Growth inhibition of *Clostridium cellulolyticum* by an inefficiently regulated carbon flow

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Carbon flow in *Clostridium cellulolyticum* was investigated either in batch or continuous culture using a synthetic medium with cellobiose as the sole source of carbon and energy. Previous experiments carried out using a complex growth medium led to the conclusion that the carbon flow was stopped by intracellular NADH. In this study, results showed that cells cultured in a synthetic medium were better able to control electron flow since the NADH/NAD⁺ ratios were in the range 0.3–0.7, whereas a ratio as high as 57 was previously found in cells cultured on a complex medium. Furthermore, a specific rate of cellobiose consumption of 2.13 mmol (g cells)⁻¹ h⁻¹ was observed on synthetic medium whereas the highest value obtained on complex medium was 0.68 mmol (g cells)⁻¹ h⁻¹. When *C. cellulolyticum* was grown in continuous culture and cellobiose in the feed medium was increased from 5.84 to 17.57 mM in stepwise fashion, there was an increase in cellobiose utilization without growth inhibition. In contrast, when the reactor was fed directly with 14.62 mM cellobiose, residual cellobiose was observed (4.24 mM) and growth was limited. These data indicate that *C. cellulolyticum* is not able to optimize its growth and carbon flow in response to a sudden increase in the concentration of growth substrate cellobiose. This interpretation was confirmed (i) by the study of cellobiose batch fermentation where it was demonstrated that growth inhibition was not due to nutritional limitation or inhibition by fermentation products but was associated with carbon excess and (ii) by the growth of *C. cellulolyticum* in dialysis culture where no growth inhibition was observed due to the limitation of carbon flow by the low rate of cellobiose diffusion through the dialysis tubing.

Keywords: *Clostridium cellulolyticum*, growth inhibition, synthetic medium, carbon flow

INTRODUCTION

Clostridium cellulolyticum was previously found to undergo an early growth stoppage when grown in batch cultures on cellobiose (Giallo *et al.*, 1983). Giallo and coworkers reported that this inhibition was neither the result of nutrient depletion, nor the result of low pH or inhibition by end products; nevertheless, complex media were used routinely (Giallo *et al.*, 1983; Gelhaye *et al.*, 1993a, b; Gehin *et al.*, 1995). From a fundamental point of view and to achieve biotechnological application, it is essential to understand the conditions that direct the increase of the bacterial population since cellulolytic

clostridia are of major importance in the anaerobic digestion of lignocellulose (Leschine, 1995).

Payot *et al.* (1998) demonstrated that, on complex media, the main product of *C. cellulolyticum* cellobiose catabolism was acetate, whereas the production of reduced compounds such as ethanol or lactate was low. Such patterns of product formation were accompanied by an accumulation of intracellular NADH leading to an NADH/NAD⁺ ratio as high as 57 (Payot *et al.*, 1998). The high values of the NADH/NAD⁺ ratio inhibited glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity which in turn limited cellobiose catabolism and cell growth. It is difficult to understand why during the course of *C. cellulolyticum* evolution such metabolic regulations have been selected. Taking into account that

Abbreviations: fd, ferredoxin; O/R, oxidation/reduction.

many natural ecosystems rarely contain all nutrients in high quantity (Koch, 1997), the aim of the present work was to study the behaviour of *C. cellulolyticum* on a synthetic medium with special attention to the effect of carbon flow on growth.

METHODS

Organism and medium. The organism used in this study was *C. cellulolyticum* ATCC 35319, originally isolated by Petitdemange *et al.* (1984) from decayed grass. Stock cultures of *C. cellulolyticum* were maintained on cellulose as described previously (Payot *et al.*, 1998) and were grown for one transfer on cellobiose before the initiation of growth experiments. The anaerobic culture technique used was that proposed by Hungate (1969) as modified by Bryant (1972).

The defined medium used in all experiments was a modification of the CM3 medium described by Weimer & Zeikus (1977) in which 5 g yeast extract l⁻¹ is replaced by oligoelement and vitamin solutions. The composition was (g l⁻¹): KH₂PO₄, 1.40; K₂HPO₄·3H₂O, 2.90; (NH₄)₂SO₄, 1.00; MgCl₂·6H₂O, 0.10; CaCl₂, 0.02; 5% (w/v) FeSO₄ in 50 mM H₂SO₄, 25 µl; trace element solution, 1.0 ml; vitamin solution, 10 ml; Na₂S, 0.50 and 0.2% (w/v) resazurin, 0.5 ml. In addition, the medium contained cellobiose in variable amounts as specified in Results. The trace element solution contained (g l⁻¹): FeSO₄·7H₂O, 5.00; ZnSO₄·7H₂O, 1.44; MnSO₄·7H₂O, 1.12; CuSO₄·5H₂O, 0.25; Na₂B₄O₇, 0.20; (Mo)₇(NH₄)₆O₂₄·4H₂O, 1.00; NiCl₂, 0.04; CoCl₂, 0.02; HBO₃, 0.03; Na₂SeO₃, 0.02; HCl 10 M, 50.0 ml. The composition of the vitamin solution was [mg (100 ml distilled water)⁻¹]: D-biotin, 10; *p*-amino-benzoic acid, 25; nicotinic acid, 15; riboflavin, 25; pantothenic acid, 25; thiamin, 25; cyanocobalamin, 10. The vitamin solution was sterilized by filtration with a 0.2 µm filter (Millipore).

Growth conditions. *C. cellulolyticum* was grown either in batch or in continuous culture. Cells were cultured aseptically in a 2 l bioreactor (LSL BioLafitte) with a 1.5 l working volume. Vitamin and trace element solutions, cellobiose and Na₂S were sequentially added to the medium for optimal reduction. Anoxic conditions were maintained by passing a stream of filter-sterilized nitrogen gas over the cultures. All tubing used was made of Viton (Du Pont) to prevent oxygen entry.

The temperature was maintained at 34 °C and the pH was controlled at 7.2 by automatic additions of 1 M NaOH. Agitation was kept constant at 50 r.p.m. For continuous fermentations, the volume was kept constant at 1.5 l by automatic regulation of the culture level. The inoculum was 10% by volume and was in exponential-growth phase. The culture was grown in batches for 15 h before the medium flow was started. A period of three to four residence times was found to be sufficient to achieve steady-state values of the biomass and of the residual cellobiose concentration.

In addition, dialysis cultures were grown in 3 l flasks and fermentations carried out in dialysis tubing (Visking, molecular mass cut-off 6000–8000 Da) as described previously (Gehin *et al.*, 1996). No alteration of the tubing was observed during the course of our experiments.

Analytical procedures. Bacterial growth was measured spectrophotometrically at 600 nm and calibrated against cell dry weight measurement. Samples (30 ml) were centrifuged (8000 g, 10 min, 4 °C), washed twice with 0.9% (w/v) NaCl

and dried at 65 °C to constant weight (48 h). A mean biomass formula of C₄H₇O₂N and C₁₆H₂₅O₉N₆ for extracellular proteins determined by elemental analysis (Service Central d'Analyses, CNRS), were used for elemental recovery calculations.

Culture supernatants (10000 g, 15 min, 4 °C) were stored at –80 °C until they were analysed. Cellobiose concentrations were determined by the method of Miller (1959). Acetate, lactate and ethanol were estimated by using the appropriate enzyme kits (Boehringer Mannheim). The fermenter effluent gas was measured by gas chromatography as described by Vasconcelos *et al.* (1994).

Extracellular proteins from the cell-free supernatant were measured by the Bradford dye method (Bradford, 1976). The quantity of amino acids present in the supernatant was measured using the procedure of Mokrasch (1967) and by ion-exchange chromatography on a cation-exchange resin with a Beckman 7300 amino acid analyser. The mean elemental amino acid composition was C₅H₁₀O_{2.5}N.

Extracellular pyruvate was quantitatively assayed using an enzymic method coupled with fluorimetric detection of NADH. Emission was measured at 459 nm after excitation at 341 nm with a fluorimeter (Hitachi F2000). The mixture contained triethanolamine buffer (0.1 M, pH 7.6), NADH (12.5 µM) and lactate dehydrogenase (10 U).

Enzymic determination of intracellular pyruvate was not possible due to significant interference by extracellular pyruvate which led to erroneous estimates of intracellular concentrations.

Levels of NAD(P)⁺ and NAD(P)H in the biomass were measured by first extracting the nucleotides from a sample of culture. NAD(P)⁺ and NAD(P)H were extracted with HCl and KOH, respectively, as described by Wimpenny & Firth (1972). Levels of coenzymes in both extracts were determined by fluorimetric measurements. NAD⁺ was assayed with a NAD(H)-specific alcohol dehydrogenase (EC 1.1.1.1) and NADP⁺ was assayed with a glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Klingenberg, 1965; London & Knight, 1966). NADH was determined using a lactate dehydrogenase (EC 1.1.1.27) assay (Klingenberg, 1965). NADPH was measured in a reaction mixture containing 100 mM triethanolamine buffer (pH 6.0), 20 mM α-ketoglutaric acid and 20 U of NADP(H)-specific glutamate dehydrogenase from *Proteus* species (EC 1.4.1.4).

Calculations. The main products of cellobiose fermentation by *C. cellulolyticum* are acetate, ethanol, lactate, H₂ and CO₂ (Giallo *et al.*, 1983). The reactions leading to the formation of the metabolites were described previously (Payot *et al.*, 1998).

$q_{\text{cellobiose}}$ is the specific rate of cellobiose used in mmol (g cells)⁻¹ h⁻¹; q_{acetate} , q_{ethanol} and q_{lactate} are the specific rates of product formation in mmol (g cells)⁻¹ h⁻¹; and extracellular q_{pyruvate} is the specific rate of extracellular pyruvate formation in µmol (g cells)⁻¹ h⁻¹.

The specific rates of NADH production and NADH consumption were calculated as described previously (Payot *et al.*, 1998).

The specific rate of H₂ production via the NADH-ferredoxin (fd)-H₂ path was calculated as follows:

$$q_{\text{NADH fd}} = q_{\text{NADH produced}} - q_{\text{NADH used}}$$

The oxidation/reduction (O/R) index was calculated according to Gottschalk (1985).

Y_{ATP} , the energetic yield of biomass, can be calculated from acetate, lactate and ethanol concentrations according to equations described previously (Payot *et al.*, 1998).

$$Y_{ATP} = \text{biomass concn} / (2\text{concn}_{\text{acetate}} + \text{concn}_{\text{lactate}} + \text{concn}_{\text{ethanol}})$$

Y_{ATP} is in g cells (mol ATP)⁻¹ produced.

$Y_{X/S}$, the molar growth yield for cellobiose, is in g cells (mol cellobiose)⁻¹ used.

Carbon recoveries were calculated from the production of biomass, fermentation products, amino acids and extracellular proteins present in the supernatant.

RESULTS

Kinetic analysis of batch cellobiose fermentation

When *C. cellulolyticum* was grown in batch culture on a defined medium with 23.4 mM cellobiose as an energy source, the growth curve (Fig. 1a) was characterized by a maximum specific growth rate of 0.18 h⁻¹ (Fig. 2a). During the first 10 h after inoculation, growth and cellobiose consumption rates accelerated (Fig. 2a) and the cells produced acetate, ethanol and pyruvate (Fig. 1b). Accelerating growth was correlated with increasing

Fig. 1. Growth and cellobiose concentration (a) and product concentrations (b) during batch fermentation of *C. cellulolyticum*. The cellobiose concentration was 23.4 mM; ammonium concentration (26.5 mM) was always in excess. ○, Biomass; ●, cellobiose; □, acetate; △, lactate; ■, ethanol; ▲, extracellular pyruvate.

Fig. 2. Specific rates of growth and cellobiose consumption (a) and product formation (b, c) during batch fermentation of *C. cellulolyticum*. The cellobiose concentration was 23.4 mM; ammonium concentration was always in excess. ○, Specific growth rate; ●, specific rate of cellobiose consumption; ▲, □, △, ■, specific rates of extracellular pyruvate, acetate, lactate and ethanol formation, respectively.

production of extracellular pyruvate (Fig. 2b), acetate and ethanol (Fig. 2c) and was followed abruptly by a decelerating growth phase (Fig. 2a). The start of lactate formation (Fig. 2c) was associated with the peak of extracellular pyruvate production and the increase in the specific rate of lactate production corresponded to the decrease in pyruvate, acetate and ethanol biosynthesis (Fig. 2b,c). Between 25 and 43 h, the q_{lactate} was stable and coincided with an almost nil pyruvate production (Fig. 2b,c). Growth ceased before the cellobiose was depleted (Fig. 1a), but cells continued to catabolize cellobiose and produce acetate and lactate.

Table 1. Substrate, biomass and product concentrations and yields for continuous cultures of *C. cellulolyticum*

During all experiments, ammonium concentration was always in excess. For experiments A–H₁, cultures were provided with cellobiose in increasing amounts from 5.84 to 20.46 mM (see Fig. 3 legend). In the experiments S, the reactor was fed directly with 14.62 mM cellobiose. Values represent mean determinations from three different samples ($\pm$ SD). All other values were determined with a mean accuracy of 10%.

Experiments	D (h ⁻¹)	Feed cellobiose (mM)	Residual cellobiose (mM)	Biomass (g l ⁻¹)	Product yield [mol (mol cellobiose) ⁻¹]			Extracellular pyruvate (μ M)	Extracellular protein (mg l ⁻¹)	Amino acids (mM)	Carbon recovery (%)
					Acetate	Lactate	Ethanol				
A	0.086	5.84	0.12 $\pm$ 0.05	0.332 $\pm$ 0.015	1.68	0.10	0.53	149.2 $\pm$ 8.2	28.9 $\pm$ 1.1	0.56 $\pm$ 0.04	84.3
B	0.083	7.31	0.23 $\pm$ 0.08	0.388 $\pm$ 0.016	1.61	0.09	0.54	172.9 $\pm$ 9.3	44.8 $\pm$ 2.5	0.69 $\pm$ 0.04	79.5
C	0.085	8.77	0.17 $\pm$ 0.07	0.475 $\pm$ 0.020	1.69	0.09	0.53	208.5 $\pm$ 9.9	57.2 $\pm$ 2.7	0.81 $\pm$ 0.05	83.4
D	0.083	10.23	0.12 $\pm$ 0.06	0.581 $\pm$ 0.024	1.78	0.11	0.56	267.4 $\pm$ 12.8	62.5 $\pm$ 2.8	0.83 $\pm$ 0.05	86.8
E	0.086	12.42	0.21 $\pm$ 0.07	0.724 $\pm$ 0.031	1.73	0.14	0.54	327.5 $\pm$ 14.7	84.0 $\pm$ 4.0	0.98 $\pm$ 0.06	85.9
F	0.084	14.62	0.26 $\pm$ 0.07	0.789 $\pm$ 0.030	1.60	0.19	0.56	372.9 $\pm$ 15.5	96.2 $\pm$ 4.1	1.27 $\pm$ 0.07	83.8
G	0.084	17.57	0.21 $\pm$ 0.08	0.863 $\pm$ 0.035	1.54	0.23	0.59	414.6 $\pm$ 18.2	132.9 $\pm$ 5.7	1.68 $\pm$ 1.01	81.4
H ₁ *	0.085	20.46	0.88 $\pm$ 0.12	0.860 $\pm$ 0.032	1.25	0.46	0.75	443.1 $\pm$ 18.9	142.6 $\pm$ 5.6	1.25 $\pm$ 0.08	82.0
H ₂ *	0.085	20.46	1.69 $\pm$ 0.14	0.741 $\pm$ 0.029	1.34	0.42	0.89	411.6 $\pm$ 17.6	166.6 $\pm$ 6.1	1.15 $\pm$ 0.06	85.5
H ₃ *	0.085	20.46	2.77 $\pm$ 0.18	0.621 $\pm$ 0.028	1.27	0.34	0.93	395.7 $\pm$ 16.4	167.4 $\pm$ 6.3	0.85 $\pm$ 0.05	80.0
H ₄	0.085	20.46	2.04 $\pm$ 0.15	0.733 $\pm$ 0.032	1.22	0.34	0.97	386.4 $\pm$ 16.3	177.1 $\pm$ 6.8	0.94 $\pm$ 0.06	80.6
S	0.083	14.62	4.24 $\pm$ 0.24	0.487 $\pm$ 0.200	1.22	0.37	0.83	267.8 $\pm$ 12.3	115.9 $\pm$ 5.1	1.11 $\pm$ 0.06	83.8

* Values of parameters are shown but were not obtained under steady-state conditions.

When the cellobiose was exhausted, cell lysis was observed despite a slight consumption of pyruvate. The stationary phase occurred before cellobiose became limiting and the growth arrest could not be attributed to limitation or inhibition by fermentation products since the same culture reinoculated allowed growth of the new inoculum (data not shown). This result indicates that growth inhibition of *C. cellulolyticum* was not due to the appearance of inhibitory compounds in the culture medium. This was confirmed by the growth of *C. cellulolyticum* in a supernatant from a culture entering stationary phase which was centrifuged and filtered or autoclaved (data not shown).

Growth of *C. cellulolyticum* with increasing amounts of cellobiose

When *C. cellulolyticum* was grown in continuous culture at a specific growth rate (μ) of approximately 0.085 h⁻¹ and the concentration of cellobiose in the feed medium was increased in stepwise fashion, there was an increase in cellobiose consumption (Table 1) without growth inhibition (Fig. 3). Cultures which were provided with cellobiose progressively from 5.84 to 17.57 mM (experiments A–G, Table 1) utilized almost all the carbon source (< 0.26 mM left) and the microbial population increased regularly whilst the values of Y_{ATP} were almost constant (Table 2). Nevertheless, when the feed cellobiose concentration was 17.57 mM, the growth parameters Y_{ATP} and $Y_{X/S}$ decreased, i.e. from 13.9 to 12.7 g cells (mol ATP)⁻¹ and from 54.9 to 49.7 g cells (mol cellobiose)⁻¹ for experiments F and G, respectively, indicating that some cellobiose and hence ATP were not associated with biomass formation. The same effects were accentuated when the cellobiose concentration was increased to 20.46 mM (experiments H₁–H₄). The residual cellobiose concentration increased from 0.88 to 2.77 mM and the biomass decreased from 0.860 to

Fig. 3. Growth of *C. cellulolyticum* in continuous culture ($D = 0.085 \pm 0.002$ h⁻¹) with increasing amounts of cellobiose from 5.84 mM (experiment A) to 20.46 mM (experiment H). The culture was sampled at the times indicated by arrows, before the addition of cellobiose concentration. Feed cellobiose (mM): A, 5.84; B, 7.31; C, 8.77; D, 10.23; E, 12.42; F, 14.62; G, 17.57; H₁–H₂–H₃–H₄, 20.46. Data from these experiments are summarized in Tables 1, 2 and 3.

0.621 g l⁻¹ (steps H₁, H₂, H₃); the steady state was recovered only in step H₄, and $Y_{X/S}$ (39.8 g cells mol⁻¹) and Y_{ATP} (10.7 g cells mol⁻¹) decreased sharply. These results indicate that (i) experiments A–G were performed under conditions of cellobiose limitation, (ii) increased carbon flow from 1.46 to 1.69 mmol cellobiose (g cells)⁻¹ h⁻¹ was balanced by the demands for biosynthesis, (iii) the increase of $q_{\text{cellobiose}}$ above 1.69 mmol (g cells)⁻¹ h⁻¹ led to substrate-sufficient cultures with imbalance between cellobiose uptake and the demands for biosynthesis.

When the reactor was fed directly with 14.62 mM cellobiose in experiment S, compared with experiment G (17.57 mM cellobiose), the residual cellobiose con-

Table 2. Specific consumption and production rates for the continuous cultures of Table 1

For experiments A–H₁, cultures were provided with cellobiose in increasing amounts from 5.84 to 20.46 mM (see Fig. 3 legend). In the experiments S, the reactor was fed directly with 14.62 mM cellobiose. Values represent mean determinations from three different samples ($\pm$ SD). All other values were determined with a mean accuracy of 10%.

Experiments	D (h ⁻¹)	Specific rate of cellobiose consumption or product formation [mmol (g cells) ⁻¹ h ⁻¹]				Extracellular q_{pyruvate} [μ mol (g cells) ⁻¹ h ⁻¹]	Y _{ATP} [(g cells) mol ⁻¹]	Y _{X/X} [(g cells) mol ⁻¹]
		$q_{\text{cellobiose}}$	q_{acetate}	q_{lactate}	q_{ethanol}			
A	0.086	1.46	2.49	0.14	0.78	38.6	14.5	58.0
B	0.083	1.51	2.44	0.13	0.82	37.0	14.2	54.8
C	0.085	1.54	2.61	0.14	0.82	37.3	13.7	55.2
D	0.083	1.44	2.57	0.15	0.81	38.2	13.6	57.5
E	0.086	1.45	2.51	0.21	0.79	38.9	14.3	59.3
F	0.084	1.53	2.45	0.26	0.86	39.7	13.9	54.9
G	0.084	1.69	2.60	0.35	1.00	40.3	12.7	49.7
H ₁ *	0.085	1.94	2.41	0.90	1.45	43.6	11.8	43.9
H ₂ *	0.085	2.15	2.88	0.91	1.91	47.2	10.1	39.5
H ₃ *	0.085	2.42	3.09	0.83	2.24	54.1	8.9	35.1
H ₄	0.085	2.13	2.62	0.73	1.99	44.8	10.7	39.8
S	0.083	1.77	2.15	0.65	1.46	45.6	12.9	46.9

* Values of parameters are shown but were not obtained under steady-state conditions.

Table 3. Oxidoreduction balance, nucleotide levels and electron flow for the continuous cultures of Table 1

For experiments A–H₁, cultures were provided with cellobiose in increasing amounts from 5.84 to 20.46 mM (see Fig. 3 legend). In the experiments S, the reactor was fed directly with 14.62 mM cellobiose. Values represent mean determinations from three different samples ($\pm$ SD). All other values were determined with a mean accuracy of 10%.

Experiments	D (h ⁻¹)	H ₂ [mol (mol cellobiose) ⁻¹]	CO ₂ [mol (mol cellobiose) ⁻¹]	H ₂ /CO ₂	O/R index	Intracellular nucleotide [μ mol (g cells) ⁻¹]				q_{hydrogen} [mmol (g cells) ⁻¹ h ⁻¹]	q_{NADH} produced / q_{NADH} used
						NADH	NADPH	NAD ⁺	NADP ⁺		
A	0.086	3.54	2.34	1.51	1.02	5.3 $\pm$ 0.9	3.2 $\pm$ 0.5	17.1 $\pm$ 3.7	ND	1.70	1.99
B	0.083	3.04	2.00	1.52	0.97	5.9 $\pm$ 0.8	3.8 $\pm$ 0.4	16.3 $\pm$ 3.5	ND	1.62	1.91
C	0.085	3.54	2.30	1.54	1.00	6.4 $\pm$ 1.2	3.6 $\pm$ 0.5	17.3 $\pm$ 3.6	ND	1.79	2.01
D	0.083	3.72	2.43	1.53	1.00	7.0 $\pm$ 1.2	3.6 $\pm$ 0.4	17.2 $\pm$ 3.4	0.06 $\pm$ 0.01	1.77	2.00
E	0.086	3.41	2.20	1.55	0.98	6.2 $\pm$ 1.1	3.4 $\pm$ 0.4	15.5 $\pm$ 3.2	0.06 $\pm$ 0.01	1.72	1.96
F	0.084	3.43	2.23	1.54	0.98	6.9 $\pm$ 1.5	3.8 $\pm$ 0.5	17.7 $\pm$ 3.5	0.04 $\pm$ 0.01	1.59	1.81
G	0.084	3.14	2.08	1.51	0.96	6.9 $\pm$ 1.3	3.7 $\pm$ 0.4	16.1 $\pm$ 3.1	0.06 $\pm$ 0.01	1.59	1.68
H ₁ *	0.085	2.86	2.06	1.39	0.94	7.3 $\pm$ 1.8	3.3 $\pm$ 0.4	13.6 $\pm$ 2.9	0.08 $\pm$ 0.01	0.96	1.25
H ₂ *	0.085	2.90	2.28	1.27	0.98	8.8 $\pm$ 1.7	3.5 $\pm$ 0.5	12.5 $\pm$ 2.6	0.06 $\pm$ 0.01	0.96	1.21
H ₃ *	0.085	2.52	2.14	1.18	0.98	8.9 $\pm$ 1.9	3.3 $\pm$ 0.4	16.3 $\pm$ 3.2	0.07 $\pm$ 0.01	0.85	1.16
H ₄	0.085	2.33	2.09	1.11	1.00	7.3 $\pm$ 1.7	3.6 $\pm$ 0.5	12.2 $\pm$ 2.6	0.06 $\pm$ 0.01	0.63	1.13
S	0.083	2.43	1.98	1.23	0.97	7.2 $\pm$ 1.2	3.4 $\pm$ 0.5	10.6 $\pm$ 2.9	ND	0.69	1.19

ND, Not detectable.

* Values of parameters are shown but were not obtained under steady-state conditions.

centration increased and the biomass decreased (38.3%). This comparison indicated that the cells were not able to regulate high carbon flow and that the growth inhibition observed in experiment S was not due to end-product toxicity since end-product concentrations were higher in experiment F (Table 1). *C. cellulolyticum* may have been unable to regulate the cellobiose uptake and growth inhibition may have been due to an inability of bacteria to deal with high concentrations of carbon compounds internally.

Extracellular pyruvate was observed in all experiments (Table 1), suggesting that the flow through the pyruvate:fd oxidoreductase was limiting. An increase in pyruvate concentration correlated with accumulation of extracellular protein and biomass. The global carbon

balance, calculated by taking into account cellobiose consumption converted into biomass, products, amino acids and peptides, was in the range 79.5–86.8% (Table 1). The specific rates of acetate, lactate, pyruvate and ethanol formation were almost stable during experiments A–G (Table 2), except that q_{lactate} and q_{ethanol} increased in experiments E–G and F–G, respectively. Their specific production rate increased sharply when cellobiose was present in excess (experiments H₄ and S), whereas q_{acetate} and q_{pyruvate} remained nearly constant.

Redox balance

For the chemostats carried out under carbon limitation, the coenzyme balance (Table 3) calculated from the known catabolic pathways producing or consuming

Fig. 4. Growth of *C. cellulolyticum* in dialysis tubing.

reducing equivalents demonstrate an excess of NADH since the ratio $q_{\text{NADH produced}}/q_{\text{NADH used}}$ was always greater than 1 (experiments A–G, Table 3). This result correlated with ratios of H_2/CO_2 greater than 1 obtained by measuring CO_2 and H_2 evolved. These ratios suggest that H_2 was produced via NADH:fd reductase and hydrogenase activities in addition to the phosphoroclastic reaction, which produces 1 mol CO_2 and 1 mol H_2 (mol pyruvate) $^{-1}$ catabolized (Mortenson *et al.*, 1963). When chemostat cultures were incubated with excess cellobiose, the main effect was an increase in the specific rates of lactate and ethanol production: the q_{lactate} and q_{ethanol} increased by a factor of 2 and the H_2/CO_2 ratios decreased from 1.5 to 1.1–1.2 (experiments H₄ and S).

The low values of q_{lactate} and q_{ethanol} under conditions of cellobiose limitation (experiments A–G) apparently were not sufficient to regenerate the NAD^+ from NADH, so the production of H_2 via NADH:fd- H_2 with $q_{\text{NADH fd}}$ from 1.59 to 1.79 mmol (g cells) $^{-1}$ h $^{-1}$ (Table 3) was more significant in these culture conditions than under cellobiose excess (experiments H₁–H₄ and S).

Taking into account the production of hydrogen gas, ethanol and lactate, the O/R index was determined to be around 1. The nucleotide levels did not vary significantly among cellobiose-sufficient or excess cultures, NADP^+ was not or almost not detectable, whereas the NADPH levels were remarkably constant (Table 3). NADH ranged from 5.3 to 7.3 μmol (g cells) $^{-1}$ and NAD^+ from 10.6 to 17.7 μmol (g cells) $^{-1}$, indicating an efficient regeneration of NAD^+ from NADH.

Dialysis culture

C. cellulolyticum was grown in dialysis culture on mineral medium with 23.4 mM cellobiose as described by Gehin *et al.* (1996) (Fig. 4). A linear growth curve was observed with a maximum generation time of 4 h, which correlated with the value found in batch culture (3.8 h). It is likely that cellobiose diffusion limited growth since

this compound was not detected inside the dialysis tubing. This may have led to a decrease in the carbon flow, and enabled an increase of the biomass formation by a factor of 3 to occur.

DISCUSSION

Previous work indicated that growth of *C. cellulolyticum* in chemostat cultures on complex medium was limited by an imbalance in the specific rate of NADH production and the specific rate of NADH consumption, leading to NADH/NAD^+ ratios as high as 57 and intracellular NADH concentrations ranging from 3 to 40 μmol (g cell mass) $^{-1}$ (Payot *et al.*, 1998). It was concluded that high intracellular concentrations of NADH detected in complex medium revealed a low rate of NADH reoxidation (Holms, 1996) and that the production of lactate, ethanol and hydrogen via NADH:fd- H_2 was rate limiting. Due to a low rate of NADH reoxidation, the cells probably maintain a sufficient NAD^+ pool size available for cellobiose catabolism.

This study shows that changing the growth condition from complex medium to synthetic medium had a strong effect on the electron flow since the NADH produced by glycolysis was well balanced by the production of hydrogen gas, ethanol and lactate, leading to NADH/NAD^+ ratios in the range of 0.3–0.7. This interpretation was supported by the results of our study on the effect of carbon flow in *C. cellulolyticum* grown in chemostat culture on synthetic medium. At low D values high H_2/CO_2 and NADH/NAD^+ ratios were observed, coinciding with low q_{lactate} and q_{ethanol} values and low *in vitro* lactate and ethanol dehydrogenase activities. In contrast, at high D values the H_2/CO_2 and NADH/NAD^+ ratios decreased sharply, whereas the q_{lactate} and q_{ethanol} values and *in vitro* lactate and ethanol dehydrogenase activities increased (data not shown).

Compared with growth on complex medium, the specific production rates of ethanol and lactate were both increased about 10-fold during growth on synthetic medium. Clearly, these data indicate a better control of the electron flow by *C. cellulolyticum*, i.e. low NADH/NAD^+ ratios with lactate and ethanol production complementing hydrogen via NADH:fd- H_2 .

In addition to a better regulation of the redox balance in cells grown in synthetic medium explaining higher specific cellobiose consumption in this medium, it is necessary to take into consideration the fact that, on complex medium, yeast extract supplies many cell constituents and cellobiose serves mainly as an energy supply, whereas on mineral salts medium, a mean value of 60% of the cellobiose consumed was converted into end products of fermentation and the rest into biomass and extracellular proteins and amino acids.

Nevertheless, the rate of cellobiose catabolism apparently exceeded the rate of pyruvate consumption, since pyruvate accumulated. This suggests that, in *C. cellulolyticum*, pyruvate consumption via pyruvate fd-oxido-

reductase is a rate-limiting step. When carbon flow was limited either by cellobiose diffusion through the dialysis tubing or by continuous culture under cellobiose limitation, no growth inhibition was observed. It may thus be concluded that growth inhibition was directly related to an inefficiently regulated carbon flow. This interpretation was supported by the results of studies of growth in cellobiose batch cultures, in which growth arrest was not due to nutritional limitation or inhibition by fermentation products since new cells reinoculated in the same culture were able to grow. The growth arrest of the first seeding could be explained by a carbon flow which leads to an accumulation of intracellular inhibitory compounds. Due to early inhibition of metabolism, the accelerating growth phase was rapidly followed by the decelerating growth phase and a $\mu_{\max}$ of 0.18 h^{-1} could not be maintained (Fig. 2). In view of these considerations, it can be argued that *C. cellulolyticum* is not adapted to use carbon source and other nutrients in excess since, on complex medium, cells were not able to induce high specific rates of ethanol and lactate production. Indeed, natural ecosystems where cellulolytic microbes proliferate rarely contain all nutrients in saturating quantities, particularly a carbon source derived from cellulose. This insoluble substrate is usually coated by other polymers, predominantly xylan and lignin, which may hinder cellulolysis and hence reduce carbon availability.

It has been found that many bacteria isolated from oligotrophic reservoirs acquire the ability spontaneously or by adaptation to develop on rich media (Kuznetsov *et al.*, 1979). However, this is not the case with *C. cellulolyticum* or with cellulolytic bacteria as a rule (Sleat *et al.*, 1984; Cailliez *et al.*, 1992; Tomme *et al.*, 1995). It is reasonable to suppose that during the course of *C. cellulolyticum* evolution, these bacteria have evolved to optimize catabolism of poorly available carbon sources. The results of this study suggest that *C. cellulolyticum* is adapted to life at low nutrient concentrations.

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