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 \( 1^{-1} \) is replaced by oligoelement and vitamin solutions. The composition was (g \( 1^{-1} \]): \( \text{KH}_{2}\text{PO}_{4}, 1.40; \text{K}_{2}\text{HPO}_{4}, 3.90; \text{Na}_{2}\text{SO}_{4}, 1.00; \text{MgCl}_{2} \cdot 6\text{H}_{2}\text{O}, 0.10; \text{CaCl}_{2}, 0.02; 5 \% (w/v) \text{FeSO}_{4} \text{ in } 50 \text{mM } \text{H}_{2}\text{SO}_{4}, 25 \mu\text{l} \); trace element solution, 10 ml; vitamin solution, 10 ml; \( \text{Na}_{2}\text{S}, 0.50 \text{ and } 0.2 \% (w/v) \text{resazurin}, 0.5 \text{ ml} \). In addition, the medium contained cellobiose in variable amounts as specified in Results. The trace element solution contained (g \( 1^{-1} \): \( \text{FeSO}_{4} \cdot 7\text{H}_{2}\text{O}, 5.00; \text{ZnSO}_{4} \cdot 7\text{H}_{2}\text{O}, 1.44; \text{MnSO}_{4} \cdot 7\text{H}_{2}\text{O}, 1.12; \text{CuSO}_{4} \cdot 5\text{H}_{2}\text{O}, 0.25; \text{Na}_{2}\text{MoO}_{4}, 0.20; (\text{MoO}_{4})_{2} \cdot \text{NH}_{4} \cdot \text{O}_{2}, 4.94\text{H}_{2}\text{O}, 1.00; \text{NiCl}_{2}, 0.04; \text{CoCl}_{2} \cdot 6\text{H}_{2}\text{O}, 0.02; \text{HBO}_{3}, 0.03; \text{Na}_{2}\text{SeO}_{3}, 0.02; \text{HCL} 10 \text{M}, 500 \text{ ml} \). The composition of the vitamin solution was (g \( 1^{-1} \): \( \text{D-biotin}, 10; \text{p-aminobenzoic acid}, 25; \text{nicotinic acid}, 15; \text{riboflavin}, 25; \text{pantothenic acid}, 25; \text{thiamin}, 25; \text{cyancobalamin}, 10 \text{. The vitamin solution was sterilized by filtration with a } 0.2 \mu\text{m 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 entry. The temperature was maintained at 34 °C and the pH was controlled at 7.2 by automatic additions of 1 M \( \text{NaOH} \). Agitation was kept constant at 50 r.p.m. For continuous fermentations, the volume was kept constant at 1.5 l.

**Calculations.** The main products of cellobiose fermentation by \( C. \ cellulolyticum \) are acetate, ethanol, lactate, \( \text{H}_{2} \) and \( \text{CO}_{2} \) (Gallo et al., 1983). The reactions leading to the formation of the metabolites were described previously (Payot et al., 1998).

\[
q_{\text{cellobiose}} = \frac{q_{\text{extracellular pyruvate}} \cdot q_{\text{ethanol}} + q_{\text{extracellular pyruvate}}}{q_{\text{extracellular pyruvate}} + q_{\text{amination}} + q_{\text{amination}}}
\]

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

The specific rates of \( \text{H}_{2} \) production via the NADH-ferredoxin (fd)-\( \text{H}_{2} \) 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 Gotschalk (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).

\[ Y_{ATP} = \frac{\text{biomass concn}}{2\times \text{concen}_{\text{acetate}} + \text{concen}_{\text{lactate}} + \text{concen}_{\text{ethanol}}} \]

\( Y_{ATP} \) is in g cells (mol ATP)^{-1} produced.

\( Y_{\text{Xcel}} \), 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 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 \( q_{\text{lactate}} \) 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–H, 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 (±sd). All other values were determined with a mean accuracy of 10%.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>D (h)</th>
<th>Feed cellobiose (mM)</th>
<th>Residual cellobiose (mM)</th>
<th>Biomass (g L⁻¹)</th>
<th>Product yield (mol mol cellobiose⁻¹)</th>
<th>Extracellular pyruvate (µM)</th>
<th>Extracellular protein (mg L⁻¹)</th>
<th>Amino acid recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.086</td>
<td>5.84</td>
<td>0.12 ± 0.01</td>
<td>0.33 ± 0.03</td>
<td>14.62 ± 2.1</td>
<td>28.9 ± 1.1</td>
<td>0.58 ± 0.04</td>
<td>94.1</td>
</tr>
<tr>
<td>B</td>
<td>0.086</td>
<td>7.31</td>
<td>0.23 ± 0.08</td>
<td>0.38 ± 0.06</td>
<td>16.1 ± 0.09</td>
<td>37.2 ± 2.4</td>
<td>0.68 ± 0.05</td>
<td>91.5</td>
</tr>
<tr>
<td>C</td>
<td>0.086</td>
<td>14.62</td>
<td>0.22 ± 0.06</td>
<td>0.30 ± 0.04</td>
<td>14.0 ± 0.14</td>
<td>37.2 ± 2.4</td>
<td>0.81 ± 0.05</td>
<td>86.8</td>
</tr>
<tr>
<td>D</td>
<td>0.086</td>
<td>17.57</td>
<td>0.21 ± 0.06</td>
<td>0.28 ± 0.03</td>
<td>15.3 ± 0.14</td>
<td>37.2 ± 2.4</td>
<td>0.85 ± 0.05</td>
<td>86.8</td>
</tr>
<tr>
<td>E</td>
<td>0.086</td>
<td>20.46</td>
<td>0.21 ± 0.06</td>
<td>0.28 ± 0.03</td>
<td>16.0 ± 0.14</td>
<td>37.2 ± 2.4</td>
<td>0.90 ± 0.05</td>
<td>85.9</td>
</tr>
<tr>
<td>F</td>
<td>0.086</td>
<td>14.62</td>
<td>0.26 ± 0.06</td>
<td>0.30 ± 0.03</td>
<td>14.0 ± 0.14</td>
<td>37.2 ± 2.4</td>
<td>0.80 ± 0.05</td>
<td>85.5</td>
</tr>
<tr>
<td>G</td>
<td>0.086</td>
<td>17.57</td>
<td>0.26 ± 0.06</td>
<td>0.28 ± 0.03</td>
<td>15.3 ± 0.14</td>
<td>37.2 ± 2.4</td>
<td>0.85 ± 0.05</td>
<td>85.6</td>
</tr>
<tr>
<td>H</td>
<td>0.086</td>
<td>20.46</td>
<td>0.26 ± 0.06</td>
<td>0.28 ± 0.03</td>
<td>16.0 ± 0.14</td>
<td>37.2 ± 2.4</td>
<td>0.90 ± 0.05</td>
<td>85.5</td>
</tr>
<tr>
<td>S</td>
<td>0.086</td>
<td>14.62</td>
<td>0.24 ± 0.06</td>
<td>0.28 ± 0.03</td>
<td>12.7 ± 0.14</td>
<td>37.2 ± 2.4</td>
<td>0.85 ± 0.05</td>
<td>85.5</td>
</tr>
</tbody>
</table>

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 reincultured 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_b 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–H₃). The residual cellobiose concentration increased from 0.88 to 2.77 mM and the biomass decreased from 0.860 to 0.085 k 0.002 g L⁻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, H₅, H₆, H₇, H₈, H₉, H₁₀, 20.46. Data from these experiments are summarized in Tables 1, 2 and 3.

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

Fig. 3. Growth of C. cellulolyticum in continuous culture (D = 0.085 ± 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, H₅, H₆, H₇, H₈, H₉, H₁₀, 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 H₁, and Y_ATP (39.8 g cells mol⁻¹) and Y_ATP (107 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 d_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-
For experiments A–H, 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 (± SD). All other values were determined with a mean accuracy of 10%.

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

For experiments A–H, 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 (± SD). All other values were determined with a mean accuracy of 10%.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>D (h⁻¹)</th>
<th>Specific rate of cellobiose consumption or product formation [μmol (g cells)⁻¹ h⁻¹]</th>
<th>Extracellular qₐacetate</th>
<th>qₐpyruvate</th>
<th>qₐetanol</th>
<th>qₐexposed</th>
<th>Yeₐ</th>
<th>Yeₐexposed</th>
<th>Yeₐetanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.086</td>
<td>1.46 × 2.49 0.14 0.78</td>
<td>38.6</td>
<td>14.5</td>
<td>58.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>0.083</td>
<td>1.51 × 2.44 0.13 0.92</td>
<td>37.0</td>
<td>14.2</td>
<td>54.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.085</td>
<td>1.54 × 2.61 0.14 0.92</td>
<td>37.1</td>
<td>15.7</td>
<td>55.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>0.083</td>
<td>1.44 × 2.37 0.15 0.81</td>
<td>38.2</td>
<td>15.6</td>
<td>57.5</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>0.086</td>
<td>1.45 × 2.51 0.21 0.79</td>
<td>38.9</td>
<td>14.3</td>
<td>59.1</td>
<td></td>
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</tr>
<tr>
<td>F</td>
<td>0.084</td>
<td>1.53 × 2.45 0.26 0.86</td>
<td>39.7</td>
<td>13.9</td>
<td>54.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.084</td>
<td>1.69 × 2.60 0.35 1.00</td>
<td>40.3</td>
<td>12.7</td>
<td>49.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H₁⁺</td>
<td>0.085</td>
<td>1.94 × 2.41 0.90 1.45</td>
<td>43.6</td>
<td>11.8</td>
<td>43.9</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>H₂⁺</td>
<td>0.085</td>
<td>2.15 × 2.88 0.91 1.91</td>
<td>47.2</td>
<td>10.1</td>
<td>39.5</td>
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<tr>
<td>H₃⁺</td>
<td>0.085</td>
<td>2.42 × 3.09 0.83 2.24</td>
<td>54.4</td>
<td>11.1</td>
<td>46.1</td>
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</tr>
<tr>
<td>H₄⁺</td>
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<td>2.73 × 2.62 0.73 1.99</td>
<td>44.8</td>
<td>10.7</td>
<td>39.8</td>
<td></td>
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</tbody>
</table>

* 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–H, 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 (± SD). All other values were determined with a mean accuracy of 10%.

<table>
<thead>
<tr>
<th>Experiments</th>
<th>D (h⁻¹)</th>
<th>H₂O (mol (mol cellobiose)⁻¹)</th>
<th>CO₂ (mol (mol cellobiose)⁻¹)</th>
<th>H₂/CO₂</th>
<th>O/R index</th>
<th>Intracellular nucleotide [μmol (g cells)⁻¹]</th>
<th>qₐexposed [μmol (g cells)⁻¹ h⁻¹]</th>
<th>Yeₐ</th>
<th>Yeₐexposed</th>
<th>Yeₐetanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.086</td>
<td>3.54</td>
<td>2.34</td>
<td>1.51</td>
<td>1.02</td>
<td>5.3 ± 0.9</td>
<td>3.2 ± 0.5</td>
<td>17.1 ± 3.7</td>
<td>ND</td>
<td>1.70 1.99</td>
</tr>
<tr>
<td>B</td>
<td>0.083</td>
<td>3.04</td>
<td>2.00</td>
<td>1.52</td>
<td>0.97</td>
<td>5.9 ± 0.8</td>
<td>3.8 ± 0.4</td>
<td>16.3 ± 3.5</td>
<td>ND</td>
<td>1.62 1.91</td>
</tr>
<tr>
<td>C</td>
<td>0.085</td>
<td>3.54</td>
<td>2.30</td>
<td>1.54</td>
<td>1.00</td>
<td>6.4 ± 1.2</td>
<td>3.6 ± 0.5</td>
<td>17.3 ± 3.6</td>
<td>ND</td>
<td>1.79 2.01</td>
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<td>2.43</td>
<td>1.53</td>
<td>1.00</td>
<td>7.0 ± 1.2</td>
<td>3.6 ± 0.4</td>
<td>17.2 ± 3.4</td>
<td>ND</td>
<td>1.77 2.00</td>
</tr>
<tr>
<td>E</td>
<td>0.086</td>
<td>3.41</td>
<td>2.20</td>
<td>1.55</td>
<td>0.98</td>
<td>6.2 ± 1.1</td>
<td>3.4 ± 0.4</td>
<td>15.5 ± 3.2</td>
<td>ND</td>
<td>1.72 1.96</td>
</tr>
<tr>
<td>F</td>
<td>0.084</td>
<td>3.45</td>
<td>2.23</td>
<td>1.54</td>
<td>0.98</td>
<td>6.9 ± 1.5</td>
<td>3.8 ± 0.5</td>
<td>17.7 ± 3.5</td>
<td>ND</td>
<td>1.99 1.81</td>
</tr>
<tr>
<td>G</td>
<td>0.084</td>
<td>3.14</td>
<td>2.06</td>
<td>1.51</td>
<td>0.96</td>
<td>6.9 ± 1.3</td>
<td>3.7 ± 0.4</td>
<td>16.1 ± 3.1</td>
<td>0.06 0.01</td>
<td>1.98 1.48</td>
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<tr>
<td>H₁⁺</td>
<td>0.085</td>
<td>2.86</td>
<td>2.06</td>
<td>1.59</td>
<td>0.94</td>
<td>7.1 ± 1.4</td>
<td>3.3 ± 0.4</td>
<td>15.6 ± 2.9</td>
<td>0.08 0.01</td>
<td>1.96 1.25</td>
</tr>
<tr>
<td>H₂⁺</td>
<td>0.085</td>
<td>2.90</td>
<td>2.28</td>
<td>1.27</td>
<td>0.98</td>
<td>8.8 ± 1.7</td>
<td>3.5 ± 0.5</td>
<td>12.5 ± 2.6</td>
<td>0.08 0.01</td>
<td>0.96 1.21</td>
</tr>
<tr>
<td>H₃⁺</td>
<td>0.085</td>
<td>2.52</td>
<td>2.14</td>
<td>1.18</td>
<td>0.98</td>
<td>9.8 ± 1.9</td>
<td>3.3 ± 0.4</td>
<td>16.3 ± 3.2</td>
<td>0.07 0.01</td>
<td>0.85 1.16</td>
</tr>
<tr>
<td>H₄⁺</td>
<td>0.085</td>
<td>2.33</td>
<td>2.09</td>
<td>1.11</td>
<td>1.00</td>
<td>7.1 ± 1.7</td>
<td>3.6 ± 0.5</td>
<td>12.2 ± 2.6</td>
<td>0.06 0.01</td>
<td>0.61 1.11</td>
</tr>
<tr>
<td>S</td>
<td>0.083</td>
<td>2.43</td>
<td>1.98</td>
<td>1.21</td>
<td>0.97</td>
<td>7.2 ± 1.2</td>
<td>3.4 ± 0.5</td>
<td>10.6 ± 2.9</td>
<td>ND</td>
<td>0.69 1.19</td>
</tr>
</tbody>
</table>

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:ferredoxin 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 qₐacetate and qₐetanol increased in experiments E–G and F–G, respectively. Their specific production rate increased sharply when cellobiose was present in excess (experiments H₁ and S), whereas qₐacetate and qₐpyruvate 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
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 \( \text{H}_2/\text{CO}_2 \) greater than 1 obtained by measuring \( \text{CO}_2 \) and \( \text{H}_2 \) evolved. These ratios suggest that \( \text{H}_2 \) was produced via NADH:fd reductase and hydrogenase activities in addition to the phosphoroclastic reaction, which produces 1 mol \( \text{CO}_2 \) and 1 mol \( \text{H}_2 \) (mol pyruvate)\(^{-1} \) catabolized (Mortenson et al., 1963). When chemostat cultures were incubated with excess cellubiose, the main effect was an increase in the specific rates of lactate and ethanol production: the \( q_{\text{lactate}} \) and \( q_{\text{ethanol}} \) increased by a factor of 2 and the \( \text{H}_2/\text{CO}_2 \) ratios decreased from 1:5 to 1:1–1:2 (experiments H1 and S).

The low values of \( q_{\text{lactate}} \) and \( q_{\text{ethanol}} \) under conditions of cellubiose limitation (experiments A–G) apparently were not sufficient to regenerate the NAD\(^+\) from NADH, so the production of \( \text{H}_2 \) via NADH:fd-H\(_2\) with \( q_{\text{XADH}} \) from 1:59 to 1:79 mmol (g cells)\(^{-1} \) h\(^{-1} \) (Table 3) was more significant in these culture conditions than under cellubiose 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 cellubiose-sufficient or excess cultures, NAD\(^+\) was not or almost not detectable, whereas the NADPH levels were remarkably constant (Table 3). NADH ranged from 5:3 to 7:3 pmol (g cells)\(^{-1} \) and NAD\(^+\) from 10:6 to 17:7 pmol (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 cellubiose 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 cellubiose 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 pmol (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 cellubiose 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 \( \text{H}_2/\text{CO}_2 \) and NADH/NAD\(^+\) ratios were observed, coinciding with low \( q_{\text{lactate}} \) and \( q_{\text{ethanol}} \) values and low *in vitro* lactate and ethanol dehydrogenase activities. In contrast, at high \( D \) values the \( \text{H}_2/\text{CO}_2 \) and NADH/NAD\(^+\) ratios decreased sharply, whereas the \( q_{\text{lactate}} \) and \( q_{\text{ethanol}} \) 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 cellubiose consumption in this medium, it is necessary to take into consideration the fact that, on complex medium, yeast extract supplies many cell constituents and cellubiose serves mainly as an energy supply, whereas on mineral salts medium, a mean value of 60% of the cellubiose consumed was converted into end products of fermentation and the rest into biomass and extracellular proteins and amino acids.

Nevertheless, the rate of cellubiose 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 \( \mu_{\text{max}} \) of 0.18 h\(^{-1}\) could not be maintained (Fig. 2). In view of these considerations, it can be argued that \textit{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 cellulyolytic 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 cellulyolysis 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 \textit{C. cellulolyticum} or with cellulyolytic 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 \textit{C. cellulolyticum} evolution, these bacteria have evolved to optimize carbolysis of poorly available carbon sources. The results of this study suggest that \textit{C. cellulolyticum} is adapted to life at low nutrient concentrations.

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