

Continuous Cultivation of *Trichoderma viride* on Cellulose

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Summary

Using ball milled cellulose as the only carbon source *Trichoderma viride* was grown in a continuous flow culture at pH = 5.0 and $T = 30^{\circ}\text{C}$. Steady-state values for cell protein, cellulose, and cellulase for different substrate concentrations (4–11 g/liter) and dilution rates (0.033–0.080 hr^{-1}) were obtained. Under steady-state conditions, 50–75% of the cellulose was consumed indicating a critical dilution rate on 0.17 hr^{-1} .

Cellulase activity (U/ml) in the fermentation broth increased slightly with increasing substrate concentration and decreased with increasing dilution rate, while the specific cellulase productivity (U/mg cell protein·hr) was fairly independent of the dilution rate, with a maximum around $D = 0.05 \text{ hr}^{-1}$.

Following step changes in substrate concentration and dilution rate, new steady-state values were reached after three to five residence times (cell protein and cellulose) and four to six residence times (cellulase activity).

INTRODUCTION

Microorganisms growing on cellulose must produce extracellular cellulolytic enzymes capable of degrading the polysaccharide to soluble sugars. The degradation products, mainly glucose and cellobiose, are used as carbon and energy sources by the microorganism. Furthermore they play an important role in the regulation of the cellulase production. A cellulose fermentation thus represents a very interesting example of a fermentation where growth, enzyme production, and enzyme reaction are closely interdependent.

Cellulose fermentations have been extensively examined in batch cultures,^{1–7} and a few reports have dealt with continuous processes.^{8–11} A theoretical approach to fermentations using a polymeric substrate has been made.¹²

In the present work growth and cellulase production by the cellulolytic fungus *Trichoderma viride* in a continuous flow culture using

cellulose as the substrate have been investigated. Responses to step changes in dilution rate, substrate concentration, and temperature were followed and steady-state values were obtained.

MATERIALS AND METHODS

Microorganism

The cellulolytic fungus *Trichoderma viride* QM9414 was used in all experiments.

Medium

The cellulose source was a pure ball milled wood cellulose (BW 200, Brown Co., Berlin, New Hampshire). Nutrient salts were added in an amount proportional to the cellulose concentration (g/10 g cellulose): $(\text{NH}_4)_2\text{SO}_4$, 1.8; KH_2PO_4 , 2.0; $(\text{NH}_2)_2\text{CO}$, 0.4; CaCl_2 , 0.3; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3, and trace metals (mg/g cellulose): FeSO_4 , 2.5; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.98; ZnCl_2 , 0.83; CoCl_2 , 1.0. In shake flasks and during the initial batch phase of the fermentor runs proteose peptone (Difco) was added (1 g/10 g cellulose). Finally the addition of 1 ml per 10 liter culture liquid of a 50% mixture of polypropylene glycol (PPG 2000) and ethanol prevented foaming.

Analysis

Cellulase was measured as international units (μmol of glucose produced per min) by a filter paper method, based on the dilution to give 2 mg of glucose from 0.5 ml of enzyme acting on 50 mg of Whatman No. 1 filter paper for 1 hr at 50°C . For preparations producing less than 2 mg of glucose in this assay $\text{U/ml} = \text{mg glucose} \cdot 0.185$.

Soluble protein was determined by the Lowry method after precipitation with 5% trichloroacetic acid, using bovine serum albumin as a standard.¹³

Dry weight was determined by filtering a sample on a glass fiber filter and drying at 102°C overnight.

Mycelial protein was extracted with NaOH. The dried sample (50–500 mg) was placed in a test tube and extracted on a boiling water bath successively with 10 and 5 ml 1N NaOH and 5 ml water for 10 min. The protein was determined on the combined extracts, by a biuret method.¹³

Cellulose was determined as total carbohydrate in the residue from the NaOH extraction by the phenol-sulfuric acid method.¹³

Cell mass was calculated as dry weight minus cellulose.

Fermentor Operation

The fermentations were carried out in a 14 liter fermentor (New Brunswick Magnaferm Model MA 114) with an operating volume of 6.1 liter. The feed vessel was a stirred 14 liter glass jar. Medium was pumped from the feed vessel to the fermentor through a 6 mm wide silicone tubing (Dow Corning Medical Tubing) using a Cole Parmer pump (Model 7017). This pump was activated every 15 min for 5 to 15 sec pumping 50-120 ml medium (cellulose suspension) into the fermentor. The amount of liquid in the fermentor was kept constant through an overflow pipe inserted through the top plate and ending at the liquid surface. The overflow pipe was connected to a second Cole Parmer pump operating continuously.

The fermentor was equipped with a bottom drive with two flat turbine impellers equally spaced. The agitation rate was maintained at 350 rpm and aeration was 2 liter/min (0.33 v/v/m). Temperature was maintained at 30°C, except for one experiment (33.5°C). pH was controlled (NBS Automatic pH Controller, Model pH-40) at 5.0 through addition of 2*N* NaOH.

The system could be operated continuously for three to four weeks, the most serious problem being contamination. Some wall growth and growth on the baffles occurred at low dilution rate but in no case was the harvest line blocked. However, the clumps of mycelium would tend to increase the surface of the fermenting liquid thereby decreasing the actual operating volume of the fermentor.

The fermentor was inoculated with 200 ml culture medium from spore inoculated shake flasks grown at 27°C for three to four days on a reciprocal shaker. After fermentor inoculation, the fungus was allowed to grow two or three days before the continuous operation was started.

Under each set of fixed conditions, the system was maintained for five to seven residence times (three to nine days). In each fermentation three different steady states were obtained, unless contamination or mechanical failure occurred.

RESULTS AND DISCUSSION

The fermentation course for the initial batch phase and transition to continuous operation is shown in Figure 1. During the batch phase, growth measured as cell protein was almost linear with a specific growth rate, μ , decreasing from 0.055 hr⁻¹ (10 hr) to 0.017 hr⁻¹ (48 hr). At that time the continuous operation was started ($D = 0.079$ hr⁻¹) and cellulase activity (E) and cell protein concen-

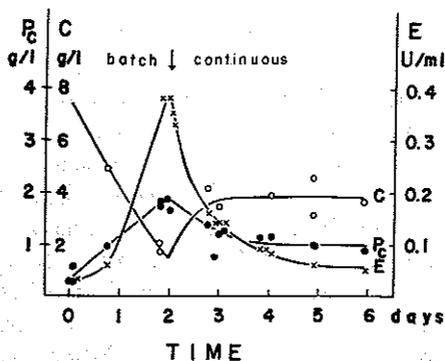


Fig. 1. Fermentation course during switch from batch to continuous operation (expt. No. 6). $C_0 = 7.5$ g/liter and $D = 0.079$ hr $^{-1}$. ●—● cell protein, P_c ; ○—○ cellulose, C ; ×—× cellulase, E .

tration (P_c) decreased while the cellulose concentration (C) increased. Cell protein and cellulose concentrations reached their steady-state values after about 48 hr (four residence times), but the cellulase activity did not reach the steady state until after 72–96 hr (six to seven residence times).

Steady-state values from four different fermentations are given in Table I. From these values parameters are derived which are necessary to describe the growth of the fungus and to show the relationship between cellulase production and growth.

The relationship between cellulose concentration and growth rate is shown in Figure 2, a reciprocal plot of μ ($\mu = D$) against the relative cellulose concentration C/C_0 , where C_0 is the cellulose concentration in the feed vessel. A straight line is obtained and for $C/C_0 = 1$ the critical dilution rate $D_{crit} = 0.17$ hr $^{-1}$. This may appear as a high value for a cellulose fermentation, and will be further discussed later. It will be assumed that $D_{crit} = \mu_{max}$.¹⁴ Using glucose as the substrate μ_{max} at pH = 4.8 and 30°C = 0.294 hr $^{-1}$ (wash out)⁹ and μ_{max} at pH = 4.0 and 28°C = 0.097 hr $^{-1}$ (batch)¹⁵ have been reported.

Thus *T. viride* grows fairly slowly and this together with the production of extracellular protein causes a decrease in the yield factor Y (g cell mass/g cellulose consumed) with decreasing growth rate. An expression relating Y to μ has been derived¹⁶ (eq. (1)):

$$Y = Y_{max} \frac{\mu}{mY_{max} + \mu} \quad (1)$$

where Y_{max} equals the yield factor for $\mu \rightarrow \infty$, and m is the maintenance coefficient (g cellulose consumed/g cell mass · hr).

TABLE I
Steady-State Values for Continuous Cultivation of *T. viride* on Cellulose^a

Expt. No.	Temperature °C	Dilution rate D hr ⁻¹	Cellulose concentration in feed C_0 g/liter	Cellulose concentration in fermentor C g/liter	Dry weight dw g/liter	Extracellular protein P_e g/liter	Cell protein P_c g/liter	Cellulase activity E U/ml
5a	30.0	0.080	5.0	2.5	4.0	0.09	0.65	0.11
5b	-	0.050	5.0	1.8	3.2	0.21	0.71	0.23
5c	-	0.033	5.0	1.2	2.8	0.48	0.95	0.34
6	-	0.079	7.5	3.8	6.3	0.04	1.0	0.06
8a	-	0.058	10.0	4.3	7.5	0.16	1.3	0.15
8b	-	0.043	10.0	3.4	6.9	0.16	1.6	0.19
8c	-	0.033	10.0	2.8	6.3	0.46	2.0	0.30
9a	-	0.038	4.0	1.0	2.5	0.18	0.61	0.22
9b	-	0.038	11.0	3.5	7.5	0.56	2.4	0.33
9c	33.5	0.038	11.0	4.0	7.2	0.32	2.0	0.24

^a pH = 5.0.

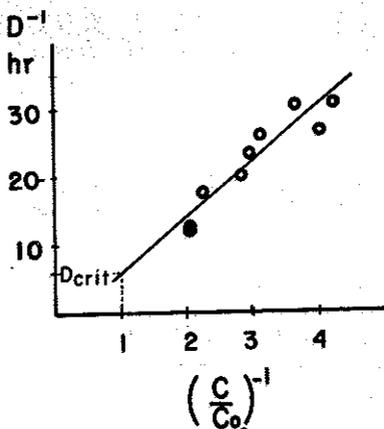


Fig. 2. Reciprocal plot of dilution rate vs. relative cellulose concentration; D_{crit} obtained for $C/C_0 = 1$. Steady-state values.

The reciprocal form (eq. (2)):

$$\frac{1}{Y} = \frac{m}{\mu} + \frac{1}{Y_{max}} \quad (2)$$

indicates that a plot of $1/Y$ vs. $1/D$ ($\mu = D$) gives a straight line (Fig. 3) from which Y_{max} and m can be obtained (Table II). In this expression the substrate used for producing extracellular protein has been included, so the constants derived will tend to be too high.

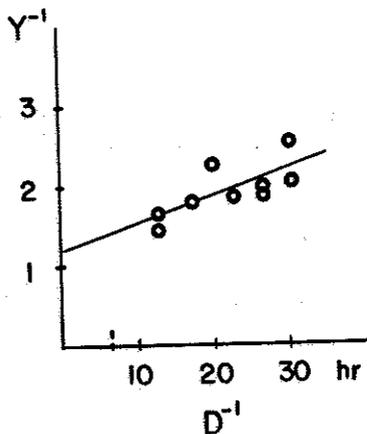


Fig. 3. Reciprocal plot of yield factor vs. growth rate. Steady-state values.

TABLE II
Growth Parameters for *T. viride* Growing on Cellulose^a

D_{crit} hr ⁻¹	0.17
Y_{max}	0.83
$Y_{(D=D_{crit})}$	0.70
m hr ⁻¹	0.034

^a pH = 5.0, T = 30°C, steady-state conditions.

The m value (0.034 hr⁻¹) is comparable to, though higher than, values reported for other fungi.^{17,18} The yield factor ranged from 0.68 to 0.39, with an estimated value $Y = 0.70$ at $D = D_{crit}$. From other cellulose fermentations $Y = 0.40$ – 0.55 have been calculated,⁴ while on glucose $Y = 0.40$ is found.¹⁵ A high value $Y = 0.67$ (biomass – carbon/glucose – carbon) for *Trichoderma lignorum*¹⁹ shows the variability of this value. The protein content of the cell mass was 40–60%, calculated from the data in Table I.

After a step change in dilution rate, the new steady-state concentrations for cell proteins and cellulose concentration were reached after three to four residence times. Longer time (four to six residence times) was required for the cellulase activity to reach its new value, and sometimes an overshoot was observed.

The effect of increasing the temperature is seen from Table I. The cell protein decreased slightly and cellulase concentration was reduced with 25%, thus the cellulase activity, E/P_c (U/mg cell protein) decreased. The steady state was now reached after two to three residence times. In shake flasks the same temperature effect has been observed.²

The response to a step change in substrate concentration, from 4 g/liter to 11 g/liter is shown in Figure 4. A rapid increase in cellulose concentration and a slower increase in cell protein was observed. The cellulase activity reached a minimum (after about 24 hr) and then rose up to a higher steady-state value. The initial drop was probably due to increased adsorption (see below) and a transient repression caused by the increased cellulose concentration. The higher cellulose concentration might be expected to increase the amount of glucose (a repressor of cellulase production²⁰) and cellobiose (under certain conditions, an inducer of cellulase production¹). The concentration of reducing sugars (assayed with dinitrosalicylic acid (DNS) reagent) was always below 0.1 mg/ml.

The effect of different cellulose concentrations (4–11 g/liter) on the steady-state cellulase activity was fairly small (Fig. 5a). To see

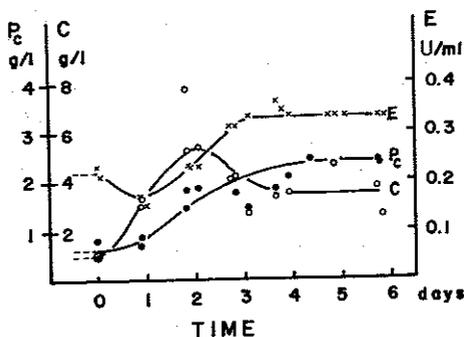
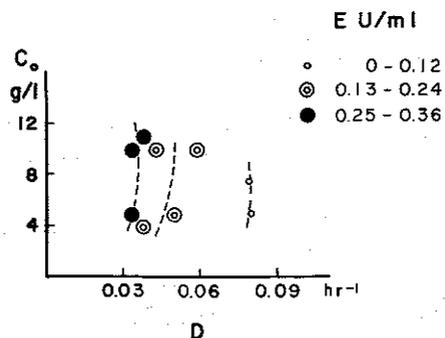


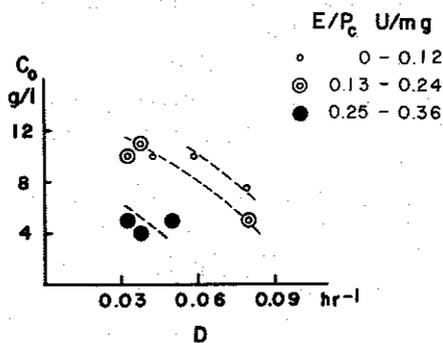
Fig. 4. Fermentation course following a step change (at $T = 0$) in cellulose concentration, C_0 4 \rightarrow 11 g/liter, (expt. Nos. 9a-9b). ●—● cell protein, P_c ; ○—○ cellulose, C ; ×—× cellulase, E .

whether adsorption might play a significant role, the amount of cellulase adsorbed at the given cellulose concentration was calculated from an adsorption isotherm.²¹ For $C_0 = 4, 5, 10,$ and 11 g/liter the amount of cellulase adsorbed was approximately 0.01, 0.03, 0.05, and 0.06 U/ml, respectively. Thus the total amount of enzyme is only slightly higher (5–20%) than the extracellular enzyme.

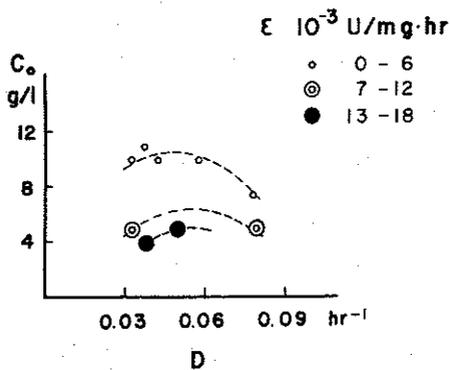
The cellulase activity E/P_c decreased with increasing dilution rate and initial cellulose concentration (Fig. 5b). The specific productivity, $\epsilon = (E \cdot D)/P_c$ (U/mg cell protein \cdot hr) was only slightly dependent on the dilution rate, with a maximum around $D = 0.05$ hr⁻¹ but decreased rapidly with increasing cellulose concentration (Fig. 5c). Since cellulases from *T. viride* are not constitutive enzymes,^{1,11,20} an inducer concentration sufficient for maximal induction must be supplied by the hydrolysis of the cellulose. With increasing cellulose concentration, increased glucose repression²⁰ might be expected, although as mentioned above the concentration of reducing sugars stayed below 0.1 g/liter. In the production of β -1,3-glucanase a sharp drop in enzyme activity per biomass was observed when the dilution rate was higher than 0.2 hr⁻¹ ($0.25 \mu_{max}$), and this was explained as catabolite repression.²² However, from the results here reported it follows that cellulase production shows little if any repression associated with high growth rate. When growing *T. viride* in a two-stage continuous fermentation using glucose in the first stage and cellulose in the second stage, a maximum specific cellulase productivity was obtained at $D = 0.02$ hr⁻¹.¹⁰ The low value was considered due to the lag period in cellulase production observed after



(a)



(b)



(c)

Fig. 5. (a) Steady-state cellulase activity, $E = f(D, C_0)$. (b) Steady-state cellulase activity, $E/P_c = f(D, C_0)$. (c) Steady-state specific cellulase productivity, $\epsilon = f(D, C_0)$.

growing on glucose. Similarly, when grown on glucose (Cerelese) alone (single stage) $D = 0.015 \text{ hr}^{-1}$ gave the highest productivity.²³

Most of the work done until now on the cultivation of *T. viride* has been aimed towards producing cellulases to be used for enzymatic hydrolysis of cellulose. In most of this work, batch cultivations have been used.^{1-4,6,11,24} Thus it is of importance to know how the results obtained in this study compare with results from batch fermentations. However, in comparing, it must be remembered that the physiological and environmental conditions are quite different. In batch fermentations the growth rate is generally low, pH is allowed to drop down between three and four and since the enzyme appears late in fermentation, more than half of the final amount of enzyme is produced after the cellulose is consumed, growth has stopped, and the cells have started to autolyze.

The fairly small effect of the different cellulose concentrations (4-11 g/liter) (see Fig. 5a) is in contrast to results from batch fermentations, where an increase in cellulose concentration gives a considerably higher cellulase activity, though not proportional.^{3,24}

A relation between substrate consumption and enzyme activity, E , is obtained by plotting E versus the relative amount of cellulose consumed $(C_0 - C)/C_0$ (Fig. 6). A straight line is obtained and by extrapolating it is seen that when less than 40% of the cellulose is consumed very little free enzyme can be expected. This occurs when $D \geq 0.10 \text{ hr}^{-1}$ which is at a considerably lower D than D_{crit} (Table

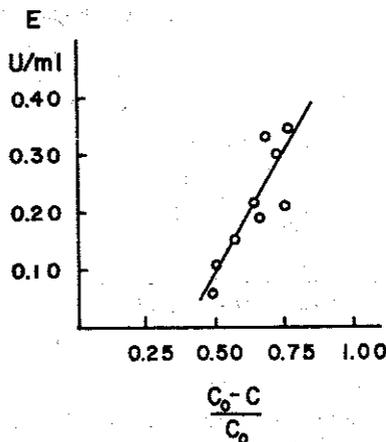


Fig. 6. Cellulase activity vs. relative amount of cellulose consumed. Steady-state values.

II). This would suggest that at higher growth rates the cellulases are either adsorbed to the cellulose, or bound to the cell surface. Many microorganisms are able to degrade cellulose, even though the cellulase activity in a culture filtrate may be quite low.¹¹

Under the best conditions here established the maximum productivity of cellulase, $E \cdot D$, is around $12-13 \times 10^{-3}$ U/ml \cdot hr (Table I, expts. 5b and 9b) corresponding to $10-20$ μ g/ml \cdot hr extracellular protein. This compares favorably with the productivity obtained in batch fermentations, 8×10^{-3} and 18×10^{-3} μ g/ml \cdot hr for 9.4 and 27 g/liter cellulose, respectively.³

However, the cellulase activity in the harvest stream from the continuous fermentation is only 10-30% of what is obtained in a batch fermentation under similar conditions, and so to be used in an industrial process the cellulase solution would have to be concentrated.

CONCLUSIONS

It has been demonstrated that *T. viride* can be grown in a continuous flow culture using cellulose as the only carbon and energy source. Under steady-state conditions, the specific cellulase productivity is fairly independent of the growth rate, with a maximum around $D = 0.05$ hr⁻¹. The overall cellulase productivity is comparable to batch fermentations using the same cellulose concentration. After a step change in dilution rate or cellulose concentration, the new steady-state concentration is reached after three to five residence times for cell protein and cellulose and four to six residence times for extracellular cellulase.

Nomenclature

C	cellulose concentration in fermentor, g/liter
C_0	cellulose concentration in feed vessel, g/liter
D	dilution rate, hr ⁻¹
D_{crit}	critical dilution rate, hr ⁻¹
E	cellulase activity, U/ml
m	maintenance coefficient, g cellulose consumed/g cell mass \cdot hr
P_c	cell protein concentration, g/liter
P_e	extracellular protein concentration, g/liter
Y	yield factor, g cell mass/g cellulose consumed
Y_{max}	Y for $\mu \rightarrow \infty$
ϵ	specific cellulase productivity, $(E \cdot D)/P_c$, U/mg cell protein \cdot hr
μ	specific growth rate, hr ⁻¹
μ_{max}	maximum growth rate, hr ⁻¹

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