

Microbial Sources of Cellulase

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The availability of high activity cellulase is the basis for a successful process for enzymatic conversion of cellulose. This depends on selection and improvement of suitable strains for enzyme production, and development of fermentation methods for producing enzyme in quantity. *Trichoderma viride* is the best present source of active cellulase. Many other actively cellulolytic organisms exist and we do not forget that variations in media or growth conditions may result in good cellulase preparations from other organisms.

Methods used in this investigation have been previously described [1, 2]. All cellulase assays were carried out at pH 4.8, and 50°C unless otherwise indicated. Cellulase units on carboxymethyl cellulose (CMC) or on filter paper (FP) equal micromoles of glucose produced per minute. Cellulase activity on filter paper equals milligrams of glucose produced in the assay described in the test. Cellulase activity on cotton equals milligrams of glucose produced by 1 ml of enzyme from 5% cotton in 24 hr.

CRITERIA FOR SELECTION OF CELLULASE SOURCE

Although many fungi and bacteria degrade cellulose, the products of growth on cellulose are microbial cells and metabolic products such as CO₂ and methane. Only a few fungi such as *Trichoderma viride*, *T. lignorum*, and *T. koningii* [1-10], *Chrysosporium lignorum* and *C. pruinatum* [6, 10], *Penicillium funiculosum* [11] and *P. iriensis* [12], and *Fusarium solani* [9] have been reported to produce high levels of enzyme capable of extensively degrading insoluble cellulose to soluble sugars in vitro. Many more organisms produce cellulase preparations that

will degrade soluble cellulose derivatives such as CMC, and a few, despite active growth on insoluble cellulose, produce very little residual cellulase of any type [1]. This situation has been explained by the fact that cellulase (EC 3214) is a complex of enzymes and enzyme-like factors and that not all members of the complex are necessarily found in the culture fluid after growth of the organism. The practical saccharification of waste cellulose requires a stable cell-free enzyme preparation with adequate levels of all essential components of the cellulase complex. Rapid growth on and decomposition of cellulose, and/or production of high levels of enzymes degrading soluble cellulose derivatives are not adequate criteria for selecting organisms to be used as a source of cellulase.

Cellulose is a polymeric insoluble substance in which the physical structure, i.e., the extent of interaction of chains varies widely. Cellulose chains which are completely free of interaction with each other as in soluble derivatives or swollen amorphous cellulose are reactive and are hydrolyzed by C_x enzymes. Cellulose chains which are highly ordered or crystalline, as in dried cotton fibers, require the presence of both C_x and C_1 enzymes for hydrolysis. Most cellulose substrates such as paper contain some loose chains or free ends which are reactive, some highly ordered crystalline cellulose, and a spectrum of partially crystalline to partially amorphous cellulose, and so exhibit limited hydrolysis by C_x requiring both C_x and C_1 components for total hydrolysis. When particle sizes are large and/or impurities are present accessibility problems may prevent total hydrolysis even when a complete enzyme complex is present.

The cellulase of *Pestalotiopsis westerdijkii* (Pw) contains high levels of C_x , but is low in C_1 . When insoluble cellulose was hydrolyzed by Pw C_x (Fig. 1), the reactive cellulose was rapidly hydrolyzed, but the rate then slowed down or leveled off as the residue was increasingly resistant. The available or reactive portion varied from less than 2% of cotton to about 24% of Sweco 270.

The cellulase of *Trichoderma viride* (Tv) contains both C_x and C_1 . When insoluble cellulose was hydrolyzed by Tv cellulase (Fig. 2), the reactive cellulose was very rapidly hydrolyzed followed by a slow hydrolysis of the more resistant portions, which continued until all available cellulose had been hydrolyzed. The extent of reaction in 48 h ranged from 6% for cotton to over 90% for Sweco 270. With long incubation even cotton will be totally hydrolyzed [6].

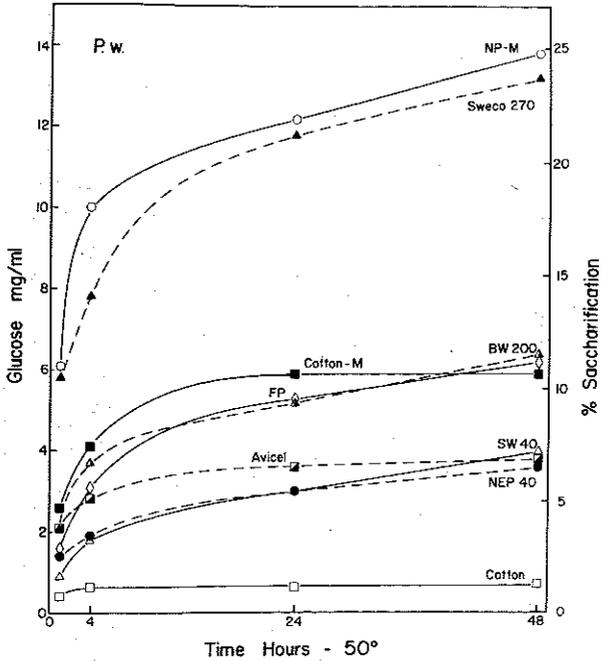


Fig. 1. Hydrolysis of insoluble cellulose by a C_x cellulase from *Pestalotiopsis westerdijkii*. 5% cellulose incubated at pH 4.8, 50°C with a filtrate of strain QM381 grown on cellulose medium. The enzyme preparation had 0.6 mg protein, 19 C_x units, 0.1 FP (filter paper) cellulase units per ml, and 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; (▲) BW200, pure cellulose pulp, ball milled, Brown Co., Berlin, N.H.; (○) Whatman No. 1 filter paper; (■) ball milled absorbent cotton; (■) Avicel PH 105, microcrystalline cellulose; (▲) pure cellulose pulp SW40, Brown Co.; (●) hammer milled newsprint NEP40, Brown Co.; (□) absorbent cotton, fibrous.

The difference between the Pw and Tv filtrates was most clearly evident when filtrates from the two organisms were diluted to equal activities on CMC (Fig. 3) or to equal protein content (Fig. 4) and allowed to act on filter paper. Initial hydrolysis by both preparations was rapid, but action by the C_x only (Pw) preparation leveled off after 30 min when the substrate for C_x was used up, while action by the C_1 plus C_x (Tv) preparation continued on the more resistant portions of the substrate, although at a slower rate.

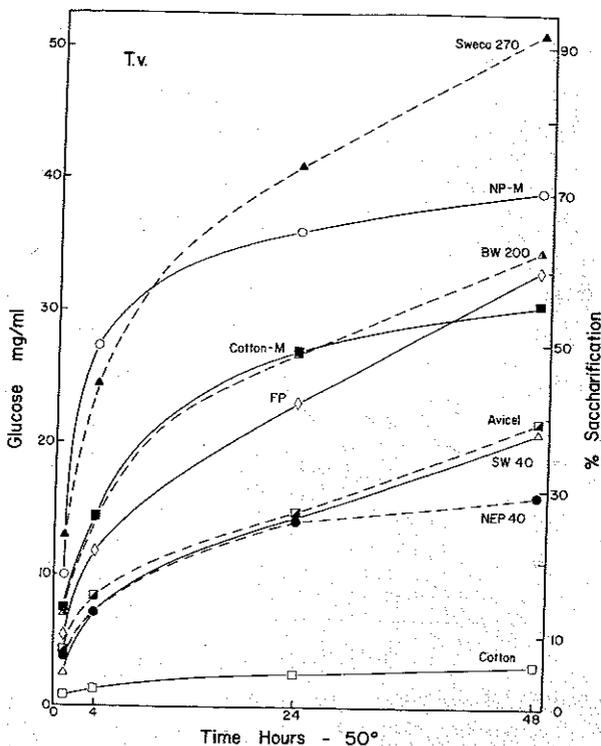


Fig. 2. Hydrolysis of insoluble cellulose by a complete cellulase from *Trichoderma viride*. 5% cellulose incubated at pH 4.8, 50° with a filtrate of strain QM9414 grown on cellulose medium. The enzyme preparation had 0.8 mg protein, 25 C_x units and 0.6 FP cellulase units per ml, and a C_x activity of 2.8 mg of glucose per 24 hours. (○) Newspaper, Sweco ball milled; (▲) pure cellulose pulp, Sweco ball milled, 270 mesh; (△) BW200, pure cellulose pulp, ball milled, Brown Co., Berlin, N.H.; (◇) Whatman No. 1 filter paper; (■) ball milled absorbent cotton; (◻) Avicel PH 105, microcrystalline cellulose; (△) pure cellulose pulp, SW40, Brown Co.; (●) hammer milled newsprint, NEP40, Brown Co.; (□) absorbent cotton, fibrous.

MEASUREMENT OF CELLULASE ACTIVITY

Measurement of cellulase activity therefore presents special problems that do not arise for enzymes hydrolyzing soluble substrates. Time is required for the enzyme to diffuse into the fiber and for the soluble products of hydrolysis to diffuse out of the fiber. The more resistant the substrate and the longer the time of exposure, the greater the difference between preparations containing only C_x and preparations

containing both C_1 and C_x . A valid cellulase assay requires incubation for a long enough time that an active preparation will hydrolyze some of the less accessible bonds. The problem is to select an appropriate substrate and to decide the extent of reaction required for meaningful values.

C_x or endo- β 1,4 glucanase is measured by reducing sugar reproduction from CMC, which has a 0.5 degree of substitution. This cellulose derivative is soluble and very reactive, but cellulase cleaves only the linkages between unsubstituted glucose units and hydrolysis is linear only to about 12% [1]. The presence of C_1 or other components does not affect this assay, hence it is satisfactory for measurement of C_x in pure, mixed, or crude preparations.

A direct measure of C_1 is more complicated since the nature of the synergism between C_x and C_1 remains controversial. An exo acting cellobiohydrolase had been identified in C_1 preparations from *T. viride*

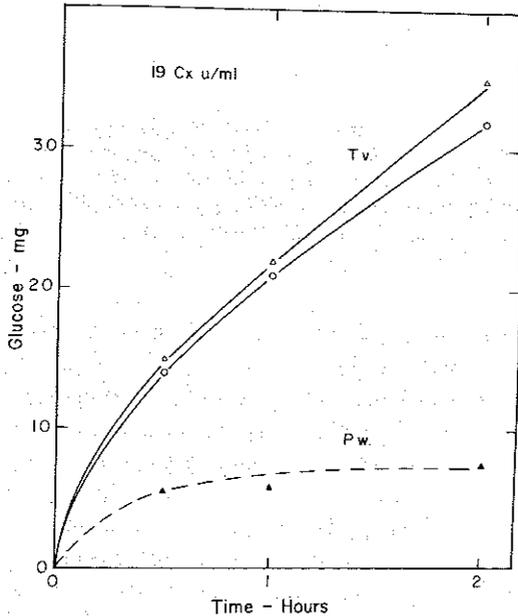


Fig. 3. Hydrolysis of filter paper by cellulase preparations from *Trichoderma viride* and *Pestalotiopsis westerdijkii* adjusted to equal activities on carboxymethyl cellulose. Culture filtrates diluted to 19 C_x units per ml. 0.5 ml enzyme + 1 ml pH 4.8 buffer + 50 mg paper; Incubated at 50°C. (Δ) Tv QM9123 culture filtrate; (\circ) Tv QM9414 culture filtrate; (\blacktriangle) Pw QM381 culture filtrate.

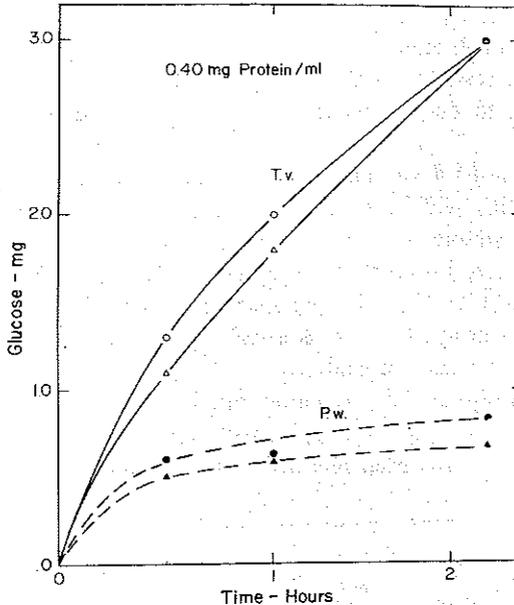


Fig. 4. Hydrolysis of filter paper by cellulase preparations from *Trichoderma viride* and *Pestalotiopsis westerdijkii* adjusted to equal protein concentrations. Culture filtrates diluted to 0.40 mg soluble protein per ml. 0.5 ml enzyme + 1 ml pH 4.8 buffer + 50 mg paper. Incubated at 50°C. (○) Tv QM9123 culture filtrate; (△) Tv QM9414 culture filtrate; (●) Pw QM381 culture filtrate A; (▲) Pw QM381 culture filtrate B.

and other organisms [3, 4, 9]. This enzyme slowly produces cellobiose from phosphoric acid swollen cellulose or Avicel, but an assay based on this reaction is useful only for pure isolated components. The original theory [6] was that C_1 converts crystalline cellulose into a substrate for C_x . The increase in alkali solubility after treatment with *T. viride* cellulase, particularly when the C_x is inhibited by methyl cellulose, supports this belief [13]. The correlation between % alkali solubility and % hydrolysis by Pw C_x for various cellulose substrates [12] suggests that alkali solubility is a fair measure of reactive cellulose. In any event, measurement of the synergism between C_x and C_1 requires an indirect assay. As we progress from the more reactive substrates such as Sweco 270 to the more resistant substrates such as cotton, the role of C_1 in the hydrolysis becomes more important (Fig. 2). C_1 therefore is measured in crude preparations using fibrous cotton as a substrate with

the understanding that C_x must also be present, and that some of the glucose produced comes from the action of C_x alone. To maximize the role of C_1 the enzyme is incubated with the cotton for 24 hr.

Filter paper is a satisfactory and easily reproducible substrate to measure total cellulase in a short time. Several measures of filter paper activity have been used [1, 7, 14] based on the glucose produced when an enzyme preparation is incubated with filter paper under standard conditions. A series of dilution curves (Fig. 5) shows the effects of

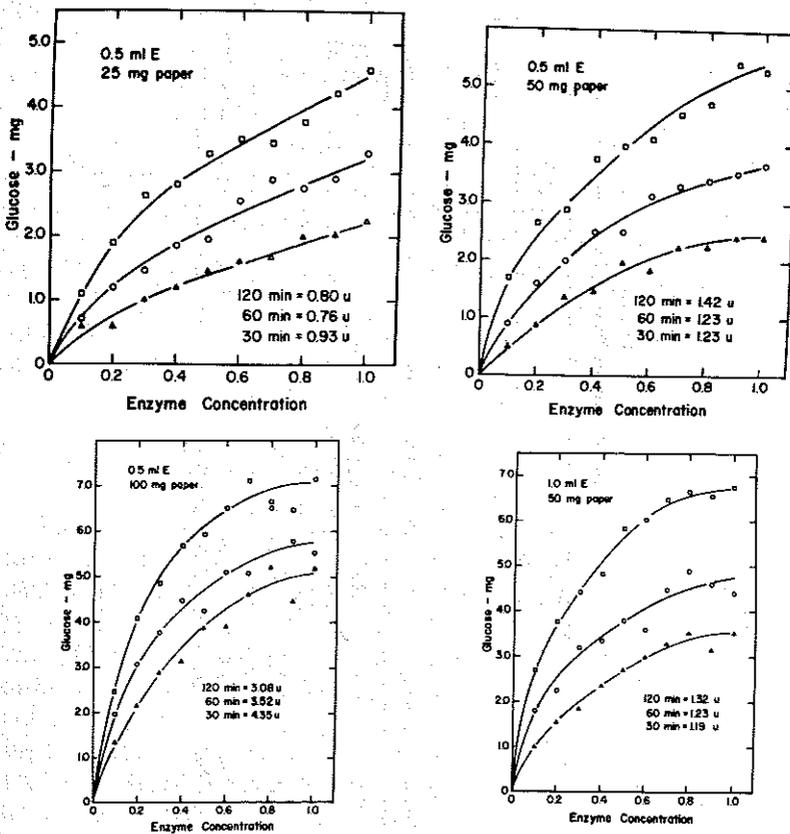


Fig. 5. Hydrolysis of filter paper by *Trichoderma viride* cellulase. Effect of assay conditions. 0.5 or 1.0 ml enzyme + 1 ml buffer pH 4.8 + 25, 50, or 100 mg Whatman No. 1 filter paper. Units = μ moles of glucose per min based on dilution to give 2 mg of glucose. (Δ) 30 min incubation 50°C; (\circ) 60 min incubation 50°C; (\square) 120 min incubation 50°C.

TABLE I
Effect of Conditions on Filter Paper Assay^a

Filter paper (mg)	Enzyme (ml)	Enzyme protein (mg)	Activity													
			Per hr						Units/ml						Units/mg	
			30 min	60 min	120 min	30 min	60 min	120 min	30 min	60 min	120 min	30 min	60 min	30 min	60 min	
25	0.5	0.7	2.29	3.30	4.60	4.58	3.30	2.30	0.93	0.76	0.80	0.66	0.56	0.59		
50	0.5	0.7	2.43	3.68	5.30	4.86	3.68	2.65	1.23	1.23	1.42	0.88	0.88	1.01		
50	1.0	1.4	3.52	4.40	6.75	7.04	4.40	3.38	1.19	1.23	1.32	0.88	0.88	0.94		
100	0.5	0.7	5.18	5.53	7.16	10.36	5.53	3.58	4.35	3.52	3.08	3.11	2.51	2.61		

^a0.5 or 1.0 ml of enzyme (culture filtrate of QM9414 grown on cellulose) plus 1 ml of pH 4.8 buffer + strip of Whatman No. 1 filter paper. Units = micromoles of glucose per minute based on the dilution to give 2.0 mg.

enzyme and filter paper concentration and time of incubation on this assay.

Increasing any of these led to an increase in sugar production, but the filter paper activity per hour fell off rapidly as incubation proceeded (Table I) because the most reactive substrate was most rapidly converted. Other factors changing as hydrolysis proceeded include available surface and product inhibition. A filter paper activity for a standardized assay is an adequate estimate of enzyme level for many purposes such as monitoring fermentations or screening new strains or mutants. The values become erratic at high glucose concentrations and so are not usable for very active preparations, enzyme concentrates, or for quantitative work. Linearity can be increased by diluting the enzyme, reducing incubation time, or increasing substrate concentration [14], but any of these changes will increase the relative contribution of the C_x component. The most satisfactory solution appears to be to retain filter paper activity as a rough indicator, but for quantitative work to also calculate enzyme units based on the same extent of hydrolysis of the filter paper, i.e., the dilution giving 2 mg of glucose. Units figured on this basis (Table I) are only moderately affected by time of incubation or the volume of enzyme used in the assay, but are strongly affected by the substrate concentration.

COMPARISON OF CELLULASES FROM ACTIVE STRAINS

Many thermophiles are extremely active in degrading cellulose. Cultures of *Chaetomium thermophilum* QM9381, *Sporotrichum thermophilum* QM9382, and *Thermoascus aurantiacus* QM9383 were given to us by Tansey as among the most actively cellulolytic fungi studied by him [15]. These and other thermophiles were tested on 3 cellulose media (Table II). Growth and cellulose decomposition were very rapid, but the cellulase activity of the culture filtrates were low compared to the filtrates of the mesophilic fungus *Trichoderma viride*. Stutzenberger [16] has described an actively cellulolytic thermophile *Thermomonospora curvata* that produces a culture filtrate containing C_x and C_1 when grown on cellulose medium. However this enzyme achieved less than 1% hydrolysis of cotton even after 30 hr incubation, and the residue was resistant to hydrolysis by fresh enzyme, suggesting that action was chiefly on a small amount of reactive cellulose rather than on crystalline cellulose.

TABLE II
Production of Cellulase by Thermophilic Cellulolytic Fungi
Compared to *Trichoderma viride*^a

QM#	Organism	Growth medium	FP units/ml	
			28°C 12 days	37°C 7 days
6a	<i>Trichoderma viride</i>	A	0.33	NG
		B	0.32	NG
		C	0.30	NG
9123	<i>Trichoderma viride</i> (mutant)	A	0.75	NG
		B	1.10	NG
		C	1.10	NG
228	<i>Humicola grisea</i>	A	NT	0.08
		B	NT	0.08
		C	NT	0.11
1192	<i>Hormiscium</i> sp.	A	NT	0.05
		B	NT	0.04
		C	NT	0.07
9381	<i>Chaetomium thermophilium</i>	A	NG	0
		B	0.08	0
		C	NG	0
9382	<i>Sporotrichum thermophilium</i>	A	0.10	0
		B	0.11	0.09
		C	0.10	0.13
9383	<i>Thermoascus aurantiacus</i>	A	0	0.05
		B	NG	0.05
		C	0	0.10

^aCultures grown on nutrient salts [1] and 0.5% cellulose, 0.1% Tween 80 plus A 0.05% peptone, 0.01% yeast extract; B 0.1% corn steep; C 0.025% phytone, 0.025% yeast extract. Culture filtrates tested for cellulase by incubating 1/2 ml with 1 ml buffer and 50 mg Whatman No. 1 filter paper for 1 hr at 50°C. NG = no growth; NT = no test.

Cellulases from thermophiles are not necessarily more heat stable than cellulases from mesophiles. The maximum temperature for growth of *Trichoderma viride* is about 34°C but the optimum temperature for *T. viride* cellulase is 50°C. For short time assays the optimum may be higher if the increased rate of reaction more than compensates for slow inactivation of the enzyme. A cellulase powder from a *Thermoactinomyces* was obtained from Dr. James Naylor of the General Electric Company as a by-product of the thermophilic fermentation of rumen fibers to produce single cell protein. It was compared to *T. viride*

cellulase on 4 substrates at assay temperatures of 50°C and 65°C (Table III). In short assays on susceptible substrates both enzymes showed high activity, frequently higher at 65°C than at 50°C. The *Thermoactinomyces* cellulase was deficient in C₁ and so showed low activity on cotton. In the 24 hr assay on cotton, the *T. viride* cellulase was inactivated at 65°C.

Most culture filtrates and enzyme preparations from mesophilic cellulolytic fungi are also low in action on cellulose fibers even though cellulose was rapidly broken down during growth (Table IV, Fig. 6). C_x activity was more common. *Penicillium iriensis* QM9624 [12] produces an active cellulase, nearly equal to that from *T. viride* when tested on filter paper. QM6a is the parent strain of *T. viride*, QM9123 and QM9414 are mutants with enhanced cellulase activity, and QM9136 is a mutant which does not produce cellulase [17, 18]. The more active mutants also secreted more protein. Homogenization of the culture did not release more enzyme from active or inactive strains. Culture filtrates are more active than acetone precipitates or the Japanese *T. viride* cellulase which is an extract from a Koji culture [1]. It is probable that the C₁ activity is adsorbed and not fully extracted from the Koji [19].

TABLE III
Cellulase from a *Thermoactinomycete* Compared to Cellulase
from *Trichoderma viride*

Testa	<i>Thermoactinomycete</i>		<i>T. viride</i> QM9123		<i>T. viride</i> QM9414	
	Powder 1.67 mg/ml		Powder 1.67 mg/ml		Culture	Filtrate
pH of assays	6.0 (PO ₄)		4.8 (citrate)		4.8 (citrate)	
Protein (mg/ml)	0.17		0.66		1.36	
Temperature (°C)	50	65	50	65	50	65
C _x (u/ml)	17.4	13.0	12.0	14.1	15.5	48.7
Walseth Act F	3.00	3.72	3.84	3.95	3.45	3.95
Walseth Act 1/5	1.80	2.58	2.00	2.70	3.15	3.52
FP (u/ml)	0.21	0.36	0.35	0.28	0.60	1.16
Cotton Act 2 ml	0.61	0.42	2.94	0.47	3.21	0.55
Cotton Act 1 ml	0.58	0.36	2.08	0.38	2.08	0.42

^aC_x 1/2 ml enzyme + 1/2 ml CMC 30 min; Walseth 1 ml enzyme + 1 ml 1.5% phosphoric acid swollen cellulose 1 hr; FP 1/2 ml enzyme + 1 ml buffer + 50 mg paper 1 hr; cotton 1 ml E + 1 ml buffer or 2 ml E + 50 mg cotton 24 hr.

TABLE IV
Production of Cellulase by *Meosiphilia Cellulolytica* Fungi^a

QM	Strain	Dry weight (mg/ml) ^b	Soluble protein (mg/ml) ^c	Total protein (mg/ml) ^d	Cellulase					
					C _x (u/ml)		FP (u/ml)		Cotton (mg/ml)	
					F	H	F	H	F	H
6a	<i>Trichoderma viride</i>	2.4	0.7	1.0	8	10	0.3	0.3	1.9	1.6
9123	<i>Trichoderma viride</i>	1.6	1.5	1.5	22	29	0.9	0.9	3.4	3.5
9414	<i>Trichoderma viride</i>	1.1	1.5	1.6	32	32	1.0	1.1	3.0	2.9
9316	<i>Trichoderma viride</i>	9.0	0	0.1	0	0	0	0	0.1	0.1
B814	<i>Streptomyces</i> sp.	2.5	0.5	0.5	2	0	0.1	0	0.3	0.1
9624	<i>Penicillium triensis</i>	1.8	0.5	1.0	14	17	0.5	0.5	1.1	1.3
9145	<i>Chrysosporium lignorum</i>	5.0	0.1	0.5	0	3	0	0.2	0.2	0.4
381	<i>Pestalotiopsis westerdijkii</i>	3.7	0.1	0.8	4	4	0.2	0.2	0.4	0.3
806	<i>Sporotrichum dimorphosporum</i>	0.8	0.8	1.0	21	22	0.2	0.3	0.3	0.5
826	<i>Chrysosporium prunosum</i>	2.4	0.6	0.8	4	3	0.2	0.3	0.5	0.6

^aCultures grown 14 days on 1% cellulose pulp, 0.1% proteose peptone, 0.2% Tween 80, 0.01% yeast extract.

^bDry weight includes residual cellulose.

^cSoluble protein, F cellulase measured on culture filtrate.

^dTotal protein, H cellulase measured on homogenate of whole culture.

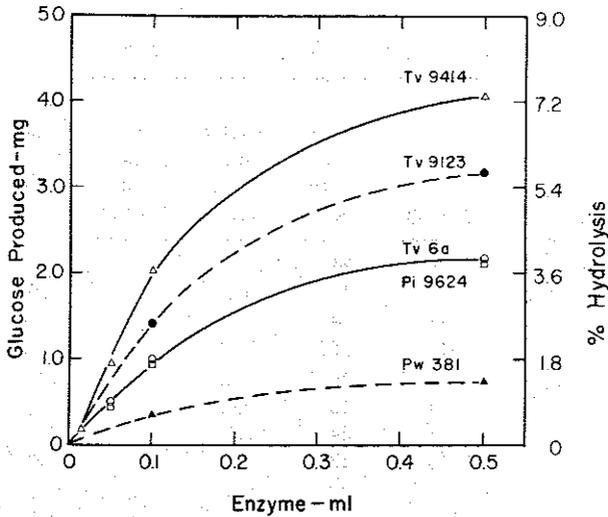


Fig. 6. Relative activity of culture filtrates on filter paper. Cultures grown on 1% cellulose for 14 days. Culture filtrate plus buffer pH 4.8 to make 1.5 ml added to 50 mg (a strip 1 × 6 cm) of Whatman No. 1 filter paper and incubated for 1 hr at 50°C.

	FP activity	FP units/ml
(○) <i>Trichoderma viride</i> QM6a	2.20	0.49
(●) <i>Trichoderma viride</i> QM9123	3.20	1.12
(△) <i>Trichoderma viride</i> QM9414	4.05	1.85
(□) <i>Penicillium iriensis</i> QM9624	2.15	0.49
(▲) <i>Pestalotiopsis westerdijkii</i>	0.75	0.14

Cultures of the active strains of *Trichoderma viride*, *Penicillium iriensis*, and a C_x producing strain, *Pestalotiopsis westerdijkii*, were grown on two media at 24 and 28°C for 14 days and the culture filtrates tested for cellulase (Table V). In general activities were high on the minimal Tv medium at 24 or 28°C. On the rich commercial medium, growth was excellent, but enzyme yields were erratic and tended to be better for 24 than 28°C, and better for the *T. viride* mutants which may be partly depressed. All 5 strains produced good levels of C_x activity under some conditions. *Pestalotiopsis westerdijkii* produces little C_1 and the filtrates were low in activity on filter paper or cotton. *Penicillium iriensis* does produce C_1 and the filtrates were equal to those of *T. viride* QM6a on filter paper, but not on cotton. *T. viride* filtrates, especially from the mutants, had the highest levels of C_1

TABLE V
Comparison of Cellulases from Active Strains^a

QM No.	Organism	Med growth	Temp growth (°C)	Cellulase filter paper (u/ml)	C _x CMC (u/ml)	5% Cotton (mg glucose/ml)	
						1.0	0.2
381	<i>Pestalotiopsis westerdijkii</i>	Pi	24	0.15	50.	0.76	0.36
		Pi	28	0.05	7.	0.40	0.18
		Tv	24	0.14	20.	0.24	0.16
		Tv	28	0.15	30.	0.32	0.22
9624	<i>Penicillium Iriensis</i>	Pi	24	0.02	0.1	0.24	0.06
		Pi	28	0.02	0.1	0.16	0.04
		Tv	24	0.77	67.	3.00	0.88
		Tv	28	0.37	81.	1.90	0.78
6a	<i>Trichoderma viride</i>	Pi	24	0.01	0.02	0.66	0.28
		Pi	28	0.01	0.1	0.35	0.04
		Tv	24	0.55	59.	3.54	1.78
		Tv	28	0.37	43.	4.90	1.92
9123	<i>Trichoderma viride</i>	Pi	24	1.15	96.	6.08	4.78
		Pi	28	0.08	5.	0.78	0.32
		Tv	24	1.68	89.	7.58	3.78
		Tv	28	0.91	93.	6.02	3.36
9414	<i>Trichoderma viride</i>	Pi	24	0.59	72.	7.08	4.02
		Pi	28	0.21	14.	5.26	2.48
		Tv	24	1.26	152.	3.58	2.60
		Tv	28	1.47	133.	7.56	5.52

^aCultures grown 14 days on Pi [12] med per liter 20 g cellulose, 20 g starch, 5 g glucose, 1 g Proflo, 4 g Pharmedia, 2.5 g Proflo oil, 15 g cornsteep, 4 g (NH₄)₂SO₄, 6 g CaCO₃ or on Tv [1] med containing nutrient salts plus 1% cellulose, 0.1% peptone, 0.2% Tween 80. Culture filtrates tested for cellulase and for cotton activity at full strength (1.0) or diluted 1:5 (0.2).

as demonstrated by action on cotton. Even when these preparations were diluted 1:5 they showed much greater activity than the undiluted *Pestalotiopsis* or *Penicillium* filtrates.

Culture filtrates produced on Tv medium were tested for hydrolysis of 5% shredded and ball milled newspaper (Figs. 7, 8). Newspaper contains some reactive cellulose and this is increased by ball milling. The 10% hydrolysis of shredded newspaper and the 29% hydrolysis of ball milled newspaper achieved by the *Pestalotiopsis* enzyme is a measure of this reactive cellulose (Figs. 7, 8). These levels of hydrolysis were achieved by 24 hr and did not increase on longer incubation. The *Penicillium* enzyme saccharified shredded newspaper 24% as compared

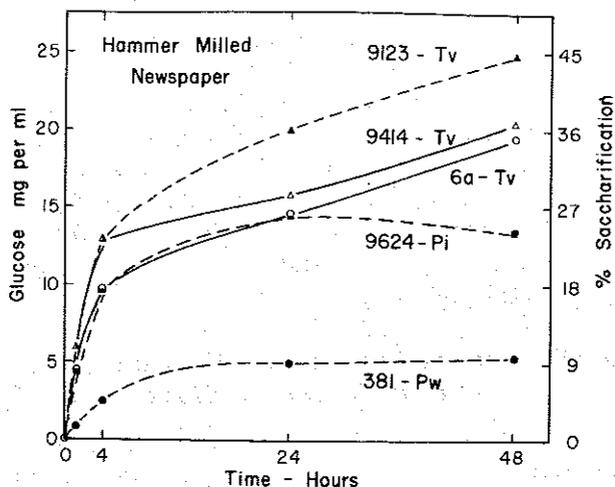


Fig. 7. Saccharification of hammer milled newspaper by culture filtrates of different fungi. 5 g hammer milled newspaper was suspended in 90 ml culture filtrate + 10 ml 0.5 M citrate buffer pH 4.8 and incubated on a shaker at 50°C. (●) *Pestalotiopsis westerdijkii* QM381; (○) *Trichoderma viride* QM6a; (▲) *Trichoderma viride* QM9123; (△) *Trichoderma viride* QM9414; (■) *Penicillium iriensis* QM9624.

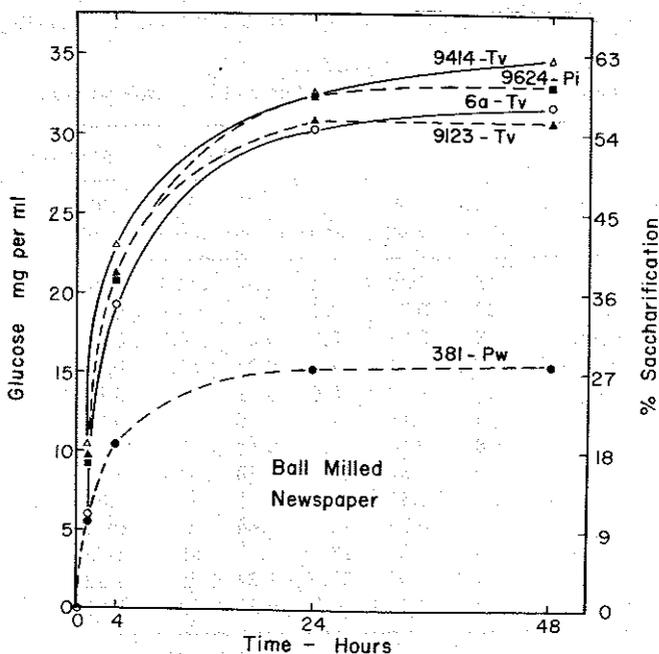


Fig. 8. Saccharification of ball milled newspaper by culture filtrates of different fungi. 5 g of Sweco ball milled newspaper was suspended in 90 ml culture filtrate + 10 ml 0.5 M citrate buffer pH 4.8 and incubated on a shaker at 50°C. (●) *Pestalotiopsis westerdijkii* QM381; (○) *Trichoderma viride* QM6a; (▲) *Trichoderma viride* QM9123; (△) *Trichoderma viride* QM9414; (■) *Penicillium iriensis* QM9624.

to 35-50% for the *T. viride* enzymes. The latter enzyme continued to slowly hydrolyze the newspaper for a 24-48 hr period. This would indicate C_1 action on the most resistant cellulose. Both the *Penicillium* and *T. viride* preparations saccharified ball milled newspaper about 60% and the reaction was virtually complete after 24 hr. Since newspaper contains 30% nonhydrolyzable "lignin," the residue was mostly lignin plus some unavailable cellulose.

EFFECT OF CARBON SOURCE ON CELLULASE PRODUCTION BY *TRICHODERMA VIRIDE*

Cellulase in *Trichoderma viride* is an induced enzyme produced when the fungus was grown on cellulose, lactose, or cellobiose. The mutants QM9123 and QM9414 appear to be partially derepressed and also produced low levels of cellulase on glucose and glycerol. The highest cellulase yields were obtained on cellulose (Table VI). Although Sophorose [20, 21] is a powerful cellulase inducer for *T. viride* active at extremely low concentrations, the levels of enzyme produced are not equal to those on cellulose, and in any case the use of this rare sugar for

TABLE VI
Effect of Substrate on Cellulase Production by *T. viride* Strains^a

Substrate growth	Temp growth (°C)	QM6a		QM9123		QM9414	
		FP (u/ml)	C _x (u/ml)	FP (u/ml)	C _x (u/ml)	FP (u/ml)	C _x (u/ml)
0.5% glucose	28	0.02	0.2	0.04	0.6	0.08	7.
1.0% glucose	28	0.05	0.9	0.15	5.	0.23	11.
0.5% cellubiose	28	0.02	0.1	0.03	0.1	0.08	1.
1.0% cellobiose.	28	0.16	7.	0.23	11.	0.25	12.
1.0% glycerol	28	0.02	0.1	0.07	1.	0.15	3.
0.5% lactose	28	0.16	2.	0.13	2.	0.33	17.
1.0% lactose	28	0.14	2.	0.13	2.	0.31	22.
0.5% cellulose	20	0.37	17.	1.00	37.	1.38	43.
0.5% SW40	25	0.52	17.	1.11	31.	1.08	44.
0.5% SW40	30	0.30	12.	0.66	21.	0.54	22.
1.0% cellulose	20	0.55	18.	1.57	55.	1.35	53.
1.0% SW40	25	0.37	23.	1.32	46.	1.30	67.
1.0% SW40	30	0.31	14.	0.91	32.	0.85	44.

^aCultures grown 14 days on nutrient salts 0.2% Tween 80 and 0.05% proteose peptone plus indicated substrate. Culture filtrates tested for cellulase.

TABLE VII
Effect of Cellulose Growth Substrate on Enzyme Production by *Trichoderma viride*^a

Substrate growth	QM6a					QM9123					QM9414					
	Protein (mg/ml)	C _x (u/ml)	FP (u/ml)	Cotton (mg/ml)	Protein (mg/ml)	C _x (u/ml)	FP (u/ml)	Cotton (mg/ml)	Protein (mg/ml)	C _x (u/ml)	FP (u/ml)	Cotton (mg/ml)	Protein (mg/ml)	C _x (u/ml)	FP (u/ml)	Cotton (mg/ml)
SW40	0.44	18.	0.23	2.8	0.60	59.	1.30	6.4	1.84	152.	1.48	2.5	1.84	152.	1.48	2.5
α cellulose	0.21	14.	0.17	1.7	0.78	48.	0.35	3.1	1.76	100.	1.48	2.8	1.76	100.	1.48	2.8
BW200	0.44	18.	0.20	2.3	0.81	44.	0.37	4.3	1.40	89.	1.11	2.4	1.40	89.	1.11	2.4
Sweco 270	0.52	21.	0.36	2.1	0.84	76.	1.30	4.9	1.56	102.	1.30	3.5	1.56	102.	1.30	3.5
Avicel	0.72	26.	0.74	2.6	1.68	78.	1.67	5.8	1.68	144.	2.04	3.5	1.68	144.	2.04	3.5
Abs Cotton	0.13	0.3	0.04	0.1	0.10	0.9	0.07	1.1	1.84	85.	1.30	4.6	1.84	85.	1.30	4.6
Shr. NP	0.75	10.	0.30	6.4	0.70	15.	0.33	6.0	1.28	24.	0.93	7.6	1.28	24.	0.93	7.6
NEP 40	1.16	16.	0.93	7.8	1.24	30.	1.11	7.0	1.44	48.	1.48	7.7	1.44	48.	1.48	7.7
BMNP	1.00	12.	0.31	6.8	1.50	41.	1.11	7.6	1.44	56.	1.11	8.6	1.44	56.	1.11	8.6
BMCP	0.25	3.	0.18	4.0	0.37	10.	0.31	4.5	0.46	22.	0.74	5.9	0.46	22.	0.74	5.9

^aGrown 13 days on *T. viride* medium plus 1% cellulose, 0.1% proteose peptone, 0.2% Tween 80. Protein: soluble, in mg/ml. C_x: 0.5 ml enzyme plus 0.5 ml 1% CMC 30 min 50°C. FP: 0.5 ml enzyme plus 1.0 ml 0.05M citrate buffer + 50 mg Whatman No. 1 filter paper. 60 min 50°C. Cotton 5%: mg glucose/ml after 24 hr at 50°C. Shr NP = shredded newspaper; BMNP = ball milled newspaper; BMCP = ball milled computer paper.

producing enzyme in quantity would not be practical. The effect of Tween 80 on cellulase production on cellulose is slight, but yields of enzyme on the sugars are much reduced in the absence of Tween 80 [22].

Cellulase was readily produced on most pure cellulosic substrates (Table VII) although growth and cellulase production on fibrous cotton were poor except for QM9414. The SW40 which is a hammer milled pulp 40 mesh was as good or superior to more finely divided or ball milled pulp. The microcrystalline cellulose, Avicel, was slightly better for filter paper activity. Waste paper products were satisfactory growth substrates, and produced cellulase preparations having low C_x activities, good filter paper activities, and high action on cotton. The 40 mesh hammer milled paper NEP40 and the ball milled newspaper appeared to be somewhat superior to the coarse fibrous shredded newspaper.

ELABORATION OF CELLULASE DURING THE GROWTH CYCLE

When *T. viride* spores were inoculated into glucose medium (Fig. 9), there was a lag of one or two days and then the glucose was rapidly consumed, yielding about 0.4 g of mycelium and 0.07 g of protein per gram of glucose. pH fell to 3.0. After the glucose was depleted, pH rose and mycelial weight fell. Only traces of cellulase were produced on the currently available glucose which is derived from cornstarch by enzymatic hydrolysis. Glucose produced by acid hydrolysis contained sophorose which induced cellulase [20]. Continuous fermentations were carried out on glucose media in 10 liter volumes in a 15 liter fermenter. We have not achieved a true steady state due to problems of clumps of mycelium adhered to walls and baffles, but the maximum growth rate was approximately 0.2 hr^{-1} with a feed of either 0.5 or 1.0% glucose. We have made many attempts to produce cellulase in continuous fermentation on glucose, varying feed rates, glucose concentration in the feed, and controlling pH at different values but have never achieved even the modest levels of cellulase obtained in shake flasks (Table VI).

When *T. viride* spores were inoculated into cellulose medium (Fig. 10) growth was much slower than on glucose, and fragments of cellulose could still be seen microscopically after 7 days. The weight of insolubles increased as peptone and possibly Tween 80 in the medium were consumed or taken up by the mycelium, and then fell as the cellulose was consumed. Mycelial weights could not be estimated from the

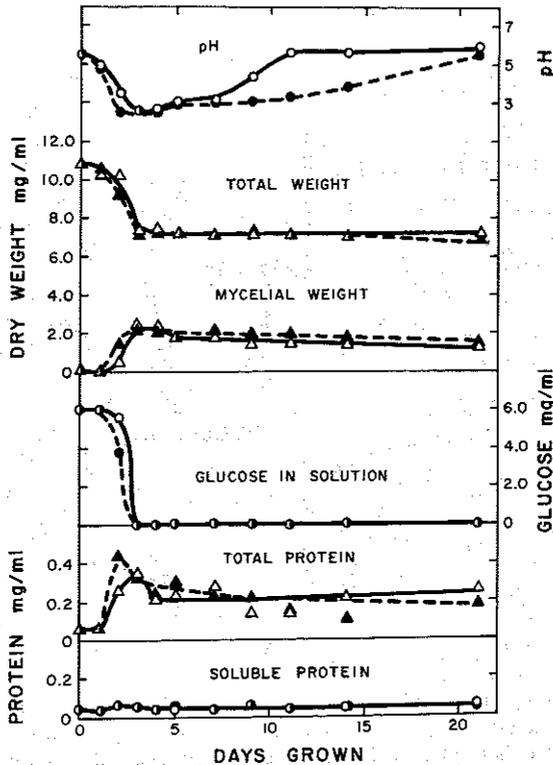


Fig. 9. Growth of *Trichoderma viride* QM6a and QM9123 in glucose medium. Cultures were grown at 28°C in shake flasks in 0.6% glucose plus 0.2% Tween 80 medium. pH, glucose in solution, and soluble protein were measured in the broth after centrifuging out solids. Total protein was measured on a homogenized aliquot of the culture (broth plus solids). Total weight was measured on an aliquot of the culture (broth plus solids) dried at 80°C. Mycelial weight was measured by filtering off the solids on a tared filter paper and drying at 80°C. (●) QM6a and (○) QM9123: pH, mycelial weight mg/ml; soluble protein mg/ml; glucose in solution mg/ml. (▲) QM6a and (△) QM9123: total weight mg/ml and total protein mg/ml.

protein values. The yield of protein per gram of cellulose was about 0.12 g for QM6a and 0.24 g for QM9123, both significantly higher than the protein yield on glucose. pH fell to around 3 and stayed low. Cellulase activity on CMC, filter paper, and cotton appeared on the 4th day, rose rapidly until day 7 and then leveled off or continued a slow rise.

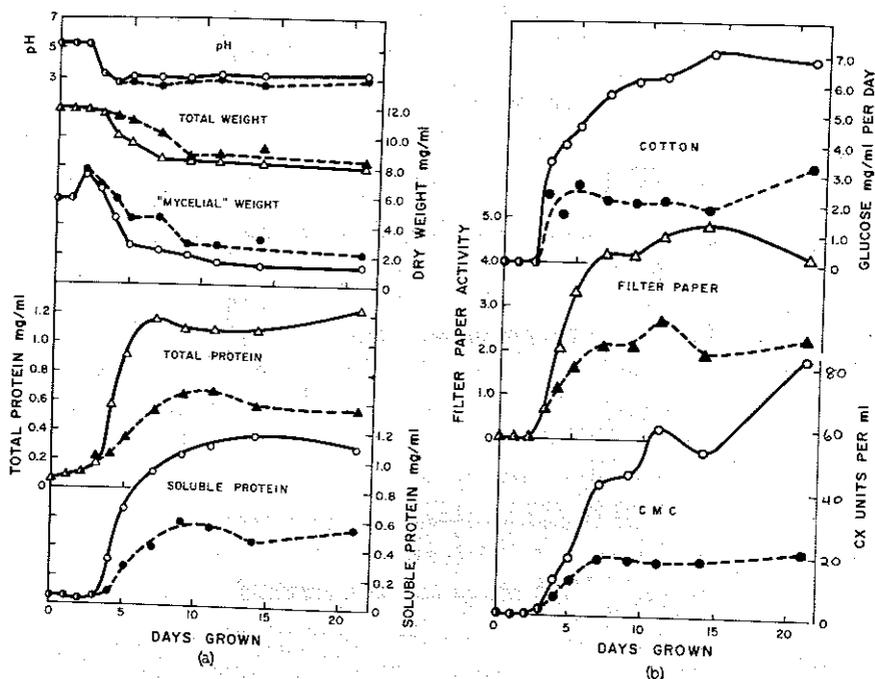


Fig. 10. Growth and cellulase production by *Trichoderma viride* QM6a and QM9123 in cellulose medium. Cultures were grown at 28°C in shake flasks in 0.5% cellulose (Solka Floc) plus 0.05% proteose peptone and 0.2% Tween 80, medium. pH, enzyme activity, and soluble protein were measured in the broth after centrifuging out solids. Total protein was measured on a homogenized aliquot of the culture (broth plus solids). Total weight was measured on an aliquot of the culture (broth plus solids) dried at 80°C. Mycelial weight (including residual cellulose), was measured by filtering off the solids on a tared filter paper and drying at 80°C. (●) QM6a and (○) QM9123: pH, mycelial weight mg/ml; soluble protein mg/ml; C_x units per ml; glucose from 5% cotton mg/ml day. (▲) QM6a and (△) QM9123: total weight mg/ml; total protein mg/ml; F.P. activity.

Growth and cellulase production on cellulose were similar in a fermenter (Table VIII). Good yields were obtained in batch culture in 5 or 6 days and the cellulase activity then continued to rise slowly. Maximum productivity (0.13-0.16 cellulase u/ml/day) was attained with 5-8 days growth. Continuous cellulose cultures have been operated. Again we have not achieved true steady state conditions, but at a dilution rate of 0.01 hr⁻¹ (5 days residence), good cellulase activities and a yield of 0.11-0.13 units/ml/day were obtained; at a dilution rate

of 0.02 hr⁻¹ cellulase activities were still good and the yield rose to 0.20-0.21 units/ml/day. At a dilution rate of 0.03 hr⁻¹ (maintained for only two days), both cellulase activity and yield decreased.

Since cellulase is adaptive there was a lag in growth and yields were low when *T. viride* was grown on pure cellulose. Addition of a readily consumed metabolite such as proteose peptone or glycerol reduced the lag and increased yields of cellulase. Peptone is the favored additive since it led to higher levels of C₁ [1] (Table IX).

Glucose represses cellulase formation [21] and metabolism of glucose can lead to inactivation of cellulase (Table X). On glucose, growth was rapid, little cellulase was produced, and little soluble protein appeared in the medium. On cellulose, growth was slower, but total protein was higher and cellulase and soluble protein were secreted in good quantity by 5 days. If 1% glucose was added to a cellulose culture initially, or any time before cellulase was secreted, growth might be stimulated, but no cellulase appeared until the glucose was consumed, and the lag before cellulase appeared was greatly increased. Secretion of soluble protein was also repressed. If glucose was added after cellulase had appeared, there was an increase in growth as shown by the increase in total protein, but

TABLE VIII
Productivity in a 10 Liter Fermenter^a

Dilution rate	Residence (days)	pH	Sol protein (mg/ml)	Cellulase concentration			Yield (u/ml day)
				FP Act	u/ml	u/mg protein	
Batch	4	3.2	0.44	1.94	0.36	0.82	0.09
Batch	5	3.2	0.84	2.97	0.70	0.83	0.14
Batch	6	3.0	1.28	3.42	0.97	0.76	0.16
Batch	7	3.5	1.16	3.06	1.00	0.86	0.14
Batch	8	3.2	1.23	3.51	1.01	0.89	0.13
Batch	10	3.4	1.25	2.90	0.76	0.61	0.08
Batch	12	3.8	1.50	3.52	1.16	0.77	0.10
Batch	13	4.5	1.40	4.52	1.28	0.91	0.10
0.01 hr ⁻¹	5	2.9-3.2	1.0-1.2	2.2-2.7	0.5-0.8	0.58	0.13
0.02 hr ⁻¹	2.5	2.8-5.4	0.6-1.0	1.7-2.9	0.3-0.7	0.79	0.21
0.03 hr ⁻¹	1.3	3.9-4.1	0.3-0.3	1.2-1.2	0.2-0.2	0.82	0.18

^aContinuous culture operated 7 days at dilution rate of 0.01 hr⁻¹, 18 days at dilution rate of 0.02 hr⁻¹ and 2 days at dilution rate of 0.03 hr⁻¹. Cellulase = FP. Growth of *T. viride* QM9414 on 0.5% cellulose pulp + 0.05% proteose peptone + 0.2% Tween 80. pH not controlled.

TABLE IX
Effect of Additives on Cellulase Production
by *Trichoderma viride* QM6a and QM9123^a

Additive	%	Cellulase activity of filtrates					
		QM6a			QM9123		
		CMC	FP	Cotton	CMC	FP	Cotton
None		7	0.9	3.9	18	2.2	7.1
Proteose	0.05	14	2.2	6.9	18	3.3	13.0
Peptone	0.10	12	1.7	5.9	11	2.7	16.4
	0.20	12	2.1	6.3	14	2.2	11.9
Glycerol	0.05	12	1.6	5.2	23	2.2	9.0
	0.10	8	1.1	2.6	19	2.2	8.7
	0.20	4	0.9	1.6	14	2.2	3.8

^aCultures were grown at 28°C in shake flasks in 0.5% Solka Floc medium for 18 days and the crude culture broths for cellulase activity at 50°, pH 4.8. CMC = C_x units per ml (30 min); FP = filter paper activity (1 hr); cotton = glucose mg/ml from 5% cotton in 3 days.

soluble protein and cellulase in the medium declined sharply. These effects were not changed by addition of 10⁻³M cyclic AMP (Table X). Attempts to detect a protease or cellulase inhibitor in the medium after glucose addition have been unsuccessful.

MUTATION TO INCREASE CELLULASE YIELDS

In the years of galloping inflation since World War II few products have declined in price despite all the marvels of technology applied to their production. Among the rare exceptions are products produced by microorganisms such as antibiotics, enzymes, and vitamins. Some have actually declined in price by one or two orders of magnitude, but this was not due to process improvements introduced by the engineers, but rather to improvements of the microbiological capability, chiefly due to genetic manipulation. For example the economic feasibility of the glucamylase process for corn starch hydrolysis rests on the development of suitable strains and conditions for producing the enzyme cheaply and in quantity.

Fundamental work on microbial genetics is based on sophisticated technology for enriching the population of mutants, and isolating

desired variants by means that prevent or retard the growth of normal strains, or identification of mutants by replica plating on various media. These procedures are not helpful in obtaining mutants with enhanced cellulase activity since growth of a fungus on cellulose does not predict enzyme secretion. High amylase producers can be detected by size of cleared zones on starch agar plates. Cleared zones on cellulose agar plates or under colonies in cellulose agar tubes [23] can be used to detect actively cellulolytic organisms but have not proved useful for separating mutants with varying levels of cellulase production. So we have worked on mutation by simple tedious old fashioned procedures. Spores from an agar slant were suspended in distilled water and exposed

TABLE X
Effect of Addition of Glucose to Cellulose Cultures^a

Glucose added	Cellulose + Glucose												
	Glucose		Cellulose		0 day		1 day		2 day		5 day		
	-	+ ^b	-	+ ^b	-	+ ^b	-	+ ^b	-	+ ^b	-	+ ^b	
Day	Filter Paper Activity												
2	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0.23	0	1.52	1.94	0	0	0	0	0	0	1.52	1.94	
6											0.54	0.57	
7	0.06	0	1.04	1.82	0	0	-0.02	0.74	0.14	0	0.48	0.51	
8									0.02	0.02	0.49	0.49	
9	0.18	0.10	1.88	1.82	0.30	0.97	0.76	1.21	1.14	0	0.49	0.55	
14	0.14	0.04	1.44	2.08	1.30	1.22	1.21	1.17	0.78	1.04	0.48	1.04	
	Soluble Protein												
2	0.09	0.08	0.02	0.02	0.08	0.09	0.07	0.10	0.02	0.02	0.02	0.02	
5	0.03	0.04	0.34	0.34	0.05	0.05	0.05	0.04	0.05	0.04	0.34	0.34	
6											0.05	0.04	
7	0.03	0	0.31	0.42	0.02	0.01	0.01	0.06	0.02	0	0.03	0.04	
8									0.03	0.02	0.06	0.05	
9	0.04	0.08	0.38	0.37	0.04	0.08	0.04	0.21	0.07	0.02	0.06	0.08	
14	0.14	0.09	0.56	0.66	0.31	0.22	0.19	0.42	0.08	0.18	0.07	0.25	
	Total Protein												
2	0.64	0.43	0.12	0.13	0.60	0.60	0.60	0.46	0.12	0.13	0.12	0.13	
5	0.62	0.54	0.64	0.60	0.75	0.70	0.60	0.56	0.31	0.46	0.64	0.60	
6											0.78	0.68	
7	0.52	0.22	0.68	0.84	0.68	0.56	0.36	0.46	0.48	0.44	0.96	0.92	
8									0.38	0.12	0.48	0.60	
9	0.28	0.20	0.54	0.56	0.23	0.48	0.33	0.52	0.44	0.17	0.60	0.60	
14	0.37	0.21	0.78	1.00	0.78	0.81	0.72	0.88	0.78	1.18	0.66	0.81	

^a*T. viride* QM6a grown on 1% glucose with no additives or 1% cellulose plus 0.1% peptone and 0.2% Tween 80. 1% glucose added to certain cultures as indicated.

^b10⁻³ cyclic AMP added to + cultures at 0 time or when glucose was added.

to mutagenic irradiation or chemicals to kill > 99%. Survivors were plated on desoxycholate agar to restrict the size of colonies [17] and individual colonies which grew up were isolated to PDA slants where they grew and sporulated normally. Pigmentation and morphological mutations were apparent on PDA but have shown no correlation with cellulase production. For screening, each isolate was grown in one flask of glucose medium with no additive and one flask of cellulose medium with peptone and yeast extracted added. Control cultures were included for reference. Flasks were harvested after 7-10 days growth and the culture filtrates assayed for cellulase. The parent culture grew well in both flasks and secreted cellulase only on cellulose. Mutants with enhanced or decreased cellulase production produced more or less cellulase than the control. Cellulase negative mutants grew on the glucose, but not on the cellulose. Auxotrophs grew on the cellulose, but not on the glucose. Although some mutants produced low levels of cellulase on glucose (Table VI), we have not yet found a constitutive mutant producing high levels of cellulase on glucose medium.

We believe that further mutation is a major avenue towards improving the economics of a practical process for enzymatic conversion of cellulose. This work would be greatly stimulated if an effective plate assay to predict high cellulase mutants were devised.

As of July 1974, the operational site of the NLABS Culture Collection of Fungi (QM) is being transferred from U.S. Army Natick Laboratories, Natick, MA, to the Department of Botany, University of Massachusetts, Amherst, MA 01002. All QM cultures will be available through this new address, ATTN: Dr. Emory G. Simmons. Our *Trichoderma strains* also are on deposit with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, where the equivalent accession numbers are:

QM = ATCC

6a = 13,631

9123 = 24,449

9136 = 26,920

9414 = 26,921

The taxonomy of this series is currently being questioned and it is probable that *T. viride* is incorrect. Possibly the species is *T. longibrachiatum*.

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