0273-2289/90/2425—0223$02.60

Cellulase Production by *Acidothermus cellulolyticus*

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ABSTRACT

Acidothermus cellulolyticus, an isolate from hot springs at Yellowstone National Park, produced cellulase enzyme when grown in cellobiose-containing medium. The cellulase production, cell growth, and cellobiose degradation rates of batch culture in 2.5, 5.0, 7.5, and 10.0 g/L of cellobiose as a substrate were studied. The specific growth rates were measured, and the μ_{max} and K_s values based on these data were 0.2 h^{-1} and 0.3 g cellobiose/L using the Monod equation. The maximum cellulase activities (21–69 U/L) and volumetric productivities (between 0.92 and 2.49 U/L-h) were proportional to the concentration of cellobiose.

Greatest cellulase production in batch culture was achieved by use of secondary cellulosic substrates. Mixed substrate systems consisting of 5 g/L cellobiose and various Avicel concentrations (5, 10, and 16 g/L) were also studied in batch culture. The maximum cellulase activities were 53, 62, and 78 U/L, respectively. The enzyme production rate could be related to Avicel using the Monod equation. Here, maximum volumetric productivity, v_{max}, was found to be 2.15 U/L-h and K_s was 7.5 g Avicel/L. Another mixed substrate system, consisting of 5 g/L cellobiose and 15 g/L Solka Floc, produced a maximum cellulase concentration of 105 U/L.

Index Entries: Cellulase; *Acidothermus cellulolyticus;* enzyme production; growth kinetics.

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INTRODUCTION

The mesophilic fungus, *Trichoderma reesei*, has been the most studied source of hydrolytic enzymes for bioconverion of cellulosic materials to fermentable sugars for ethanol production. However, cellulase enzymes from thermophilic bacteria offer a variety of potential advantages over cellulolytic enzymes from mesophilic and thermotolerant fungi in industrial applications (*1–3*). Among the thermophilic, aerobic bacteria, only a few actinomycetes are actively cellulolytic, notably *Thermomonospora curvata* (*4*), *Thermomonospora fusca* (*5*), and *Thermoactinomyces cellulosae* (*6*).

Acidothermus cellulolyticus, a thermophilic, acidophilic, cellulolytic, aerobic bacterium, was isolated from acidic hot springs at Yellowstone National Park, Wyoming (*7*). This culture was found to produce cellulolytic enzymes when grown in cellobiose and/or cellulose at pH 5.2 and 55°C. *A. cellulolyticus* produces filter paper degrading enzymes that have been reported to have the highest thermostability noted to date (*3,8*); this characteristic is the key to industrial interest when total saccharification of cellulose is the target. The specific growth rate and cell density achieved by thermophilic aerobic bacteria are potentially greater than those parameters obtained with mycelial cultures or anaerobic, thermophilic, clostridial cellulase producers (*9*). Consequently, if the specific productivity of cellulase activity were even equivalent to other systems, *A. cellulolyticus* would economically compete as a source of cellulase enzymes with desirable thermotolerant properties. Hence, the objectives of this study were: (1) to investigate the growth kinetics and cellulase production of the culture in a wide variety of sugars and cellulose sources; (2) to obtain the kinetic parameters for cell growth and cellulase production in different initial concentrations of cellobiose; and (3) to observe cellulase production from mixing cellobiose with Avicel and Solka Floc as secondary substrates.

METHODS

Microorganism

A. cellulolyticus 11B, which was isolated from the upper Norris Geyser basin in Yellowstone National Park by Mohagheghi et al. (*8*) and which has been deposited with the American Type Culture Collection, Rockville, Maryland (ATCC 43068), was used in this study. The strain was maintained frozen at −70°C after the addition of 77 μL dimethyl sulfoxide/mL culture suspension.

Culture Media

The culture was prepared with a low-phosphate basal salts medium (LPBM) that contained the following, in grams per liter: NH_4Cl, 1.0;

KH_2PO_4, 1.0; $Na_2HPO_4 \cdot 7H_2O$, 0.1; $MgSO_4 \cdot 7H_2O$, 0.2; and $CaCl_2 \cdot 2H_2O$, 0.2. The medium was also supplemented with the following, in grams per liter: yeast extract (Difco Laboratories, Detroit, MI), 1.0; D-cellobiose, 5.0 (if not mentioned otherwise), and 1.0% (v/v) Wolin trace mineral solution (*10,11*). The cellulosic substrates used included carboxymethyl cellulose (Sigma Chemical Co., St. Louis, MO), Solka Floc BW-200 NF (James River Co., Berlin, NH), and Avicel, 90 micron average particle size (Brinkmann Instruments Inc., Des Plaines, IL). All media were adjusted to pH 5.2 and sterilized by autoclaving at 121°C for 20 min.

Inoculum

The frozen culture from −70°C was inoculated immediately in a shake flask containing 20 mL of medium. After overnight incubation at 55°C and 120 rpm agitation on a rotary shaker, a 10 mL aliquot of cell suspension was transferred to a 500 mL baffled shake flask containing 200 mL of medium. After growth under similar conditions, 200 mL of cell suspension was inoculated into fermentors with working volumes of 2.5 or 1.0 L.

Fermentations

Fermentors (B. Braun Models Biostat V and Biostat S) of 2.5 or 1.0 L working volume were used. The medium was maintained at pH 5.2 during fermentation by the addition of 1.0*N* NH_4OH and 1.0*N* H_3PO_4. Temperature of the medium was maintained at 55°C and dissolved oxygen was maintained at 40% of saturation by increasing the agitation rate and/or supplying pure oxygen as needed.

Assays

Filter paper assays and residual reducing sugar determinations were conducted according to the recommendations of the Measurement of Cellulase Activities prepared for the IUPAC (*12*). International units of filter paper degrading activity were reported. Cell density was measured by absorption at 600 nm with a Spectronic 20 (Bausch & Lomb, Inc., Rochester, NY). Dense cultures were diluted with distilled water to determine absorption values. Cell dry weight was determined gravimetrically after drying at 100°C for 12 h. A relationship was established whereby 1.0 $OD_{600\ nm}$ = 0.58 g/L cell dry wt.

Cultures containing cellulose were extremely turbid; in this case, growth was estimated by a modified Lowry protein assay (*13*). The cell pellets were suspended in Lowry reagent A, and cellulose and cell debris were removed by centrifugation after lysis, induced by heating to 60°C for 20 min. The lysates were cooled and centrifuged, the supernatants were mixed with Lowry reagent B, and the chromophore was developed (*13*). ery high correlation values (0.99) related 1 g/L cellular protein, determined in this manner, to 1.61 g/L cell dry wt.

Table 1
Growth and Cellulase Production by Cells Grown on Different Carbon Sources

Carbon Source, w/v	Specific Growth rate, h^{-1}	Max. cell dry wt., g/L	Cellulase activity, U/L
0.50% CMC	0.10	0.49	6.5±0.8
0.50% glycerol	0.08	0.32	0
0.50% L-arabinose	0.09	0.50	0
0.25% L-arabinose	0.17	0.34	0
0.50% D-xylose	0.06	0.24	0
0.25% D-xylose	0.14	1.38	4.0±0.5
0.25% D-mannitol	0.12	0.40	0
0.25% D-mannose	0.17	1.47	0
0.50% D-sorbitol	0.12	0.41	0
0.25% L-sorbose	0.08	0.49	0
0.25% lactose	0.16	0.48	0
0.25% D-galactose	0.22	0.55	0
0.25% D-maltose	0.22	0.49	0
0.25% D-trehalose	0.16	0.48	0
0.25% sucrose	0.22	1.58	0
0.25% D-fructose	0.18	1.94	0
0.25% D-cellobiose	0.18	1.46	20.0±1.5
0.25% D-glucose	0.19	1.60	0

RESULTS AND DISCUSSION

A. cellulolyticus 11B is a thermophilic, acidophilic, cellulolytic, aerobic bacterium. It can utilize D-glucose, D-cellobiose, cellulose, xylan, D-galactose, maltose, sucrose, raffinose, D-mannose, D-mannitol, D-sorbitol, D-fructose, L-arabinose, D-xylose, or glycerol as the sole source of carbon and energy, and ammonium ion or amino acids can serve as sources of nitrogen (*8*). To investigate the cell growth kinetics and cellulase production from different substrates, either 5.0 or 2.5 g/L of monosaccharides, disaccharides, sugar alcohols, or cellulose was used as a substrate with media containing 2× LPBM, Wolin trace mineral solution, and yeast extract. All fermentations were conducted with control of pH (5.2), dissolved oxygen (>40% of saturation), and temperature (55°C).

The characteristics of specific growth rate, maximum cell dry weight, and cellulase activity in the broth for the different carbon sources are shown in Table 1. *A. cellulolyticus* grew very well in the initial 10 h of fermentation on all substrates; the specific growth rates varied from 0.08 to 0.22 h^{-1}. The culture stopped utilizing the various carbon sources, and large amounts of sugars were left in the medium after two or three generations of growth on all the substrates except D-glucose, D-cellobiose, D-

fructose, D-mannose, D-xylose, and sucrose. The maximum cell dry weights were low, in the range of 0.24 to 0.55 g/L. In glucose, fructose, cellobiose, sucrose, xylose, cellobiose fermentations, all the sugars were consumed in 24 h and the maximum cell dry weights were between 1.5 to 2.0 g/L.

Considering the chemical structure of these substrates, where D-glucose and D-mannose are epimers, with respect to carbon atom 2, D-glucose and D-galactose are epimers, with resepct to carbon atom 4, and D-xylose and L-arabinose, as well as D-fructose and L-sorbose, are epimers with respect to carbon atom 4; these substrate utilization data may begin to elaborate metabolic pathways. Possibly, this microorganism may metabolize the epimers on carbon 2 completely, but may not completely utilize the carbon 4 epimers. The only difference between D-mannose and D-mannitol is in carbon atom 1, the latter having an alcohol group, but cell growth on those two substrates was entirely different. The cultures that grew on those incompletely utilized substrates may accumulate some metabolites that inhibit cell growth.

A. cellulolyticus could not grow on lactose and L-sorbose when the concentration of sugars was increased from 2.5 to 5.0 g/L. Cell growth and maximum cell density of the culture on 2.5 g/L xylose were higher than on 5.0 g/L xylose. This microorganism could not grow on 20 g/L glucose. In virtually all microorganisms examined to date, the synthesis of cellulases is induced by the presence of cellulose and repressed by the presence of glucose or other readily metabolized sugars in the growth medium. The inducers include cellulose, cellobiose, sophorose, lactose, and some other sugars (*14*). In Table 1, *A. cellulolyticus* produced cellulase when grown in cellobiose, xylose, and CMC. Lactose induces cellulase in *T. reesei* and a few other fungi, but not in this microorganism. Cellobiose is known to induce cellulase synthesis in *T. reesei* (*15*), but is effective only at a high concentration (*16*). Cellobiose is the principal product of cellulase activity and is also one of the carbon sources with which cellulase can be produced by *A. cellulolyticus*. To obtain the kinetic parameters for cell growth and cellulase enzyme production, different concentrations of cellobiose, 2.5, 5.0, 7.5, and 10.0 g/L, with 2×LPBM, Wolin trace mineral solution, and yeast extract, were used in batch fermentations. The time courses for these fermentations were similar; the 5.0 g/L fermentation is shown in Figure 1.

Parameters of cell growth and cellulase enzyme production in cellobiose fermentations by *A. cellulolyticus* are shown in Table 2. Cell growth was rapid on cellobiose; the specific growth rate was enhanced by increasing the concentration of cellobiose. However, the differences in specific growth rates between all four fermentations were small and in the range of 0.18 to 0.20 h^{-1}. The μ_{max} and K_s values for cell growth, based on these data, were 0.2 h^{-1} and 0.3 g cellobiose/L using the Monod equation. The maximum cell dry weight was proportional to the concentration of cellobiose.

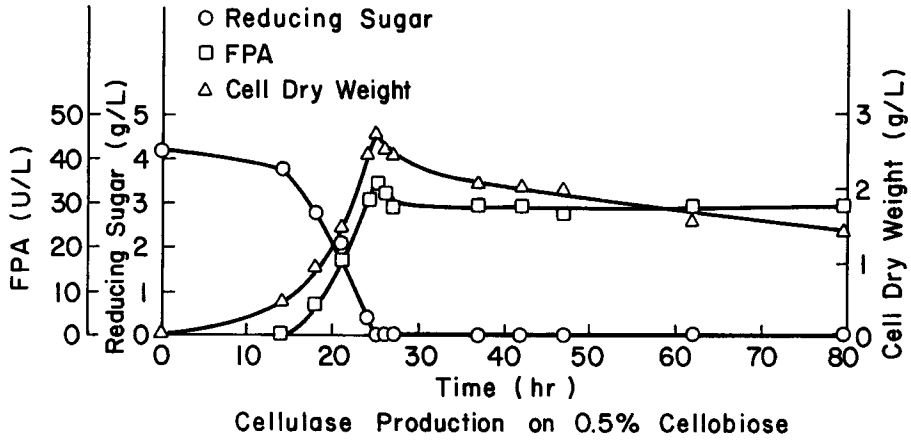


Fig. 1. Time courses of substrate consumption (○), cell mass production (△), and cellulase production (□) in batch culture of *A. cellulolyticus* on 5 g/L cellobiose.

Table 2
Product Formation Parameters for Cellobiose (CB) Fermentations

Parameter	Units	0.25% CB	0.5% CB	0.75% CB	1.0% CB
Maximum cell dry weight	g/L	1.45	2.73	3.77	5.10
Maximum enzyme concentration	U/L	21	35	69	67
Time to maximum FPA	h	23	25	26.5	29
Volumetric enzyme productivity[a]	U/L h	0.92	1.40	2.49	2.16
Specific enzyme productivity	U/g dry weight h	0.63	0.51	0.66	0.42
Specific growth rate, μ	h^{-1}	0.18	0.19	0.19	0.20

[a]Volumetric productivities were calculated as follows:

maximal FPA (U) / L × 1 / fermentation time to achieve max. FPA (h)

Cellulase production was growth associated in cellobiose fermentations. The cellulase activity increased progressively during the exponential growth phase and in proportion to the rate of utilization of cellobiose, as measured by reducing sugars. Cellulase concentration correlated strongly (>0.98) to the cell density, as measured by $OD_{600\ nm}$, which was proportional to cell dry weight and total cell protein. The time period for cell growth and cellulase production (i.e., between 17 and 29 h), occurred earlier than observed for fungi. Maximum cellulase activities obtained in

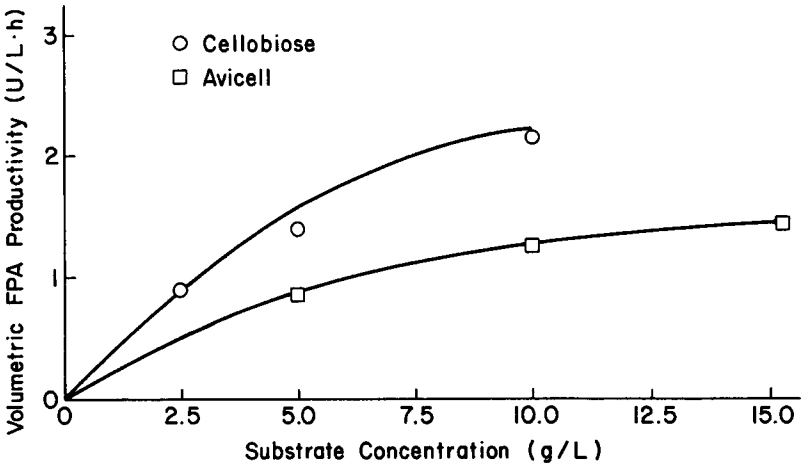


Fig. 2. Volumetric productivities of *A. celluloyticus* as functions of initial cellobiose concentration (○) and initial Avicel concentration (□), the latter mixed with 5 g/L cellobiose in batch cultures at pH 5.2 and 55°C. Data from Tables 2 and 3.

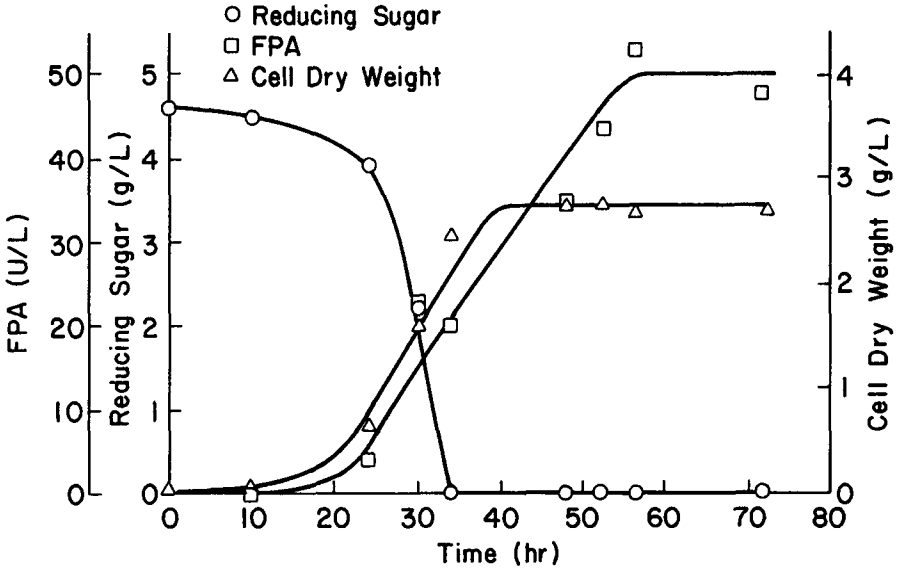
the supernatants from the culture grown at 2.5, 5.0, 7.5, and 10.0 g/L of cellobiose were 21, 35, 69, and 67 U/L, respectively.

The concentration of cellobiose in the medium was directly related to the maximum levels of cellulase enzyme activities detected, except in the case with 10.0 g/L cellobiose as a substrate. In this case, cellulase activity was lower than in the 7.5 g/L cellobiose fermentation. Cellulase volumetric productivities of this microorganism are related to the substrate concentration in Fig. 2. From these data, the enzyme maximum volumetric productivity, v_{max}, was calculated to be 5.1 u/L-h, and the K_s was 11.4 g cellobiose/L by the Monod-type relationship. The specific enzyme productivities were in the range of 0.42 to 0.66 U/g dry wt-h and showed no consistent relationship to other parameters.

The results of cellobiose batch systems indicate that increasing the biomass may enhance cellulase production. Batch production of cellulase by *T. reesei* or other microorganisms is characterized by relatively low productivities and enzyme titers owing to the necessary low substrate concentration to prevent repression (*17*). Using fed-batch fermentation, it should be possible to keep concentrations of nutrients in suitable ranges during the cultivation. This method is useful not only for cultivation of microorganisms exhibiting substrate-inhibited kinetics for growth, but also for obtaining high densities of biomass within short incubation times.

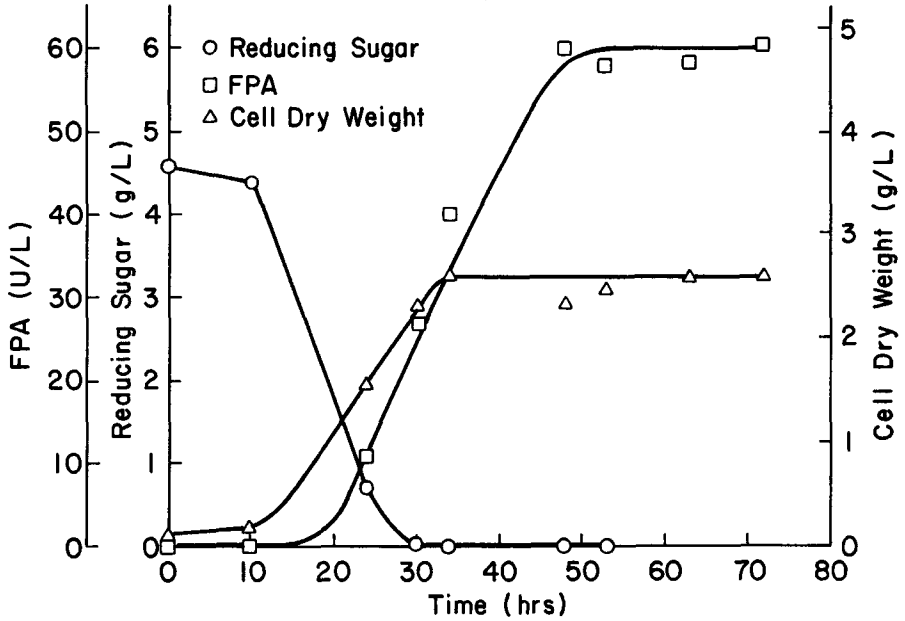
In fed-batch experiments with initial 5 g/L cellobiose concentrations, the culture grew rapidly, and cell mass reached 26 g dry cell weight/L after 51 h fermentation when 1 L of nutrient solution containing 200 g/L cellobiose had been added. Cellulase enzymes started to be produced after 27 h, and the maximum activity was 70 U/L. Cell concentration was

A



Cellulase Production on 0.5% Cellobiose and 0.5% Avicel

B



Cellulase Production on 0.5% Cellobiose and 1% Avicel

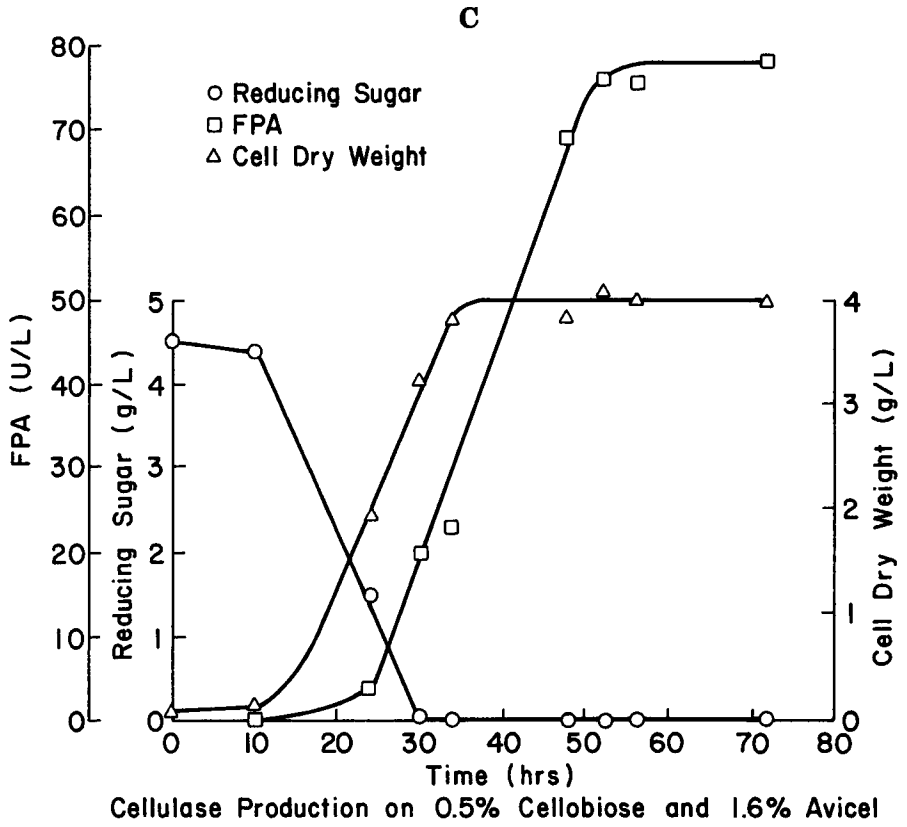


Fig. 3. Time courses of substrate consumption (○), cell mass production (△), and cellulase production (□) in batch cultures of *A. cellulolyticus* on 5 g/L cellobiose mixed with Avicel at various concentrations: (**A**) 5 g/L; (**B**) 10 g/L; and (**C**) 16 g/L.

increased five times compared to batch fermentations at the same initial substrate concentration, but enzyme levels were not increased.

A wide range of cellulosic materials, both microcrystalline (Sigmacell 50 and α-cellulose) and amorphous (filter paper, phosphoric acid swollen cellulose), were also utilized as substrates (*8*). Combining cellobiose with a secondary cellulosic substrate improved enzyme productivity. Mixing 5 g/L cellobiose and various Avicel concentrations, (5, 10, and 16 g/L) as initial substrates in batch systems was studied. The kinetic parameters of cell growth and cellulase production are given in Table 3, and the time course of cell mass synthesis, enzyme formation, and reducing sugar utilization in these three experiments are shown in Fig. 3. Cell dry weight increased with the reducing sugar utilization rate. Maximum cell dry weights were obtained after 34 h of fermentation for the three levels of Avicel. The specific growth rates, between 0.15 and 0.18 h^{-1} (Table 3), were lower than those found when only cellobiose was used as a substrate (Table 2).

Table 3
Product Formation Parameters for Cellobiose (CB) with Avicel Mixed Substrate Fermentations

Parameter	Units	0.5% CB + 0.5% Avicel	0.5% CB + 1.0% Avicel	0.5% CB + 1.6% Avicel
Maximum cell dry weight	g/L	2.80	2.71	6.29
Maximum enzyme concentration	U/L	53	62	78
Time to maximum FPA	h	52.5	48	52.5
Volumetric enzyme productivity[a]	U/L h	0.86	1.25	1.45
Specific enzyme productivity	U/g dry weight h	0.31	0.46	0.23
Specific growth rate, μ	h^{-1}	0.15	0.17	0.18

[a] Same as Table 2.

The maximum cell dry weight increased in proportion to the concentration of Avicel; therefore, the culture utilized cellobiose and Avicel simultaneously to build up cell mass (Table 3). Cellulase production started in the log phase of cell growth and continued even when the cell growth stopped. In cellobiose fermentations, there was no cellulase synthesis after the cells stopped growing. Autolysis of cells, which was observed with cellobiose fermentations, was not obvious in the mixed cellobiose and Avicel fermentations. Avicel may play an important role in maintaining cell viability as an energy source after the depletion of cellobiose. Moreover, Avicel may be an inducer of the cellulase synthesis, because more cellulase was produced and secreted in the stationary phase. Furthermore, increased levels of Avicel increased the measured cellulase activities. The maximum cellulase activities from low to high concentrations of Avicel were 53, 62, and 79 U/L, respectively (Table 3). The enzyme production rate could be related to Avicel using the Monod-type relationship (Fig. 2), where the maximum volumetric productivity, v_{max}, equals 2.15 U/L-h and K_s is 7.5 g Avicel/L. Cell growth on cellobiose and the cellobiose–Avicel mixture were similar, but the specific enzyme productivity on 5 g/L cellobiose (0.51 U/g dry wt-h) was only slightly higher than on mixture of 5 g/L cellobiose and Avicel (average 0.33 U/g dry wt-h). Lower enzyme production rates in the mixed fermentations may be caused by the differences in efficiency of induction by cellobiose and Avicel. However, the maximum enzyme activity in the mixture (5 g/L CB+16 g/L Avicel) was 23% higher than in cellobiose (7.5 g/L CB) fermentation because of sustained culture viability.

A mixture of cellulose and cellobiose substrates for high productivities of cellulase enzymes of *A. cellulolyticus* seemed a promising refinement of

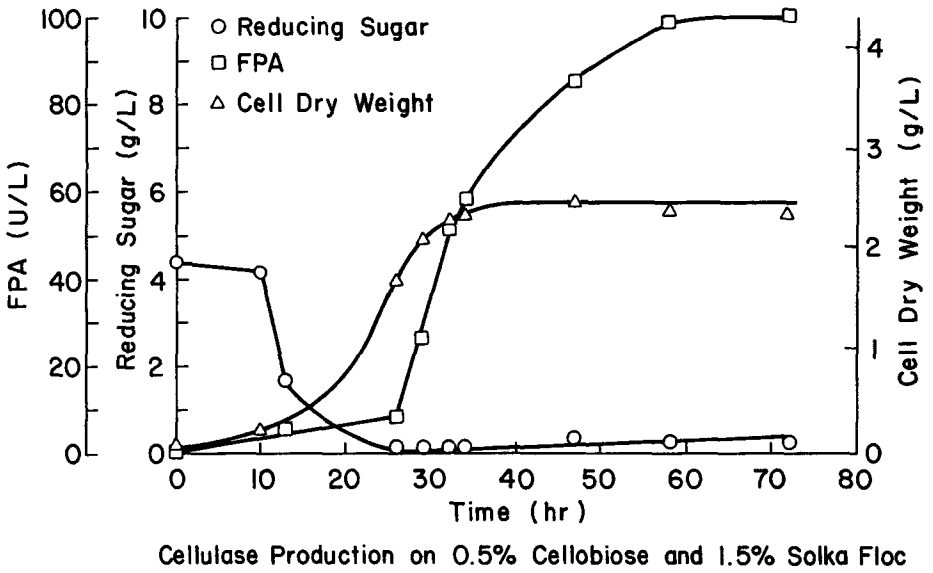


Fig. 4. Time courses of substrate consumption and production (○), cell mass production (△), and cellulase production (□) in batch culture of *A. cellulolyticus* on 5 g/L cellobiose mixed with 15 g/L Solka Floc.

the initial cellobiose fermentation. Avicel is a microcrystalline cellulose produced from highly purified spruce and hemlock pulp. A less expensive cellulose source, Solka Floc, which is composed of crystalline and amorphous cellulose, was chosen for further study.

Using 5 g/L cellobiose and 15 g/L Solka Floc as a mixed substrate, the time course of cell mass synthesis, cellulase enzyme formation, and reducing sugar utilization by *A. cellulolyticus* are presented in Fig. 4. Growth of the microorganism on cellobiose alone was much faster than on the mixture of cellobiose and Solka Floc; the specific growth rates were 0.19 (Table 2) and 0.13 h^{-1}, respectively. However, the volumetric enzyme production rate of the culture grown on cellobiose with Solka Floc was 1.46 U/L-h, which was the same as cellobiose alone (1.40 U/L-h) and cellobiose with 16 g/L Avicel (1.45 U/L-h). When using cellobiose with Solka Floc as mixed substrate, the maximum cellulase activity was 105 U/L.

From the analysis of cell mass data, it appears that *A. cellulolyticus* can use cellobiose and Solka Floc for cell growth at the same time, because more cell mass was produced on the mixed substrate. Cellulase synthesis started in the log phase of growth, and the rate of enzyme production slowed down after the cell growth reached the stationary phase. Reducing sugar from cellulase hydrolysis of Solka Floc accumulated in the broth during the stationary phase. Although cellobiose remained in the medium, cellulase production rates were lower than after the depletion of cellobiose. Cellobiose may repress and Solka Floc may induce cellulase production by *A. cellulolyticus;* in any case; this inducible activity was greater than that produced by Avicel. Certainly, the induction of cellulase in *A. cellulolyticus* needs further study.

Table 4
Cellulase Productivities of Representative Mesophilic Fungal and Thermophilic Bacterial Systems

Organism	Cellulosic substrate	Maximal FPA, U/L	Time, h	Productivity, U/L h
Trichoderma reesei L27	8% Avicel	18,000	192	93.7
Trichoderma reesei QM 6a	2% Solka Floc	5,000	333	15.0
Thermomonospora fusca	Avicel	150	29	5.1
Clostridium thermocellum	1% Solka Floc	140	72	1.9
Acidothermus cellulolyticus	1.5% Solka Floc	105	70	1.5
Thermomonospora curvata	Cellulose	100	72	1.4

The productivity of cellulases by *A. cellulolyticus* is compared with other thermophilic bacteria in Table 4, which has been adapted from a recent review by Margaritus and Marchant (*2*). Fermentation times of 72 h and productivities greater than 1.0 FPA U/L-h were typical of these systems. *T. fusca* (listed as *Thermoactinomyces sp.* XY in (*2*) and another *Thermoactinomyces sp.* N-35 exhibit productivities of 5.1 and 27.5 U/L-h, respectively. The latter value is similar to those exhibited by mesophilic fungi (*2*). The wildtype *T. reesei,* QM 6a, produces 15 U/L-h (*18*), and derivatives thereof produce between 20 and 40 U/L-h. The highest productivity reported to date is 94 U/L-h by *T. reesei* L27 on 80 g/L Avicel (*18*). The strain development and culture techniques that allow use of such high solids concentrations result in the differences between QM 6a and L27 productivities. *A. cellulolyticus* 11B, reported here, is a wildtype strain. Strain selection and culture development have demonstrated increasingly higher productivities, which will be reported in subsequent publications.

ACKNOWLEDGMENTS

The authors wish to acknowledge the support for this work from the Biochemical Conversion Program of the DOE Biofuels and Municipal Waste Technology Division through SERI subcontract No. XK-7-07031 and from the Colorado State University Experiment Station under series no. 383.

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