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GROWTH AND CELLULASE PRODUCTION

BY TRICHODERMA

Mary Mandels, David Sternberg and Raymond E. Andreotti

Food Sciences Laboratory,

U.S. Army Natick Laboratories,

Natick, MA 01760

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U.S. ARMY NATICK DEVELOPMENT CENTER
NATICK, MA 01760

SUMMARY

Trichoderma species, especially the two mutant strains QM9123 and QM9414, when grown on cellulose are excellent sources of saccharifying cellulase suitable for practical applications. Conditions for maximum cellulase production are not the same as conditions for optimum growth. When the fungus metabolizes soluble carbohydrates there is a rapid fall in pH closely associated with the disappearance of the sugar. When cellulose is the substrate, a similar very rapid fall in pH occurs which suggests that metabolism of the cellulose may be as rapid as that of the sugars. However, a continuous culture on cellulose can not be maintained in this rapid growth phase. This is probably because cellulose is a multiple substrate with rapid metabolism only of the amorphous fraction. The crystalline fraction is metabolized very slowly and only after considerable preliminary enzyme (C₁?) action to convert it to a more reactive form. Rapid metabolism of sugars represses cellulase production and also causes disappearance of preformed cellulase, the latter effect being related to the low pH conditions induced. β -Glucosidase is more sensitive to low pH than is saccharifying cellulase. This may be a means of feedback control. All of these effects create special problems in continuous fermentation production of cellulase.

INTRODUCTION

Trichoderma species (*viride*, *lignorum*, *koningii*) are the best pres-

ent sources of active cellulase suitable for a practical process for saccharification of cellulose (5, 9, 14, 17, 18, 23). Much fundamental work has been done on induction of cellulase (7, 8, 10), on the properties and interrelationships of the components of the cellulase complex (16, 24), and on optimization of media and growth conditions, and production of the enzyme on pure and waste cellulose (5, 13, 15, 18). Stable mutants QM9123 and QM9414 with enhanced cellulase production (11) have been developed (Table 1) and have been distributed to investigators all over the world.

Table 1. *Trichoderma* strains in Natick Collection. Cellulase activity in shake flasks grown on cellulose media. These strains are available from Dr. Emory G. Simmons, NLABS Culture Collection of Fungi (QM), Dept. of Botany, University of Massachusetts, Amherst, MA 01002 USA, or from The American Type Culture Collection (ATCC) 12301 Parklawn Drive, Rockville, MD 20852 USA.

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

Trichoderma cellulase has been used to carry out extensive saccharification of various cellulosic substrates in 5...20% slurries with a 50% yield (70...90% conversion of the cellulose) of soluble sugars in 6...24 hours (1, 2, 3, 12, 13, 23) (Table 2). Waste "cellulose" also contains hemicelluloses and other polysaccharides. Fortunately *Trichoderma* readily produces various hemicellulases and other

Table 2. Hydrolysis of cellulose by *Trichoderma viride* cellulase. QM9414 cellulase 1.2 FP units per ml, saccharification at 50°C, pH 4.8.

Substrate	% Saccharification			
	1 h	4 h	24 h	48 h
<u>PURE CELLULOSE</u>				
Cotton- fibrous	1	2	6	10
Cotton- ball milled	14	26	49	55
Cellulose pulp SW 40	5	13	26	37
Cellulose pulp- ball milled	23	44	74	92
<u>WASTE CELLULOSE</u>				
Bagasse	1	3	6	6
Bagasse-ball milled	14	29	42	48
Rumen fibers	7	12	16	--
Rumen fibers- ball milled	35	49	51	--
Newspaper- Mighty Mac Mulcher	10	24	31	42
Newspaper- ball milled	18	49	65	70
Corrugated fibreboard- Mighty Mac Mulcher	11	27	43	55
Corrugated fibreboard- ball milled	17	38	66	78
Black Clawson fibers	5	11	32	36
Black Clawson- ball milled	13	28	53	56
Bureau of Mines Cellulose	7	16	25	30
Bureau of Mines- ball milled	13	31	43	57

carbohydrases so that these carbohydrates are broken down, usually more rapidly than the cellulose since they are less crystalline and less recalcitrant. The products of enzyme action therefore are mixtures of sugars, chiefly glucose, cellobiose, and xylose in proportions depending on the nature of the substrate, the make-up of the crude enzyme and the extent of hydrolysis. In most cases, it will be desirable to produce the enzyme on the same substrate that is to be hydrolyzed to ensure the proper mix of enzymes, and to produce it on the site for hydrolysis to eliminate transpor-

tation and processing costs. Cellulase is commercially available from Japan but the cost is economically prohibitive except for research use (Table 3). Currently enzyme can be produced with 0.5 to 2.0 units of saccharifying cellulase per ml of broth, and this

Table 3. Commercial sources of *Trichoderma* cellulase. These cellulases are used at 1...5% concentration for saccharification.

Type	Source	Price - US \$ per kg
Meicellase	Meiji Seika Kaisha Ltd 8 2-Chome Kyodashi Chud Hu Tokyo, Japan	30 (1971)
Onozuka SS P 1500	Kanematsu Goshō Ltd CPO Box 141 Tokyo 100-91	115 (1971)
Pancellase	Kanematsu Goshō (USA) Inc 1 World Trade Center Suite 4811 New York, NY 10048	170 (1974)

is adequate so that the broth can be used directly for saccharification without concentration. Any improvement in enzyme yield will improve the economic outlook of the process. Today I would like to discuss growth and enzyme production of *Trichoderma* and relate it to the objectives of maximizing enzyme yield in submerged fermentation.

Measurement of growth in a cellulose culture is not simple. Consumption of cellulose (decrease in weight) or production of a cellular component such as protein or RNA can be used, but results tend to be unsatisfactory because of the difficulty in obtaining representative samples of a mycelial suspension, especially from a fermentor. More consistent results are obtained with soluble

products of growth such as extracellular protein or enzyme. Since our objective is to produce enzyme, we feel that it makes sense (and is easier) to optimize the fermentation for enzyme production.

METHODS

Methods used in these experiments have been described elsewhere in detail (9, 13). The basic medium for growth and enzyme production contained, per liter, KH_2PO_4 2.0 g, $(\text{NH}_4)_2\text{SO}_4$ 1.4 g, Urea 0.3 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.3 g, CaCl_2 0.3 g and Trace Metals; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 5 mg, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 1.6 mg, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 1.4 mg, CoCl_2 2.0 mg, plus 5...10 g of dextrose or cellulose. Cellulose medium usually also contained 0.05...0.10% proteose peptone (Difco) and 0.2% Tween 80 (polyoxy ethylene sorbitan mono oleate, Atlas Chemical Industries). SW 40 (hammer milled pure cellulose pulp) and BW 200 (ball milled pulp prepared from SW 40) were obtained from Brown Co., Berlin, NH. *Trichoderma* cultures were maintained on Potato Dextrose Agar (PDA) slants. Spore suspensions for inoculum were prepared using 10 ml of water for a 10 ml PDA slant to give 10^5 ... 10^7 spores per ml. 100 ml cultures in shake flasks received 0.1...0.2 ml and a 10 liter fermentor 10 ml of such a spore suspension for inoculum. After growth the solids were separated by filtration through a dispo plug and enzyme assays made on the filtrate. Enzyme substrates were carboxymethylcellulose (DS 0.5) for endo- β -glucanase, Whatman No. 1 Filter Paper for saccharifying cellulase, and cellobiose for β -glucosidase. Units are micromoles of glucose (or reducing sugar as glucose) produced per minute in the standard assay. Soluble carbohydrate was determined by the phenol sulfuric method, reducing sugar by the dinitrosalicylic acid method, and glucose by glucostat (Worthington). Filter paper (FP) activity is the mg of reducing sugar (as glucose) produced in 1 hour when 50 mg of filter paper is incubated with 0.5 ml culture filtrate at pH 4.8, 50°C. FP units were determined by diluting culture filtrates to a FP activity of 2.0 (0.37 units/ml). Filter paper activities would be higher if one ml of enzyme were used instead of 0.5 ml, and both FP activities and units would be higher if the quantity of filter

paper in the assay were increased (5, 14) (Table 4). Total protein was measured on a homogenate of the whole culture, broth plus solids, and extracellular soluble protein on the culture filtrate, by the Folin procedure after precipitation with 10% trichloroacetic acid.

Table 4. Effect of conditions on filter paper assay. 0.5 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 (14). Units = micromoles of glucose per minute based on the dilution to give 2.0 mg.

Filter paper mg		25	50	50	100
Enzyme ml		0.5	0.5	1.0	0.5
Enz. protein mg		0.7	0.7	1.4	0.8
Activity	30 min	2.29	2.43	3.52	5.18
	60 min	3.30	3.68	4.40	5.53
	120 min	4.60	5.30	6.75	7.16
Activity	30 min	4.58	4.86	7.04	10.36
per	60 min	3.30	3.68	4.40	5.53
hour	120 min	2.30	2.65	3.38	3.58
Units/ml	30 min	0.93	1.23	1.19	4.35
	60 min	0.76	1.23	1.23	3.52
	120 min	0.80	1.42	1.32	3.08
Units/mg	30 min	0.66	0.88	0.88	3.11
	60 min	0.56	0.88	0.88	2.51
	120 min	0.59	1.01	0.94	2.61

Submerged culture experiments of 5 and 10 liter volumes were conducted with a New Brunswick Scientific Co. Magnaferm Fermentor Model MA-114. This fermentor utilizes a 15 liter glass vessel equipped with a magnetically driven double propeller impeller and a mechanical foam breaker. Both temperature and pH were continuously recorded throughout the experiments. Two independently controllable peristaltic pumps fitted with silicone rubber tubing were

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used for feeding and harvesting the culture. A 15 minute timer with microswitches was used to control the cycling of the pumps during the continuous culture experiments. Every 15 minutes the harvest pump was turned on for a 5...10 s pulse, depending upon the dilution rate required, allowing a delivery of 25...50 ml of culture suspension, followed by an identical pulse for the nutrient or feed pump to deliver the replacement volume. This rapid pulse feeding gave much better results than slow continuous feeding for suspensions of cellulose or mycelium. Growth in the fermentors was at 28°C, with impeller speed at 100...200 rpm and aeration at 0.2...0.4 liters of air per liter of culture per minute.

EXPERIMENTAL

Trichoderma grows readily on many substrates (7) but at different rates. The fungus is a vigorous acid producer when consuming carbohydrate above a minimal rate with NH_4^+ as the nitrogen source. The rate of acid production is directly related to the rate of carbohydrate consumption (Fig. 1). For sugars which are more slowly consumed, e.g. lactose and L-arabinose, not only are the lag times longer but consumption is slower after the lag, and the rate of acid production is correspondingly less. If carbohydrate is very slowly consumed (as is D-arabinose), the onset of the drop in pH is considerably later than the beginning of carbohydrate consumption and the amount of acid produced is less than on the more rapidly metabolized carbon sources. A significant feature of all pH patterns is that as soon as the carbohydrate is consumed the pH abruptly rises. Thus, the organism has the ability to neutralize or reverse the acid condition it has produced, perhaps by secretion of NH_4^+ .

Assuming that acid production is not enhanced when the organism grows on cellulosic media, the pH changes of the medium during growth can be used as a simple means of determining the rate of cellulose consumption (Table 5). Under unfavorable conditions, *Trichoderma* will grow slowly on cellulose, producing low levels of cellulase; in such cases the pH of the medium drops very little (similar to D-arabinose).

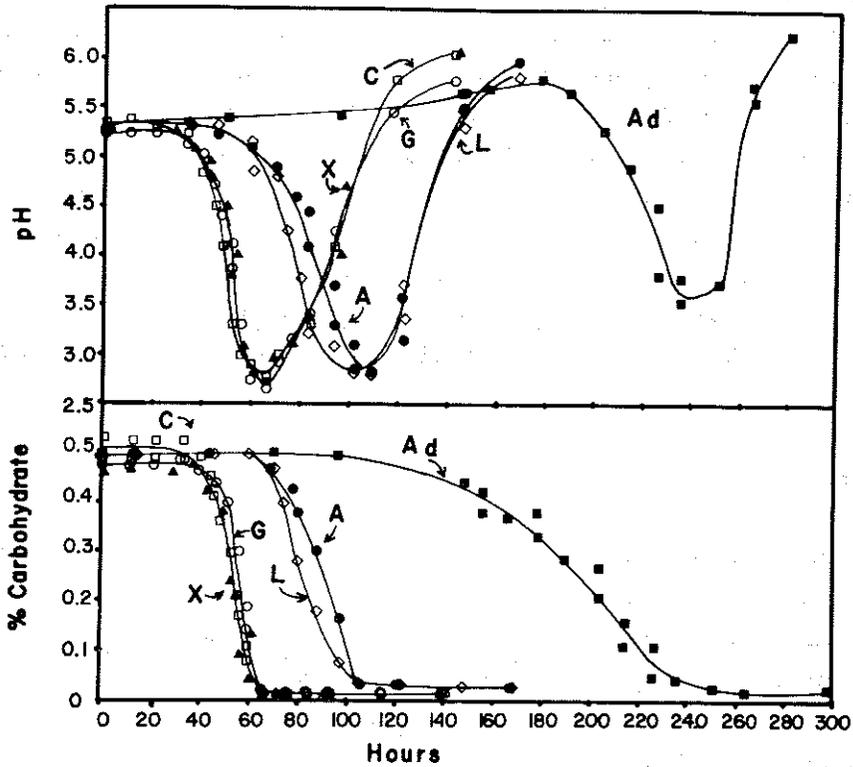
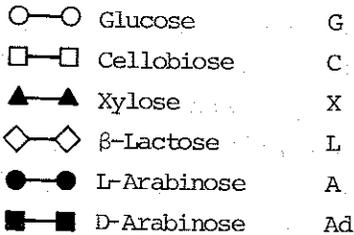


Fig. 1. Growth of *Trichoderma* on soluble sugars. QM9414 on 0.5% sugars, carbohydrate by phenol sulfuric method, spore inoculum.



Under favorable conditions large quantities of cellulase are produced and the rate of acid production is greater than with glucose. (Fig. 2) indicating that during early growth the organism may be capable of consuming cellulose as rapidly as glucose. However, when grown on cellulose much higher levels of extracellular protein are produced than when grown on glucose. Therefore the rate of NH_4^+

Table 5. Relation of substrate consumption and pH fall.
 $d \text{ sugar} = \text{sugar consumption (mg/ml/h)}$,
 $d \text{ pH} = \text{pH change (units/h)}$.

Carbon source	Slope $d \text{ sugar}$	Slope $d \text{ pH}$
Xylose	0.357	0.180
Glucose	0.253	0.156
Cellobiose	0.225	0.175
Lactose	0.139	0.076
L-Arabinose	0.100	0.063
D-Arabinose	0.060	0.055
Cellulose	--	0.246

consumption and consequent pH fall may also be greater. During the rapid fall in pH, saccharifying and endo-cellulase are induced - an observation which indicates that cellulase synthesis is not brought about by a slow feed of carbohydrate but rather that induction occurs during a time of high metabolic activity.

Acid production during growth on cellulose appears to have a regulatory function. Since the main cellulolytic pathway is the release of cellobiose as the soluble product and its subsequent hydrolysis to glucose by β -glucosidase, the activity of β -glucosidase may control the flow of glucose from cellulose into the cell. Rapid metabolism and the fall in pH profoundly affect the activities of β -glucosidase and cellulases. If 0.5% glucose is added to the medium of a culture growing on cellulose and thus having the array of cellulase enzymes, the pH rapidly drops to about 2.5 as the glucose is consumed, with a concomitant disappearance of β -glucosidase activity and a significant loss in saccharifying cellulase. If cellulose is still present in the culture, the enzymes will reappear after the glucose is consumed, but if all the cellulose has been consumed the enzymes will not recur (8) (Table 6).

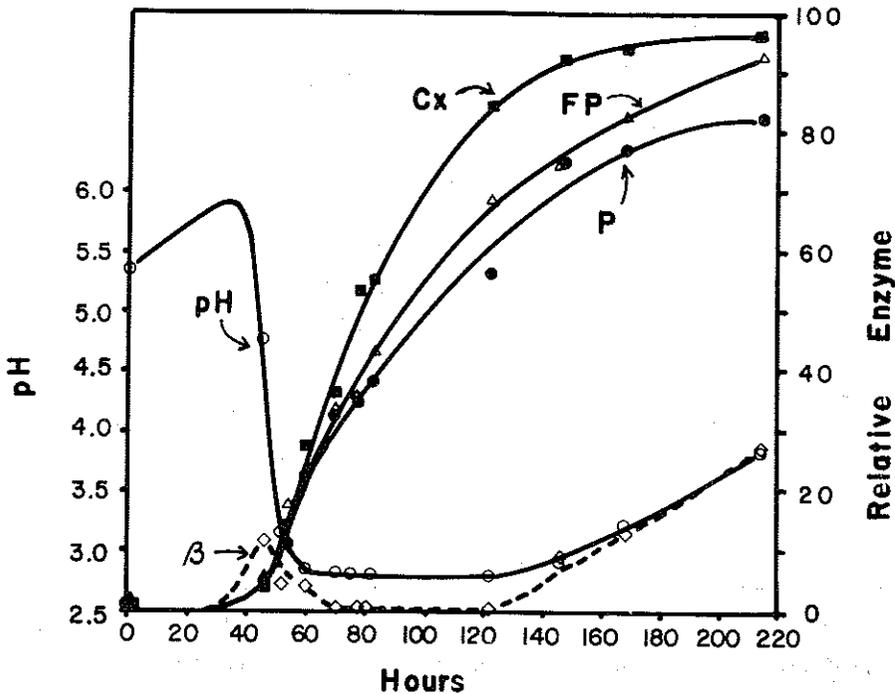


Fig. 2. Growth and enzyme production of *Trichoderma* on cellulose. QM9414 on 0.75% BW 200 (ball milled pulp) + 0.075% proteose peptone, 0.2% Tween 80, spore inoculum.

- pH
- △—△ Saccharifying cellulase (FP) 100 = 1 unit/ml
- Endo-cellulase (C_x) 100 = 55 units/ml
- ◇—◇ β -glucosidase (β) 100 = 0.10 unit/ml
- Extracellular protein (P) 100 = 2 mg/ml

However, if 0.5% glucose is added after the cellulose is consumed and the pH is controlled at about 5.0, both enzyme activities remain stable (Table 7). Thus, the apparent "glucose effect" is related to pH - an observation confirmed by adjusting pH by adding HCl to a cellulase-induced culture (Fig. 3). As the pH drops from 4.0 to 3.0, most of the β -glucosidase activity is lost; sacchari-

Table 6. Effect of addition of glucose to cellulose cultures.
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.

Day	1% Glucose	1% Cellulose	1% Cellulose + 1% Glucose added at			
			0 day	1 day	2 day	5 day
Filter paper activity						
2	0	0	0	0	0	0
5	0.12	1.73	0	0	0	1.73
6	NT	NT	NT	NT	NT	0.56
7	0.03	1.43	0	0.38	0.07	0.50
8	NT	NT	NT	NT	0.02	0.49
9	0.14	1.85	0.64	0.99	0.57	0.52
14	0.09	1.76	1.26	1.19	0.91	0.76
Soluble protein mg/ml						
2	0.09	0.02	0.09	0.09	0.02	0.02
5	0.04	0.34	0.05	0.05	0.05	0.34
6	NT	NT	NT	NT	NT	0.05
7	0.02	0.37	0.02	0.04	0.01	0.04
8	NT	NT	NT	NT	0.03	0.06
9	0.06	0.38	0.06	0.13	0.05	0.07
14	0.12	0.61	0.27	0.31	0.13	0.16
Total protein						
2	0.54	0.13	0.60	0.53	0.13	0.13
5	0.58	0.62	0.73	0.58	0.39	0.62
6	NT	NT	NT	NT	NT	0.73
7	0.37	0.76	0.62	0.41	0.46	0.94
8	NT	NT	NT	NT	0.25	0.54
9	0.24	0.55	0.36	0.43	0.31	0.60
14	0.29	0.89	0.80	0.80	0.98	0.74

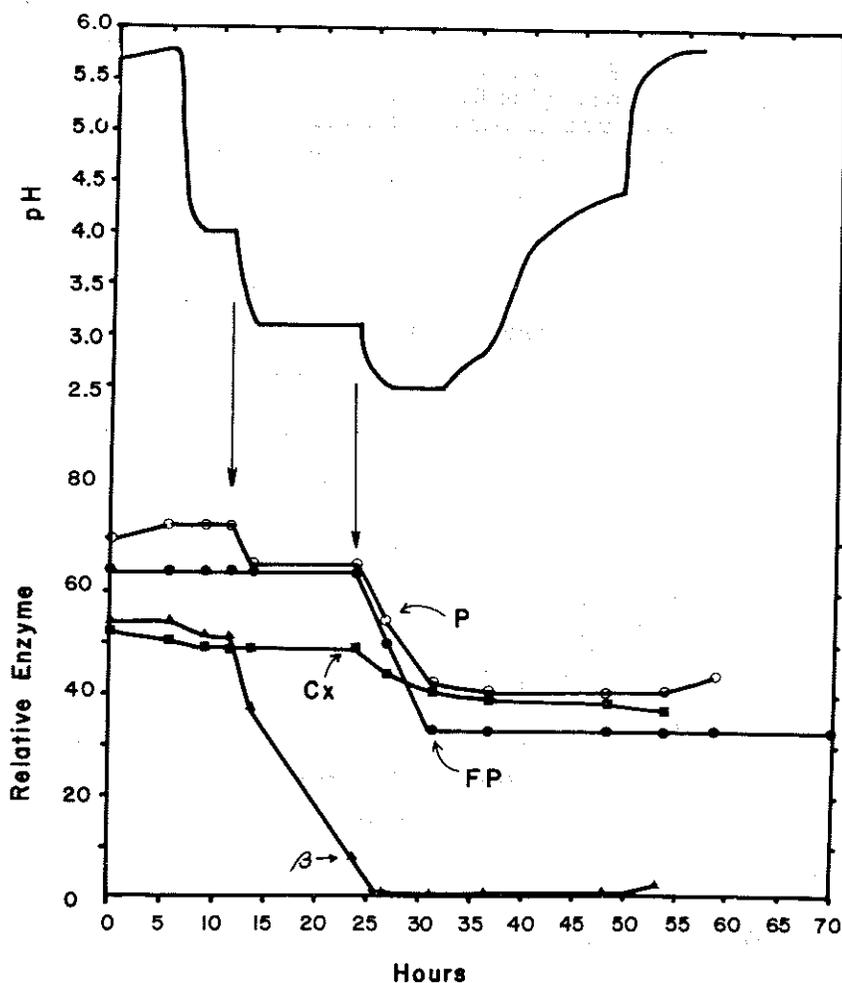


Fig. 3. Effect of pH level in a culture on enzymes of the cellulase complex. QM9414 was grown in a 10 liter fermenter on 0.5% BW 200. pH was not allowed to drop below 5.0. After cellulose consumption, the pH rose naturally, then was lowered to 4.0, 3.1, and 2.5 with HCl and raised gradually with NaOH as indicated. Aeration, stirring, and temperature control at 28°C were continued.

- Saccharifying cellulase (FP) 100 = 1 unit/ml
- ▲—▲ β -Glucosidase (β) 100 = 0.5 units/ml
- Endo- β -glucanase (C_x) 100 = 50 units/ml
- Extracellular protein (P) 100 = 2 mg/ml

Table 7

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Table 7. Effect of addition of glucose to a cellulase induced culture with and without pH control. QM9414 was grown in a 10 liter fermenter on 0.5% BW 200 with pH not allowed to drop below 4.0. After pH rose (cellulose consumed), 0.5% glucose was added and pH controlled not to fall below 5.0. Glucose was rapidly consumed. After pH rose again, 0.5% glucose was added, pH was not controlled and fell to 2.4 as glucose was more slowly consumed.

	<u>Hours after glucose addition</u>				
	<u>pH uncontrolled</u>		<u>pH controlled at 5.0</u>		
	<u>0</u>	<u>22</u>	<u>0</u>	<u>22 h</u>	
β -Glucosidase:	0.146	0	0.183	0.159	units per ml
Saccharifying cellulase:	0.48	0.32	0.77	0.75	units per ml

ifying activity remains stable until pH drops to 2.5, at which point about 45% activity is lost. Endo-cellulase appears the most stable, decreasing only about 20% overall. In this starved culture, none of the enzyme activities are recovered by increasing the pH. Extracellular protein level falls in a manner reflective of saccharifying cellulase. Similar results are obtained when a culture filtrate (free of mycelium) is incubated at the appropriate pH (Fig. 4). The amount of TCA-soluble Folin reacting material increases as TCA-insoluble decreases, suggesting proteolysis. If an acid protease is involved, it presumably cannot hydrolyze the enzymes at higher pH levels where enzyme activity and stability are maximum. As pH is lowered, the enzymes would be in a slightly denatured state and therefore more susceptible to proteolysis (22).

The more rapidly cellulose (or any carbon source) is consumed, the greater the rate of acid production, which in turn leads to a loss in cellulase activity - first β -glucosidase then saccharifying cellulase. By limiting β -glucosidase and cellulase activities, the uptake of glucose is limited. When the rate of glucose consumption decreases, pH will rise (as seen in Figs. 1 and 2), permitting further enzyme accumulation. This system may allow for a negative feedback control over enzymes which exist outside the cell. Such

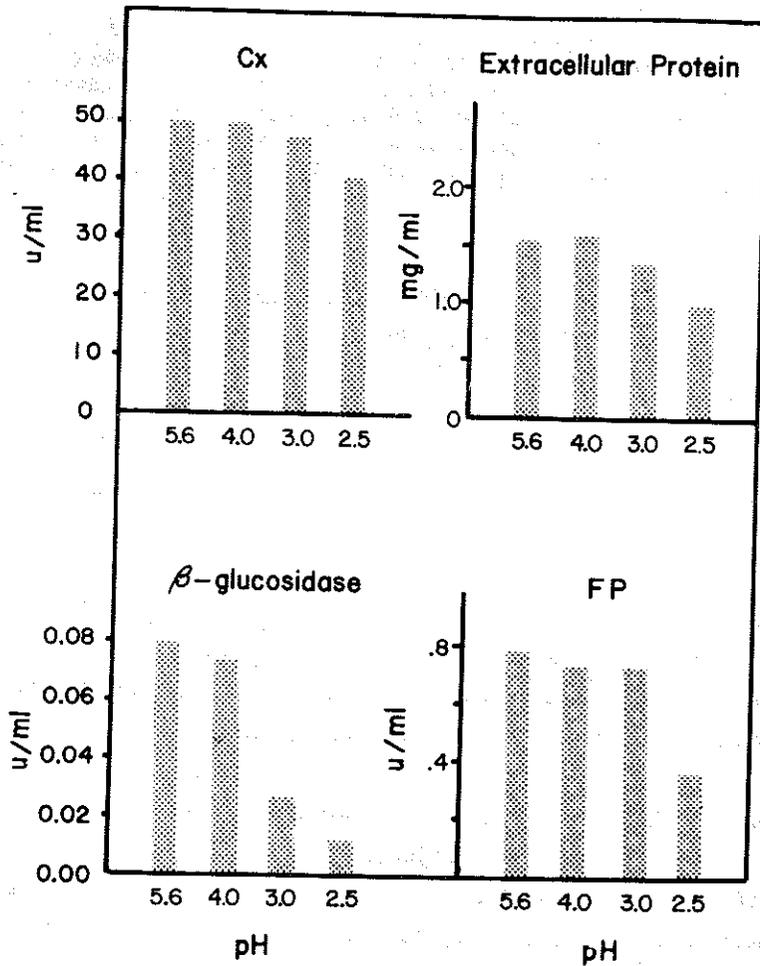


Fig. 4. Effect of pH level in a culture filtrate on enzymes of the cellulase complex. QM9414 was grown on 0.75% BW 200 in a shake flask, harvested at 13 days and the mycelium separated from the culture broth. The filtrate initially at 5.6 was adjusted with HCl as indicated, incubated 24 hours at 27°C on the shaker and then readjusted to pH 5.6 before assay.

a system may operate to ensure that the rate of cellulolysis does not exceed the cell's need for carbohydrate. This control mechanism acting on already synthesized enzymes should not be confused with glucose repression of enzyme.

It has been shown that in a glucose cellulose culture, the rate of cellulolysis is not limited by the rate of cellulose hydrolysis, but by the rate of glucose uptake. This is in contrast to the rate of cellulose hydrolysis in a cellulose culture, where the rate of cellulolysis is limited by the rate of cellulose hydrolysis. This is because in a glucose cellulose culture, the rate of cellulolysis is limited by the rate of glucose uptake, while in a cellulose culture, the rate of cellulolysis is limited by the rate of cellulose hydrolysis. This is because in a glucose cellulose culture, the rate of cellulolysis is limited by the rate of glucose uptake, while in a cellulose culture, the rate of cellulolysis is limited by the rate of cellulose hydrolysis.

To investigate this further, an experiment was conducted where the concentration of glucose in the culture medium was varied. The results of this experiment are shown in Figs. 5, 6, and 7.

It has been suggested that cellobiose is the true inducer in a cellulose culture but that yields are low when *Trichoderma* is grown on cellobiose because the cellulase is repressed and/or inactivated by the rapid metabolism of the sugar (8, 20). In a culture growing on cellulose, cellobiose is slowly released so that repression and inactivation do not occur. Similarly, some cellulolytic fungi that produce acetyl esterases produce cellulase when they are grown on cellobiose octa acetate, as cellobiose is slowly released from this insoluble substrate and slowly consumed. It has even been suggested that slow growth on other sugars derepresses the cellulase forming mechanism (6). However the enzyme levels reported in these experiments were extremely low. In our laboratory, despite numerous efforts in shake flasks and fermentors, we have never achieved significant cellulase yields by slow addition of glucose or cellobiose to a growing culture, nor by maintaining continuous cultures on low levels of glucose or cellobiose. We have achieved very good yields of cellulase on cellobiose by using a high cellobiose concentration (1% or more) and slowing metabolism by decreased aeration, suboptimal temperature, a marginal nutrient deficiency, or a marginally toxic excess of trace metals (8). Or excellent yields of cellulase on cellobiose can be attained by adding surfactants such as Tween 80 or sodium oleate to a 1% or more cellobiose culture (21). If we use lower concentrations of cellobiose, around 0.5%, we can not achieve good yields by these means. Thus the question remains open as to whether cellobiose is in fact the true inducer or whether an inducer is produced from cellobiose, perhaps by a transferase. It is of interest to note that sophorose, which is a powerful inducer at low concentrations, has maximum inducing activity with washed mycelium at pH 2.8 (10). Perhaps at higher pH, β -glucosidase hydrolyzes the sophorose, at lower pH, conditions are too unfavorable to the fungus.

To investigate the relationships between growth and cellulase production, an experiment was carried out in shake flasks using two concentrations of cellulose with and without addition of glucose or peptone and incubating the cultures at three temperatures (Table 8, Figs. 5, 6). Growth was good in all cultures as shown by fall in pH,

Table 8. Relationships between growth and cellulase production of *Trichoderma* on cellulose media. QM9414 grown on SW 40 (hammer milled pulp) + 0.2% Tween 80. Soluble protein and cellulase measured on culture filtrate, total protein on homogenate of entire culture (broth + solids).

Cellulose	Add	Temp °C	pH		Dry weight		Total protein		Soluble protein mg/ml	Cellulase FP u/ml
			3	6	3	6	3	6		
					mg/ml		mg/ml			
0.5%	--	24	5.2	2.8	4.75	2.30	0.06	1.10	1.56	1.1
	--	28	--	3.0	--	0.95	--	1.25	1.44	1.2
	--	30	4.1	3.3	5.25	1.85	0.08	0.78	0.92	0.8
0.05% Peptone		24	4.0	3.2	4.70	1.90	0.64	1.86	1.76	1.7
		28	3.5	5.6	2.30	0.90	0.92	1.76	1.68	1.4
		30	3.8	6.3	3.20	0.85	0.81	1.18	1.24	1.1
0.1% Peptone		24	6.2	4.3	4.60	1.45	0.72	1.96	1.32	1.8
		28	6.3	5.7	4.05	1.00	0.92	1.96	1.92	1.8
		30	5.2	6.5	3.00	1.00	0.92	1.25	1.44	1.2
0.05% Glucose		24	5.7	3.8	5.50	2.20	0.13	1.10	1.12	1.4
		28	3.6	2.8	5.10	2.45	0.32	1.25	1.28	1.1
		30	3.2	3.1	4.45	1.55	0.34	0.92	0.92	0.7
0.1% Glucose		24	5.2	2.8	5.85	2.00	0.14	0.88	1.20	1.2
		28	4.7	2.9	5.15	2.05	0.29	1.25	1.20	1.1
		30	3.6	3.3	4.25	1.15	0.23	0.81	0.92	0.7
1.0%	--	24	5.1	2.7	9.35	6.10	0.05	1.04	1.32	0.8
	--	28	3.3	2.8	9.10	4.90	0.13	1.10	1.16	0.9
	--	30	3.2	2.9	9.10	4.65	0.13	0.66	0.62	0.4
0.1% Peptone		24	3.3	2.8	7.45	4.15	0.62	1.76	2.08	1.8
		28	3.1	2.9	5.85	3.00	1.00	1.96	2.50	1.9
		30	3.2	3.2	5.50	2.65	0.64	1.18	1.50	1.2
0.2% Peptone		24	4.0	3.2	8.30	3.25	0.92	2.16	2.88	2.1
		28	4.3	3.7	7.10	2.15	1.18	1.96	2.50	2.0
		30	3.6	6.2	5.65	2.00	0.88	1.96	1.50	1.4
0.1% Glucose		24	4.3	2.6	9.60	7.15	0.13	0.96	1.12	0.8
		28	3.2	2.8	8.90	5.65	0.28	0.84	0.92	0.7
		30	3.1	2.9	8.75	4.95	0.37	0.62	0.44	0.3
0.2% Glucose		24	3.5	2.7	9.05	5.30	0.16	0.96	1.28	0.8
		28	3.3	2.9	8.70	4.65	0.28	0.75	0.92	0.7
		30	3.1	2.9	7.00	4.40	0.50	0.72	0.48	0.3

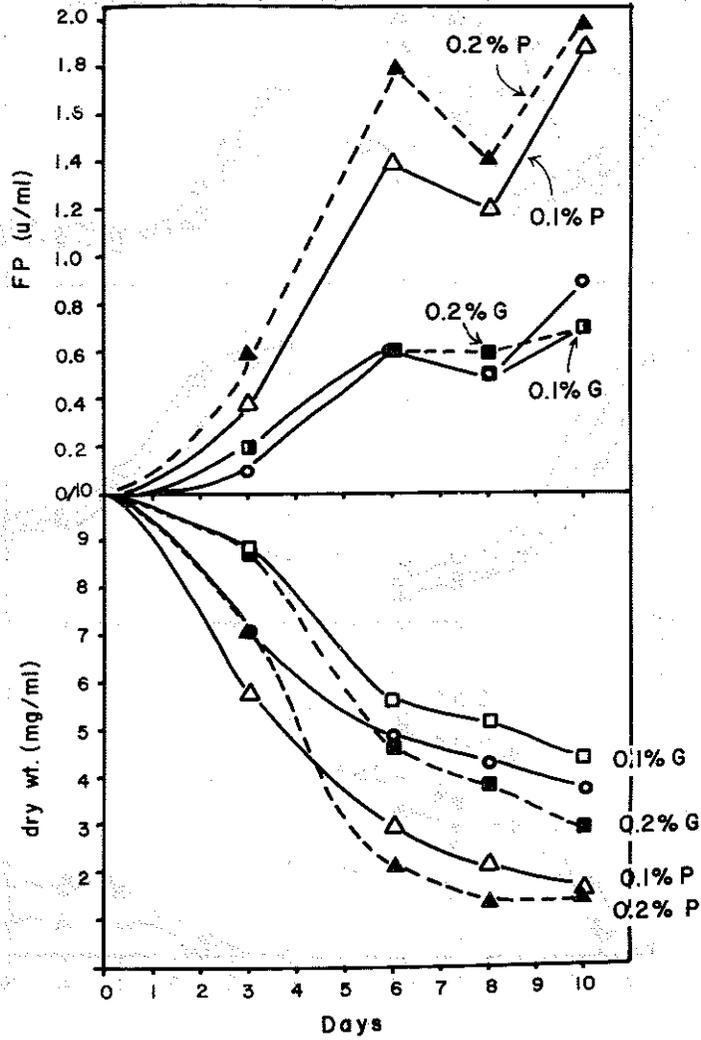


Fig. 5. Effect of addition of a soluble substrate on growth and cellulase production in cellulose culture. QM9414 grown on 1% SW 40 (hammer milled pulp) + 0.2% Tween 80 at 28°C.

- No additive
- △—△ + 0.1% peptone
- ▲---▲ + 0.2% peptone
- + 0.1% glucose
- + 0.2% glucose

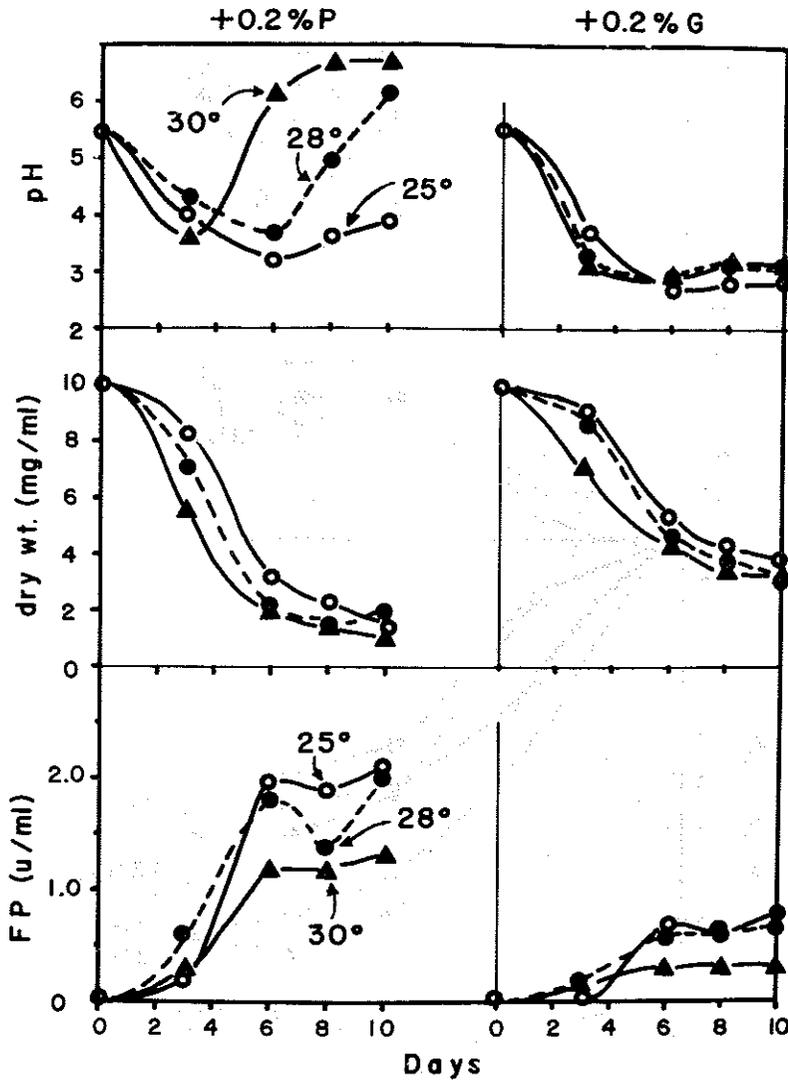


Fig. 6. Effect of incubation temperature on growth and cellulase production in cellulose cultures. QM9414 grown on 1% SW 40 + 0.2% Tween 80. 0.2% peptone or 0.2% glucose added to medium.

- grown at 25°C
- grown at 28°C
- ▲—▲ grown at 30°C

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decrease in cellulose (dry weight) and production of protein, although there was a lag in cultures grown at 25°C or with no soluble substrate added. Maximum total protein was reached by 6 days, but cellulase and soluble protein, which were closely correlated, continued to increase slowly until 10 days. By 10 days essentially all of the protein was extracellular (soluble).

Cellulose concentration had little effect on enzyme production. The addition of glucose decreased the lag in growth and increased the fall in pH as compared to cultures with no additive but it was not particularly favorable to either disappearance of cellulose or enzyme production. The addition of peptone decreased the fall in pH but increased growth as measured by protein or disappearance of cellulose, and greatly increased enzyme production. Cultures grew most rapidly at 30°C as shown by shorter lags and more extensive disappearance of the cellulose but total protein production was lower than in cultures grown at lower temperatures. Enzyme production was maximum in cultures grown at 25°C.

The inoculum can have a marked effect on growth and cellulase production in cellulose media. The use of mycelial inoculum shortens the lag that occurs when spores are used. It has been suggested that productivity in a continuous fermentor could be increased by continuous inoculation from a glucose grown culture (15). A series of cultures was grown on cellulose with and without addition of peptone, and omitting Tween 80. Each 100 ml culture was inoculated with 0.25 or 1.0 ml of a spore suspension (about 10^7 spores/ml) prepared from a PDA slant, or with 1 or 5 ml of a culture grown 26 to 144 hours on glucose, or with 1 or 5 ml of a culture grown 72 to 144 hours on cellulose (Table 9, Fig. 7).

Cultures inoculated with spores showed a lag, especially on medium with no peptone. The lag was reduced when glucose grown mycelium was used for inoculum and the peptone effect was also reduced but cellulase yields at 7 days were not greater than for the spore cultures. There was not much effect of the quantity of spores or age of the glucose grown mycelium, but there was a longer lag

Table 9. Effect of inoculum on cellulase production. QM9414 grown on 0.75% SW 40 - no Tween 80 at 28°C. Spore inoculum - conidia from a PDA slant - suspension contained about 10^7 spores/ml, mycelial inoculum at 1 or 5% from a culture grown on 0.5% glucose or on 0.75% SW 40 for 26, 72, 96, or 144 hours as indicated.

Inoculum		Filter paper activity							
		2		3		4		7 days	
Age, quantity		-*	+*	-	+	-	+	-	+
Spore suspension									
0.25 ml/100 ml		0	0	0	0.7	0	1.9	1.2	2.3
1.0 ml/100 ml		0	0	0	0.7	0.2	1.3	2.0	--
Glucose grown mycelium									
26 h	5%	0.4	0.7	1.2	2.1	2.0	1.9	1.9	2.0
72 h	1%	0	0.6	0.7	1.5	1.0	0.8	2.2	2.4
72 h	5%	0.4	1.4	1.5	1.8	1.8	1.7	2.6	1.7
144 h	5%	0.3	1.0	1.2	1.8	1.4	2.0	2.0	2.2
Cellulose grown mycelium									
72 h	5%	1.4	2.0	2.6	2.7	2.1	2.7	1.9	3.7
96 h	1%	0.9	1.2	1.7	1.8	1.4	1.9	1.9	2.8
96 h	5%	1.7	1.7	2.4	2.6	2.1	2.8	2.0	3.8
144 h	5%	1.3	1.7	2.2	2.2	1.9	1.8	2.2	3.1

* + 0.075% peptone. - No peptone

with the 1% mycelium as compared to the 5%. Cellulose grown mycelium was markedly superior with more rapid and greater enzyme production. The age of the mycelium was not important, but the 5% inoculum was superior to the 1% and the addition of peptone increased enzyme production. Overall yields in this experiment were reduced by the omission of Tween 80 from the medium.

When *Trichoderma* was grown continuously on glucose, the culture could be maintained at a rapid growth rate with a μ Max of about 0.2 h^{-1} and a yield of 0.4...0.5 mg of mycelium per mg of glucose consumed (14) but a continuous culture on cellulose could not be

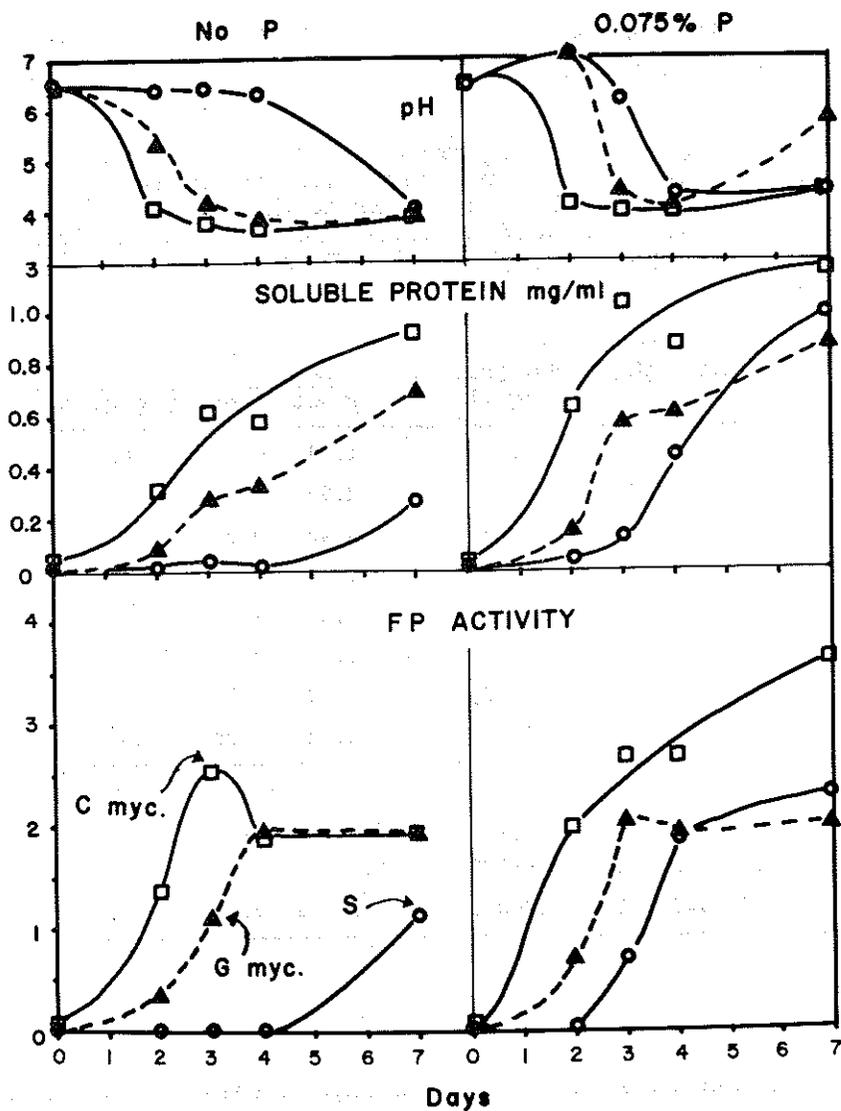


Fig. 7. Effect of inoculum on growth and cellulase production. QM9414 grown on 0.75% SW 40 as Table 9, P = peptone.

- | | | |
|-----|--------------------|----------------|
| ○—○ | Spore inoculum | 0.25 ml/100 ml |
| ▲—▲ | Glucose mycelium | 26 h old 5% |
| □—□ | Cellulose mycelium | 72 h old 5% |

maintained at such a growth rate. Maximum cellulase productivity was attained at a dilution rate of about 0.02 h^{-1} (Table 10).

Table 10. Productivity in a 10 liter fermenter. Continuous culture operated 7 days at dilution rate of 0.01 h^{-1} , 18 days at dilution rate of 0.02 h^{-1} and 2 days at dilution rate of 0.03 h^{-1} (14). Growth of *T. viride* QM9414 on 0.5% cellulose pulp + 0.05% proteose peptone + 0.2% Tween 80. pH not controlled.

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
"	5	3.2	0.84	2.97	0.70	0.83	0.14
"	6	3.0	1.28	3.42	0.97	0.76	0.16
"	7	3.5	1.16	3.06	1.00	0.86	0.14
"	8	3.2	1.23	3.51	1.01	0.89	0.13
"	10	3.4	1.25	2.90	0.76	0.61	0.08
"	12	3.8	1.50	3.52	1.16	0.77	0.10
"	13	4.5	1.40	4.52	1.28	0.91	0.10
Continuous							
0.01 h^{-1}	5	2.9-3.2	1.0-1.2	2.2-2.7	0.5-0.8	0.58	0.13
0.02 h^{-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 h^{-1}	1.3	3.9-4.1	0.3-0.3	1.2-1.2	0.2-0.2	0.82	0.18

When the dilution rate was increased beyond this level, soluble protein and cellulase in the effluent declined rapidly and dry weight (cellulose) rose (14). In a cellulose batch culture inoculated with spores, there was a lag of 30 or more hours before active growth began as indicated by pH fall (Figs. 2, 8). The lag was greater when trace metals were omitted from the medium even though final yields were not reduced. If mycelial inoculum was used, this lag was reduced to 6...12 hours. The steep fall in pH lasted about 12...24 hours and it was another 12...24 hours before pH began to rise indicating that the cellulose was fully consumed. If 1...10% of a culture that had consumed cellulose was replaced by fresh

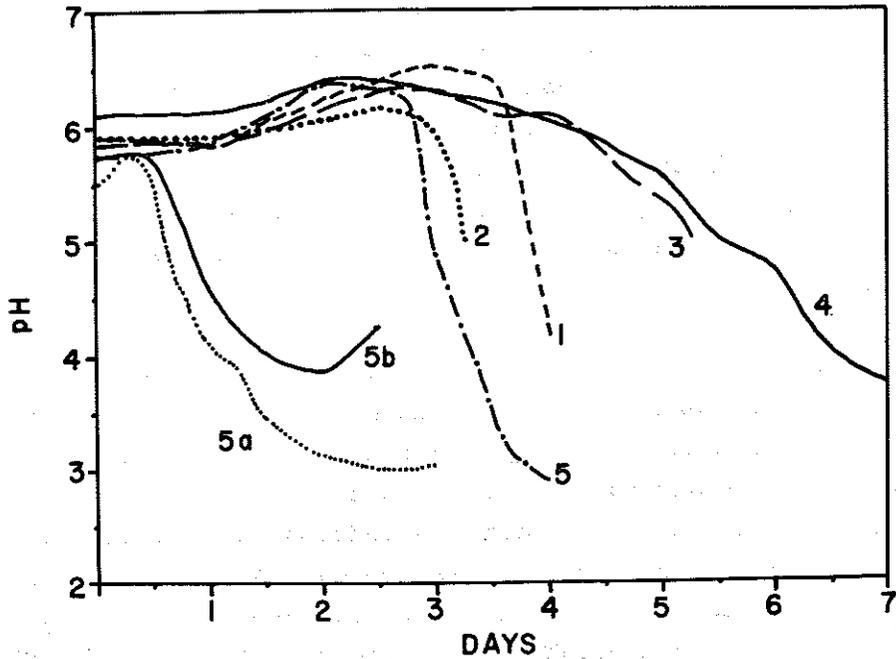


Fig. 8. Growth of *Trichoderma* on cellulose in submerged fermentation. QM 9414, volume 10 liters, all media had peptone at 1/10 the cellulose level and 0.2% Tween 80.

Run	Inoc.	Substrate	Water	Trace metals	FP (1) Act. (day)	FP (2) Act. (day)	Comments
1	Spore	0.5% BW 200	Dist.	+	2.0 (4)	3.4 (10)	pH control 4.0 Continuous D = 0.02 h ⁻¹
2	Spore	0.75% BW 200	Dist.	+	1.8 (3)	3.4 (6)	pH control 5.0 Batch
3	Spore	0.5% BW 200	Tap	-	2.3 (6)	2.5 (7)	pH control 5.0 Batch
4	Spore	0.75% BW 200	Tap	-	0.9 (7)	3.0 (11)	pH not controlled Batch
5	Spore	0.75% SW 40	Dist.	+	2.1 (4)	3.1 (6)	pH not controlled Continuous D = 0.02 h ⁻¹
5a	Myc ⁽³⁾	0.5% SW 40	Dist.	+	3.3 (3)	4.0 (4)	pH not controlled Batch
5b	Myc ⁽³⁾	0.5% SW 40	Tap	-	2.9 (2)	2.9 (3)	pH not controlled Batch

- (1) FP Activity at end of pH data shown.
- (2) FP Activity and conditions after end of pH data shown.
- (3) Culture pumped out and replaced with fresh media.

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- 0.09
- 0.14
- 0.16
- 0.14
- 0.13
- 0.08
- 0.10
- 0.10

- 0.13
- 0.21
- 0.18

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medium, the steep pH fall was complete in 4...6 hours and the rise began again in 10...12 hours. If 25% of the culture was replaced with fresh cellulose medium, the fall was complete in 10...12 hours and the rise began in 22...24 hours. This suggests that the rapid metabolism of cellulose indicated by the steep fall in pH represents an amorphous or reactive form of the cellulose, but complete consumption of the crystalline cellulose is much slower, probably limited by the slow rate of C_1 action on the crystalline cellulose.

A culture was grown on hammer milled cellulose with peptone and Tween 80. Spores were used for inoculum, and pH was not controlled (Fig. 9). The pH rose initially to 6.5 and then began to fall at 60 hours. Twenty-four hours later when the pH had reached 3.0 and dry weight had fallen to 4.5 mg/ml, a continuous feed and harvest with a dilution rate of 0.02 h^{-1} was begun. At first the dry weight continued to fall and the cellulase level rose to one unit per ml, but then pH and weight rose while cellulase level fell, indicating that the dilution rate was exceeding the growth rate. Soluble protein closely paralleled the cellulase level. After 6 days of continuous culture we harvested the entire culture and replaced it with fresh feed medium. The mycelium that remained clinging abundantly to baffles and probes served as inoculum. Growth as indicated by pH fall, began rapidly and continued vigorously. By 4 days the dry weight was less than 2 mg/ml and cellulase level was 1.5 units/ml. Again we harvested the entire culture and replaced it with fresh medium. Again growth was excellent and cellulase level at 3 days was $0.8 \mu/\text{ml}$. Productivities in units per ml per day were 0.10 for the initial 4 day batch culture, 0.22...0.37 (average 0.27) for the continuous culture, 0.39 for the subsequent 4 day batch culture, and 0.30 for the final 3 day batch culture.

DISCUSSION

Growth of a fungus on the insoluble substrate cellulose is more complicated than growth on a simple soluble carbohydrate. Cellulose



Fig.

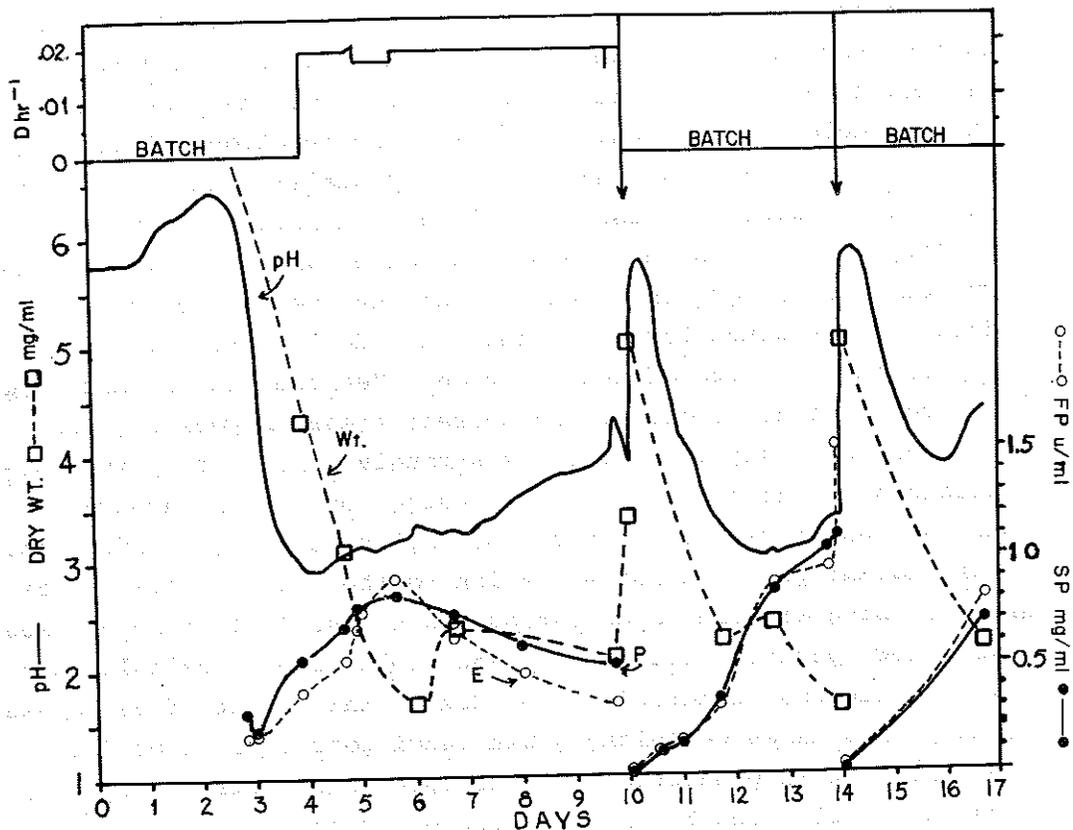


Fig. 9. Batch and continuous fermentation of *Trichoderma* on cellulose media. QM9414 grown 4 days in batch culture (10 liters volume) on 0.75% SW 40, 0.075% peptone, 0.2% Tween 80, then 6 days in continuous culture (5 liters volume) with a feed of 0.5% SW 40, 0.05% peptone, 0.2% Tween 80. At 235 hours the continuous culture was ended and 250 ml of culture was pumped out and replaced with fresh feed medium. At 240 hours the whole culture (4850 ml) was pumped out and replaced with fresh feed medium. At 14 days the whole culture was pumped out again and replaced with fresh feed medium, but prepared using tap water and omitting trace metals.

- $D \text{ h}^{-1}$
- pH (recorder tracing)
- Cellulase (FP) units per ml
- Soluble protein mg per ml
- Dry weight mg per ml

contains only one monomer and only one linkage but differences in the physical relationships between the chains and their degree of interaction make it a multiple substrate which is broken down to soluble sugars and finally glucose by a series of reactions catalyzed by a complex of enzymes. Some of these reactions almost certainly are involved in changing the more crystalline and resistant forms of the cellulose to less crystalline and more susceptible forms. Only the final soluble products can be absorbed by the fungus and utilized for growth. The enzymes involved in cellulose breakdown are not constitutive; they are induced directly or indirectly by the products of their action. Yet the fungus must grow and produce mycelium before it can secrete these enzymes and the synthesis of cellulolytic enzymes is strongly repressed by the soluble products of their action, levels being controlled through a feedback mechanism related to rapid metabolism and acid production. There are advantages of this repression to the fungus since excess sugars are not available to competing organisms. Cellulase enzymes have very low specific activities. The yield of extracellular protein in a cellulose culture is often 20% of the weight of cellulose consumed. This makes cellulose grown fungi poor candidates for single cell protein production. It is more profitable to grow organisms on soluble substrates and produce intracellular protein.

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

So the situation in a continuous culture does not favor rapid growth

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and enzyme production on cellulose. If the cellulase level is high, the amorphous portion of added cellulose is rapidly hydrolyzed, producing sugars that repress further enzyme production. The crystalline portion of the added cellulose is then broken down very slowly, but adsorbs enzyme and carries it off with the harvest insolubles. Controlling pH at higher levels leads to more rapid consumption of the cellulose but not to sustained high yields of enzyme. Less enzyme is required to produce sugar at pH 5, optimum for the enzyme, than at pH 2.8 where enzyme action is low. The addition of peptone to the feed is favorable even when abundant mycelium is present, perhaps because the amino acids can be directly incorporated into the enzyme protein. We have noted that non-proteolytic contaminants in a cellulose culture do not decrease enzyme production, and may even increase it, perhaps by scavenging soluble sugars. A constitutive or non-repressible cellulase producing mutant would be valuable.

So fermentation production of cellulolytic enzymes offers many challenges for productive basic and applied research. Fortunately this symposium and others held recently or planned for the near future are evidence that there is increasing interest in and support for this research that offers such interesting possibilities (4, 19).

ACKNOWLEDGMENT

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