⁵ A related problem is that the hydrolysis conditions may promote microbial contamination of the saccharification vessel. If biocides are used, they must neither inactivate the enzymes, nor interfere with the later use of the sugar syrup. This paper reports on factors affecting the stability of the crude "cellulase" enzymes as found in culture filtrates under conditions that are used in the enzymatic hydrolysis process.

METHODS

The cellulase preparation used was a cellulose culture filtrate of *Trichoderma reesei* QM 9414.⁶ The cellulase complex contains three types of components:

1) Endo- β -1,4-glucanase (Cx) measured against CMC (0.5%) for 30 min.

2) Cellobiohydrolase (CBH), an exoenzyme measured by incubating acid-treated Avicel pH 102 (1.25%) with enzyme for 60 min. The pretreatment involves boiling for 30 min with 1N HCl, washing and blending for 5 min in a Waring blender, and repeating the process a second time.

There is no means for directly measuring CBH in the presence of Cx; i.e., there is no substrate for CBH that is not also a substrate for Cx. While Avicel, because of its crystallinity, is a poor substrate for pure CBH or for pure Cx, it is a fairly good substrate for a

mixture of the two (i.e., a synergistic effect). When a compound is found to have no effect on Cx activity, yet strongly affects Avicel hydrolysis, we assume the action is on the CBH component of the enzyme mixture. It is in this context that "Avicelase" and "CBH" are used in the text.

3) β -Glucosidase (Cellobiase) measured against salicin (0.5%) for 30 min.

In these assays, 1 vol enzyme was incubated at 50°C with 1 vol substrate at pH 4.8. The enzymes were diluted so that the reducing sugar produced during hydrolysis was about 0.50 mg/ml as glucose.

For enzyme inactivation, 1 vol enzyme (6 mg/ml) was incubated (unshaken) at 50°C, pH 5.0, with 1 vol inactivating compound for one to seven days. At the end of the incubation period, the enzyme was diluted 1:30 in citrate buffer prior to assaying (thus diluting out the inactivating factor).

For the determination of the effective biocide concentration, 1 vol biocide was added to 4 vol peptone broth inoculated with a 24 hr culture of *Bacillus coagulans*. (This organism is a thermophile isolated from a contaminated cellulose hydrolysis batch, and tentatively identified as *Bacillus coagulans* by H. S. Levinson.) Incubation was under conditions to be used for the cellulose hydrolysis (e.g., pH 5.0, 50°C, for one to seven days), even though this organism grows much better at higher pH values.

RESULTS AND DISCUSSION

Stability of Cellulase Components

All stability experiments were carried under the conditions contemplated for the enzymatic conversion of cellulose to glucose (50°C, pH 5.0). Effects on enzyme alone were first examined. It was found (Fig. 1) that of the three cellulase components, CBH, measured in this case against cotton, was rapidly inactivated, whereas endoglucanase (Cx), and β -glucosidase were stable.

This development led to an evaluation of the incubation conditions, and it was found that the merthiolate used as a preservative was responsible for much of the adverse effect (Fig. 2). The action was on Avicelase and not on the endo- β -glucanase; and at 50°C, but not at lower temperatures. The same merthiolate concentrations had no effect on enzyme stored in the refrigerator for years; nor could any effect be observed during short incubation periods (30–60 min, 50°C) used in assaying the activities. It seems that, at 50°C,

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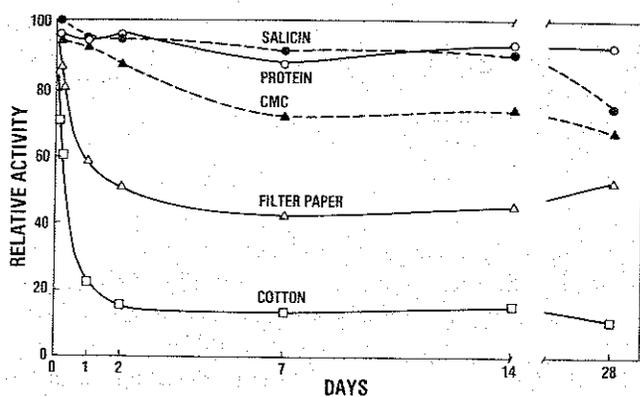


Fig. 1. Stability of cellulase components of *T. reesei* QM 9414. Enzyme (2.5 mg protein/ml) was incubated at pH 4.8, 50°C (in the presence of 0.01% merthiolate for times shown, then assayed).⁴ (●—●) β -Glucosidase; (▲—▲) endo- β -glucanase (Cx); (△—△; □—□) combined action of Cx and CBH.

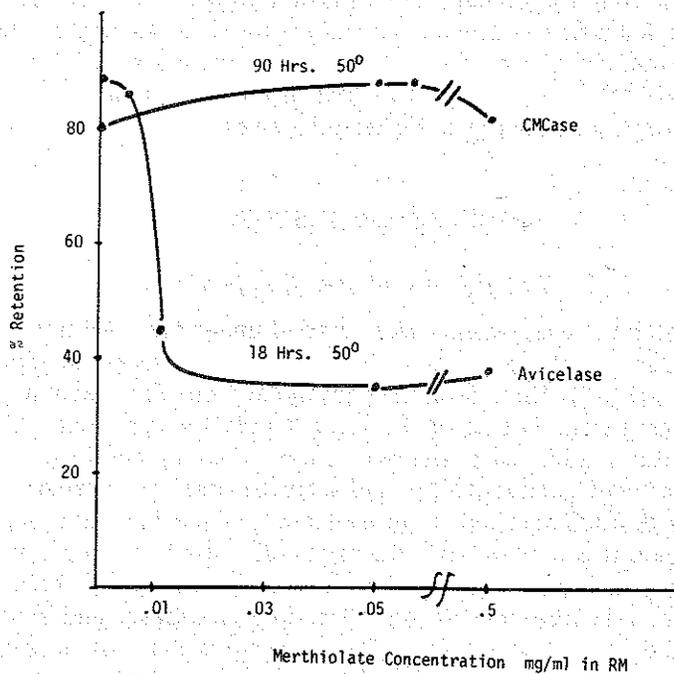


Fig. 2. Effect of merthiolate on stability of cellulase of *T. reesei*. Merthiolate was added to enzyme (3 mg protein/ml) and incubated under hydrolysis conditions (e.g., 50°C, pH 5.0), after which the sample was diluted before assay.

the configuration of the affected enzyme may be changed in such a way as to make it more susceptible to the merthiolate. A chromatogram of a preparation partially inactivated by merthiolate does indeed show the disappearance of *one* of the two CBH peaks,¹⁵ thus possibly accounting for the leveling off of the curve at high merthiolate concentrations. Other mercury compounds (Table I)

TABLE I
Inactivation of Cellulase Components of *T. reesei* QM 9414

Compound	<i>I</i> ₅₀ (20 hr, 50°C, pH 5.0) ^a		
	Avicelase (mg/ml)	CMCase (mg/ml)	Biocide conc ^b (mg/ml)
A) Greatest inactivation of Avicelase			
<i>p</i> -Chloro-Hg benzoate	0.002	> 1.0	-
HgCl ₂	0.003	> 0.2	-
Merthiolate	0.01	> 1.0	0.002
K-Iodoacetate	0.10	> 2.0	1.25
Quaternary ammonium (Roccal)	0.20	> 3.0	0.008
Pentachlorophenol	0.3	> 1.0	0.005
Dithiothreitol	0.5	> 5.0	-
<u>Mercaptoethanol</u>	<u>0.8</u>	> 5.0	> 2.5
Zn-Dimethyldithiocarbamate	0.4	> 1.0	-
Glutaraldehyde	0.5	> 1.0	2.0
Formaldehyde	1.0	> 10.0	0.05
<i>p</i> -NO ₂ -phenol	1.0	> 1.0	0.01
Sodium lauryl sulfate	0.05	0.2	0.04
B) Less inhibitory			
Hydrogen peroxide	1.2	> 1.0	0.05
Na-Azide	6	> 20.0	1.0
Tween 80	20 +	> 20.0	> 10
C) No inactivation in three days			
Toluene (satd) (0.5 mg/ml)	-	-	> satd
Thymol (satd) (1.0 mg/ml)	-	-	> satd
Succinic anhydride (2.0 mg/ml)	-	-	2.0
Butyraldehyde (10 mg/ml)	-	-	-
Diethylcarbonate (10 mg/ml)	-	-	> 10

^a *I*₅₀ is the concentration of chemical required to give 50% inactivation of enzyme (under conditions shown).

^b Concentration of biocide required to prevent growth of *B. coagulans* in broth after seven days at 50°C (pH 5.0); (-) = not tested.

*I*₅₀ = 1.5 / 154 = 3mM 50° 20hr
 [RNase (yeast) = 5mM 37° 2hr] ←
 BB 23: 953

Why have we overlooked this reduction of -S-S- bonds by dithiothreitol etc

resemble merthiolate in this action. The presence of a number of disulfide linkages^{3,7} in CBH may explain this effect.

The sensitivity of cellulase components to heat and to merthiolate, lead to an investigation of a means of improving the stability, and to a search for a biocide effective in preventing contamination of the hydrolysis mixtures without impairing the action of the enzyme. If the hydrolytic conversion of cellulose to glucose is done batchwise, for 24 hr at 50°C, addition of a biocide may be unnecessary. If, however, a continuous process is envisioned, contamination with thermophiles may be a problem. In anticipation of this, we have examined potential biocides for their effects on the cellulolytic enzymes (Table I). We found that Avicelase is generally much more sensitive to these compounds than is endoglucanase or β -glucosidase. I_{50} values for β -glucosidase, very similar to those for CMCase, were found for those compounds tested; e.g., mercury compounds, iodoacetate, azide, toluene, and formaldehyde. The data indicate that most of the biocides inactivate CBH at concentrations required for prevention of microbial growth. Even 0.1% azide inactivates at 50°C, if the time is long enough. Toluene, thymol, and azide (0.01%) appear promising, in that they showed no inactivation of enzyme over a seven-day period under the conditions of digestion.

The difference in susceptibility between Avicelase and endoglucanase is reflected in the I_{50} values (concentration of inhibitor required to give 50% inactivation of enzyme; Table I). Compounds at the top of the table show the greatest differences between the two enzymes. These are agents that react with the—SH or —NH₂ protein groups. Included are two thiols that may be acting on the disulfide linkages to modify the conformation of CBH. The aldehydes react with—NH₂ groups; glutaraldehyde being much more effective than butyraldehyde. The quaternary ammonium compounds (cationic surfactants) precipitate the enzymes at 50°C, pH 5.0 (at pH 7.0 precipitation occurs at room temperature). The non-ionic surfactant (Tween 80) has no effect on the enzyme activity.

A series of antibiotics was also tested for their effect on Avicelase. Only oxytetracycline showed any inactivation at a concentration of less than 1 mg/ml. Under the test conditions, this antibiotic quickly turned brown, had an I_{50} versus Avicelase of 0.3; and in these properties was quite unlike the two other tetracyclines tested (Table II).

The biocides were then tested for their ability to inhibit bacterial growth at room temperature ($\pm 25^\circ\text{C}$). Toluene, thymol, and azide

TABLE II
Antibiotics for Use in Cellulase Hydrolysis Systems

Antibiotic	I_{50} Avicelase ^a (mg/ml)	Biocide conc ^b (mg/liter)
Tetracycline	> 1.0	2
Chlortetracycline (aureomycin)	> 1.0	2
Oxytetracycline (tetracycline)	0.3	50
Polymyxin B sulfate	> 1.0	5
Magnamycin	> 1.0	8
Streptomycin-sulfate	> 1.0	40
Cycloheximide	> 1.0	400
Mycostatin	> 1.0	400+
Endomycin	> 1.0	600
Penicillin G	> 1.0	500

^a I_{50} is the concentration of chemical required to give 50% inactivation of enzyme (under conditions shown).

^b Concentration of biocide required to prevent growth of *B. coagulans* in broth after seven days at 50°C (pH 5.0).

were effective at concentrations showing no inactivation of enzyme. However, when tested against the thermophilic *B. coagulans* at 50°C (pH 5.0), these agents did *not* prevent growth. Several antibiotics do look promising (Table II). Of these, two tetracyclines are effective at very low concentrations, and at low cost (about 0.01 cents/liter; tetracycline at 35-40 dollars/kg).

Protection of Avicelase

During these tests, some compounds seemed to protect Avicelase; i.e., they *reduced* the rate of inactivation at pH 5.0, 50°C (Table III), to about $\frac{1}{2}$ that of unprotected enzyme, but under no condition was complete protection achieved. At first, it was thought that bacterial growth might be responsible for inactivation, and indeed the addition of *B. coagulans* cells did increase the inactivation. However, when bacteria are kept from growing, the protective effect of the compounds still remains.

Cycloheximide and mycostatin are antifungal antibiotics. But this does not explain their protective action. There seems to be no correlation between the antibiotic and protective actions of these compounds.

The other "protectants" of Avicelase are substrate (cellulose), and product (cellobiose) analogs. Methocel is a highly substituted methyl ether of cellulose. Glucostilic acid is a cellobioside of a

TABLE III
Compounds that Protect Avicelase against
Heat Inactivation

Compound	Protection ^a (mg/ml)
Cycloheximide	0.4
Mycostatin	0.1
Methocel DS 1.89	0.04
Ustilagic acid	0.5
Glucoustilic acid	2.0
Lactose	2.0

^a Protection is the amount of compound required for 50% reduction in inactivation at 50°C, pH 5.0, 24 hr, unshaken. Enzyme protein = 3 mg/ml.

fatty acid; ustilagic acid is a modification of glucoustilic acid in which the cellobiose units are esterified. Lactose is a dimer differing from cellobiose only in the position of one hydroxyl group.

Methocel, the substrate analog, has long been recognized⁸ as a potent inhibitor of endo- β -1,4-glucanase (Cx). It also inhibits Avicelase activity; but it also *protects* the enzyme against inactivation (Fig. 3). This effect is observed because the subsequent assay for activity is done at a dilution of 30-fold or more; i.e., the methocel is diluted to such a low concentration that its effect in the assay becomes negligible. The decreased "protection" at high methocel concentrations is probably attributable to the fact that after dilution for assay, these solutions still contain sufficient methocel to *inhibit* the activity.

The protectants that are modifications of cellobiose are assumed to react with CBH much as cellobiose itself. But they are more resistant to hydrolysis by the *T. reesei* enzymes, and so remain active for a much longer time. Cellobiose and methyl- β -cellobioside, which are readily hydrolyzed, show less protection; glucoustilic acid, lactose, and ustilagic acid, which resist hydrolysis, show greater protection (Table III).

Unfortunately, these compounds are like methocel in that they not only protect Avicelase against inactivation, but they also inhibit its activity. As a result, while they may be useful in preserving concentrated enzyme solutions, they do not have much promise as enzyme protectants during cellulose hydrolysis.

Many other compounds were tested as protective agents for Av-

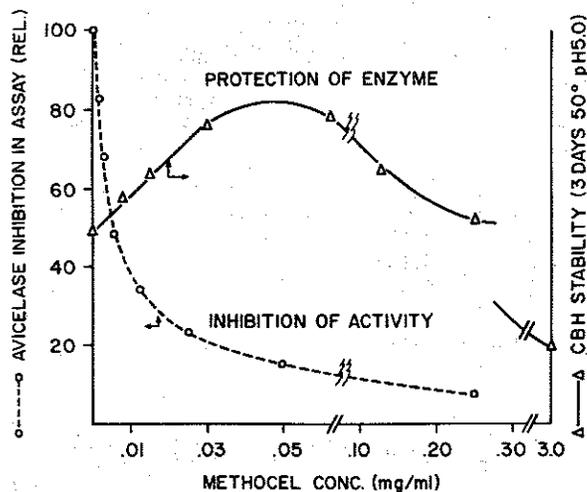


Fig. 3. Effect of methocel (DS 1.89) on (○—○) activity during the Avicel assay (i.e., 50°C, pH 4.8, 60 min); and on (△—△) stability over a prolonged period (i.e., three days, 50°C, pH 5.0). Enzyme concentration during the stability test is 3 mg protein/ml; concentration in Avicel assay is 0.05 mg protein/ml.

icelase under the hydrolysis conditions. These included metals (Ca, Co, Mn, Cu, Fe, Zn), proteins, polysaccharides, sugars, EDTA, and Tween 80. None had any significant effect at the concentration tested (e.g., 1 mg/ml).

Strain Differences in Enzyme Stability

We have a number of mutants from the parent strain *T. reesei* QM 6a, developed at U.S. Army Natick Research & Development Command, and at Rutgers University.^{9,10} A comparison of these for heat stability under unshaken conditions (Table IV) reveals that the most stable Avicelase is that of Rutgers strain C 30; the next, Rutgers NG 14. The Natick mutants QM 9414 and MC G77 show no increase in stability over the parent. Fortunately, the C 30 strain is also more productive.

Avicelase of the *T. viride* strain used in Japan (Onozuka preparations) was found to be less stable than any of the above.

While the Avicelase of strain C 30 is more stable than others, it reacts similarly with chemical reagents (Table I). Merthiolate and dithiothreitol strongly inactivate it; methocel offers some protection.

TABLE IV
Stability of Avicelase of Various Strains of *T. reesei*

Strain		Enzyme inactivation ^a (pH 5.0, 50°C)	
		2 days	3 days
(%)			
C 30	Rutgers	14	28
NG 14	Rutgers	20	36
6a	Parent strain	30	45
9414	Natick	36	48
MCG 77	Natick	33	44

^a Unshaken.

Enzyme Modification

Another approach is to increase stability by chemical modification of enzymes. It is well known that immobilization often increases stability. But in our case, with an insoluble substrate, immobilization is not the answer. One needs to modify the enzyme yet retain its solubility so that it is free to move to its site on the *insoluble* cellulose. Having previously used glutaraldehyde for immobilization, we decided to try it under milder conditions to produce a modified yet *soluble* component. The entire enzyme complex of *T. reesei* was treated at pH 5.0, 25°C, with 1% glutaraldehyde for various times. The result was to greatly increase the molecular weights, from ca. 34000 to over 500000 (gel permeation).

Undoubtedly, various cross-linkages between the different enzymes were formed. The *pI* values were also determined (isoelectric focusing) on original and treated samples. The *pI* value of the endo- β -1,4-glucanase of *T. reesei* was lowered from 4.7 to 3.9; the β -1,3-glucanase components from 6.7 and 8.2 to 3.9 and 4.1. Such a lowering of the isoelectric points is to be expected of reagents acting on amino groups. Other changes were also observed; e.g., a slight downward shift in the optimum pH of the endoglucanase; and a slight increase in its absorptability on clay. Unfortunately, there was no increase in heat stability; and the ability to degrade *solid* celluloses (i.e., CBH) was impaired to a greater extent than the action on the soluble cellulose derivatives (i.e., Cx).

Enzyme Stability during the Hydrolysis Reaction¹¹

The preceding experiments on stability were carried out using buffered solutions under static conditions in the *absence* of sub-

1/100
Pat Drumm
finds that The Rutgers mutants
are deficient in protease
as a main correlation
most stable: least protease
(but not for shaking
inactivation)

strate. The experiments in this section were done in the presence of substrate (10% Avicel pH 102) and included both shaken and unshaken suspensions (Table V). Results with chemical inhibitors were similar to those obtained in the absence of substrate. Merthiolate caused 31% inhibition of Avicel hydrolysis at 0.005 mg/ml; the quaternary ammonium compound (Roccal) 67% inhibition at 0.5 mg/ml; and mercaptoethanol 11% inhibition at 1 mg/ml; all in shaken flasks. The reduction in enzymatic hydrolysis was accompanied by a reduction in the amount of Avicelase remaining in solution after three days hydrolysis. Again antibiotics showed no adverse effects.

The most surprising effect was the inactivation of C 30 cellulase due to shaking (Table VA). Little hydrolysis of Avicel by this enzyme occurred in shaken flasks after the first day and, by three days, hydrolysis in shake flasks was only half that in unshaken flasks. The cellulase of QM 9414 was less affected by shaking. These data are in contrast to those of unshaken tests (above) where C 30 enzyme was shown to be more stable than QM 9414 enzyme. This is still apparent when residual cellulase and protein in the

TABLE V
Effect of Shaking on Enzymatic Hydrolysis of Cellulose (Avicel)^a

Cellulase source	Atmosphere	Hydrolysis (%)			
		22 hr		70 hr	
		shaken	unshaken	shaken	unshaken
A)					
<i>T. reesei</i> QM 9414	air	31.5	29	42	49
	CO ₂	32	31	43	50
<i>T. reesei</i> C 30	air	22	30	26	50
	CO ₂	22	30	23	51
		Residual Avicelase ^b		Residual protein ^b	
B) 70 hr results		shaken	unshaken	shaken	unshaken
<i>T. reesei</i> QM 9414	air	21	32	44	49
	CO ₂	21	50	43	56
<i>T. reesei</i> C 30	air	1	49	16	64
	CO ₂	0	57	13	67

^a Reaction conditions: 10 g Avicel (pH 102) + 25 ml enzyme solution (0.025M citrate, pH 5.0) of about 1.2 fp units⁴/ml (*T. reesei*), and 2.2 cellobiase μ /ml (*Aspergillus phoenicis* QM 329); tetracycline (0.01 mg/ml) as a preservative; flask size 125 ml, with rubber stopper; shaker: rotary 120 rpm, 3 cm diam; 50°C temperature.

^b Enzyme and protein remaining in solution in the digest. Subsequently precipitated with 2 vol acetone, and redissolved in buffer. Values are percent of initial enzyme; i.e., before addition of substrate.

digests were measured after three days (Table VB). Under unshaken conditions more of the C 30 enzyme (than QM 9414) remained active. Under shaken conditions, the amounts of both enzymes were reduced, with that of C 30 enzyme being reduced to a much lower level.

A carbon dioxide atmosphere was used to replace air in some flasks in view of Eriksson's claim¹² that an oxidative mechanism is sometimes involved in cellulose digestion. CO₂ had no effect on hydrolysis rate with either enzyme, under shaken or unshaken conditions. On the other hand, CO₂ did have a favorable effect in unshaken flasks on the amount of Avicelase and protein remaining in solution at the end of the incubation (Table V).

The optimum hydrolysis of Avicel in these tests occurred when *T. reesei* C 30 enzyme (with added β -glucosidase) was used under unshaken conditions at pH 5.0, 50°C, with an antibiotic such as tetracycline as a preservative. The use of a CO₂ atmosphere preserved more enzyme for further digestion and recovery.

There are many aspects of these problems yet to be considered. In view of the results presented here on adverse effects of merthiolate and shaking, many of the earlier published results may need reinterpretation. However, the present results obtained in flask scale and with "nearly pure" cellulose must be extended to larger-scale reactors and to more complex cellulosic substrates.

Mutant strains must be evaluated not only for cellulase productivity but also for enzyme stability and performance in saccharification. As enzyme stability is increased, it may be possible to: a) hydrolyze at higher temperatures and thus speed up the process and b) use less enzyme and so reduce enzyme costs. The value of increased β -glucosidase has previously been demonstrated.¹³ For complex cellulose substrates, additional enzymes (such as xylanase) may increase cellulose hydrolysis by removal of interfering polymers.¹⁴

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