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Metabolic flux in cellulose batch and cellulose-fed continuous cultures of *Clostridium cellulolyticum* in response to acidic environment

Mickaël Desvaux, Emmanuel Guedon and Henri Petitdemange

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*Clostridium cellulolyticum*, a nonruminal cellulosolytic mesophilic bacterium, was grown in batch and continuous cultures on cellulose using a chemically defined medium. In batch culture with unregulated pH, less cellulose degradation and higher accumulation of soluble glucides were obtained compared to a culture with the pH controlled at 7.2. The gain in cellulose degradation achieved with pH control was offset by catabolite production rather than soluble sugar accumulation. The pH-controlled condition improved biomass, ethanol and acetate production, whereas maximum lactate and extracellular pyruvate concentrations were lower than in the non-pH-controlled condition. In a cellulose-fed chemostat at constant dilution rate and pH values ranging from 7 to 6, maximum cell density was obtained at pH 7.0. Environmental acidification chiefly influenced biomass formation, since at pH 6.4 the dry weight of cells was more than fourfold lower compared to that at pH 7.0, whereas the specific rate of cellulose assimilation decreased only from 11.7 to 10.1 milliequivalents of carbon (g cells)\(^{-1}\) h\(^{-1}\). The molar growth yield and the energetic growth yield did not decline as pH was lowered, and an abrupt transition to washout was observed. Decreasing the pH induced a shift from an acetate-ethanol fermentation to a lactate-ethanol fermentation. The acetate/ethanol ratio decreased as the pH declined, reaching close to 1 at pH 6.4. Whatever the pH conditions, lactate dehydrogenase was always greatly in excess. As pH decreased, both the biosynthesis and the catabolic efficiency of the pyruvate-ferredoxin oxidoreductase declined, as indicated by the ratio of the specific enzyme activity to the specific metabolic rate, which fell from 9.8 to 1.8. Thus a change of only 1 pH unit induced considerable metabolic change and ended by washout at around pH 6.2. *C. cellulolyticum* appeared to be similar to rumen cellulosolytic bacteria in its sensitivity to acidic conditions. Apparently, the cellulosolytic anaerobes studied thus far do not thrive when the pH drops below 6.0, suggesting that they evolved in environments where acid tolerance was not required for successful competition with other microbes.

**Keywords:** cellulosolytic bacteria, flux analysis, environmental pH, cellulose degradation, chemostat

**INTRODUCTION**

Biological degradation of cellulosic materials is signifi-

\begin{itemize}
  \item Abbreviations: AADH, acetaldehyde dehydrogenase; AK, acetate kinase; ADH, alcohol dehydrogenase; ATP-Eff, efficiency of ATP generation; Fd, ferredoxin; G1P, glucose 1-phosphate; G6P, glucose 6-phosphate; LDH, lactate dehydrogenase; meq C, milliequivalent of carbon; PFO, pyruvate-ferredoxin oxidoreductase; PTA, phosphotransacetylase; \( R \), ratio of specific enzyme activity to metabolic flux.
\end{itemize}

cant in swamps, marshes, sediments, composts and anaerobic waste treatment, and in the intestinal tracts of herbivorous animals and insects (Ljungdahl & Eriksson, 1985). Within the biosphere, cellulosolytic clostridia participate significantly in this process (Bayer & Lamed, 1992; Leschine, 1995; Tomme *et al.*, 1995), which is strongly linked to the global carbon cycle (Wolin & Miller, 1987).

High concentrations of fermentation acids and, as a
result, low pH conditions are often found in anaerobic habitats (Ljungdahl & Eriksson, 1985; Goodwin & Zeikus, 1987). Yet due to their particular pattern of intracellular pH regulation (Huang et al., 1985) compared with other low-G+C Gram-positive anaerobes, mainly the so-called lactic acid bacteria, the clostridial-type bacteria are generally considered as restricted to less acidic ecological niches (Russell et al., 1996).

Cellulolytic clostridia digest cellulose through extracellular multi-enzyme complexes (Bégum & Lemaire, 1996; Bayer et al., 1998). These cellulosomes are found at the surface of the bacteria and allow both cell adhesion to cellulose fibres (Bayer et al., 1996) and very efficient degradative activity against crystalline cellulose due to a high synergism of the different cellulase components (Boisset et al., 1999).

Clostridium cellulolyticum is a low-G+C Gram-positive nonruminal cellulolytic mesophilic bacterium belonging to the clostridial group III, and also classified in family 4, genus 2, of a new proposed hierarchical classification of metabolic fluxes (Guedon et al., 1993a, b). A few studies, however, have focused on the metabolism of this bacterium on cellulose: recent investigation of cellulose fermentation performed in batch culture (Desvaux et al., 2000) indicated (i) variation of metabolite yields as a function of initial cellulose concentration, and (ii) an early growth inhibition related to pyruvate overflow as with cellulose-degrading bacteria are generally considered as restricted to less acidic ecological niches (Russell & Dombrowski, 1980; Russell & Wilson, 1996; Russell & Diez-Gonzalez, 1998), little is known of how these conditions affect the metabolism of cellulolytic clostridia (Duong et al., 1983; Mitchell, 1998). The aim of the present work was to investigate the cellulose degradation and metabolic changes of C. cellulolyticum on insoluble cellulose caused by environmental pH conditions. Since conditions in natural environments most likely resemble those somewhere between a closed batch culture and an open continuous culture system (Kovárová-Kovar & Egli, 1998), both types of culture systems were used to study the effects of pH on growth and metabolism of C. cellulolyticum.

**METHODS**

**Organism and medium.** Clostridium cellulolyticum ATCC 35319 was isolated from decayed grass by Petitdemange et al. (1984). Germination of stocks of spores and anaerobic cell culture were performed as described by Desvaux et al. (2000). The defined medium used in all experiments was a modified CM3 medium as described by Guedon et al. (1999a) containing various amounts of cellulose MN301 (Macherey-Nagel) as specified in Results.

**Growth conditions.** Clostridium cellulolyticum was grown either in batch or in continuous culture with cellulose as sole carbon and energy source. All experiments were performed in a 1.5 l working volume fermenter (LSL Biotafitte) as previously described (Guedon et al., 1999a, b); the temperature was maintained at 34 °C and the pH was controlled by automatic addition of 3 M NaOH or 1 M HCl as specified in Results. The inoculum was 10% (v/v) from an exponentially growing culture.

Batch cultures were prepared as previously described (Desvaux et al., 2000). The chemostat system used was a segmented gas–liquid continuous culture device as described by Weimer et al. (1991b) with some modifications (Desvaux et al., 2001). The cultures were maintained for a period of eight to nine residence times (Desvaux et al., 2001); for each condition the data were the mean of at least three samples.

**Analytical procedures.** Biomass, cellulose concentration, gases, extracellular proteins, amino acids, glucose, soluble cellobiose, glycogen, acetate and lactate extracellularly produced were assayed as described by Desvaux et al. (2001). Pyridine nucleotides, coenzyme A (CoA), acetyl-CoA, GTP and G6P were extracted and fluorimetric determination performed as previously described (Desvaux et al., 2001). ATP and ADP were measured using the luciferin–luciferase luminescence system (Microbial Biomass Test Kit, Celsis Lumac) (Guedon et al., 2000a).

**Enzyme assays.** Cell extracts were prepared and enzyme assays performed as previously described (Guedon et al., 2000a). Pyruvate-ferredoxin (Fd) oxidoreductase (PFO) (EC 1.2.7.1), lactate dehydrogenase (LDH) (EC 1.1.1.27), phosphotransacetylase (PTA) (EC 2.3.1.8), acetate kinase (AK) (EC 2.7.2.1), acetaldehyde dehydrogenase (AADH) (EC 1.2.1.10) and alcohol dehydrogenase (ADH) (EC 1.1.1.1) were assayed as described by Desvaux et al. (2001).

**Calculations and carbon flux analysis.** The metabolic pathways and equations of cellulose fermentation by C. cellulolyticum, expressed as n hexose equivalents (hexose eq) corresponding to n glucose residues of the cellulose chain, were previously reported (Desvaux et al., 2000).

The $q_{cellulose}$ is the specific rate of hexose residue fermented in mmol (g cells)$^{-1}$ h$^{-1}$. $q_{product}$ and $q_{lactate}$ are the specific rates of product formation in mmol (g cells)$^{-1}$ h$^{-1}$. $q_{extracellular\ pyruvate}$ formation in mmol (g cells)$^{-1}$ h$^{-1}$ is the specific rate of extracellular pyruvate formation and $q_{NADH}$ consumed are the specific rates of NADH production and NADH consumption respectively in mmol (g cells)$^{-1}$ h$^{-1}$ and were calculated as follows: $q_{NADH}$ consumed = $q_{pyruvate}$ and $q_{NADH}$ produced = 2 $q_{ethanol} + q_{lactate}$. The specific rate of acid production (O’Sullivan & Condon, 1999), was calculated as follows: $q_{acid} = q_{product} - q_{NADH}$ consumed + $q_{extracellular\ pyruvate}$.

The molar growth yield ($Y_{m}$) is expressed in g cells (mol hexose eq fermented)$^{-1}$. The energetic yield of biomass ($Y_{ATP}$)
was (Desvaux et al., 2000): $Y_{\text{ATP}} = \text{conc}_{\text{biomass}}/(1.94 \text{ conc}_{\text{acetate}} + 0.94 \text{ conc}_{\text{ethanol}} + 0.94 \text{ conc}_{\text{extracellular pyruvate}})$. $Y_{\text{ATP}}$ is expressed in g cells (mole ATP produced)$^{-1}$. $q_{\text{ATP}}$ is the specific rate of ATP generation in mmol (g cells)$^{-1} \cdot h^{-1}$, calculated by the following equation (Desvaux et al., 2000): $q_{\text{ATP}} = 1.94 q_{\text{acetate}} + 0.94 q_{\text{ethanol}} + 0.94 q_{\text{extracellular pyruvate}}$. The energetic efficiency (ATP-Eff) corresponding to the ATP generation in cellulose catabolism is given by the ratio of $q_{\text{ATP}}$ to $q_{\text{cellulose}}$ (Miyagi et al., 1994).

Distribution of the carbon flow by stoichiometric flux analysis (Papoutsakis, 1984; Desai et al., 1999a, b) was determined by adapting the model developed by Holms (1996) to C. cellulolyticum metabolism (Desvaux et al., 2001).

At steady state, the carbon flux through each enzyme of the known metabolic pathway, as indicated in Fig. 1, was calculated in milliequivalents of carbon (meq C) (g cells)$^{-1} \cdot h^{-1}$, as follows:

- $q_{\text{G1P}} = 0.63 q_{\text{cellulose}}$
- $q_{\text{Glucose}} = 0.37 q_{\text{cellulose}}$
- $q_{\text{biosynthesis}} = q_{\text{biomass}} - q_{\text{glycogen}} + q_{\text{extracellular protein}}$
- $q_{\text{G6P}} = q_{\text{biosynthesis}} + q_{\text{pyruvate}}$
- $q_{\text{phosphoglucomutase}} = q_{\text{G6P}} - q_{\text{glucose}}$
- $q_{\text{extrapolysaccharide}} = q_{\text{G1P}} - q_{\text{phosphoglucomutase}} - q_{\text{glycogen}}$
- $q_{\text{Acetyl-CoA}} = q_{\text{acetate}} + q_{\text{ethanol}}$
- $q_{\text{carbon dioxide}} = (q_{\text{acetate}} + q_{\text{ethanol}})/2$
- $q_{\text{pyruvate}} = q_{\text{acetate}} + q_{\text{ethanol}} + q_{\text{lactate}} + q_{\text{extracellular pyruvate}} + q_{\text{carbon dioxide}}$

The turnover of a pool (h$^{-1}$) was calculated from specific rate and pool size expressed in moles or in carbon equivalents (Holms, 1996). The ratio $R$ corresponded to the ratio of specific enzyme activity to metabolic flux (Holms, 1996; Desvaux et al., 2001).

**RESULTS**

**Kinetic profile in batch cellulose fermentation**

C. cellulolyticum was grown in a bioreactor on defined medium with 6-7 g cellulose l$^{-1}$ under non-pH-controlled and pH-controlled conditions (Fig. 2). In the non-pH-controlled run, the pH dropped to 5-3 after 6 d culture (Fig. 2a). Compared to the batch culture controlled at pH 7-2, the final biomass attained in the non-pH-controlled condition was lower, as was the percentage of cellulose degradation, which reached 67%, as against 86% in the pH-controlled fermentation (Figs 2b and 2Ib). The maximum specific growth rate, however, was similar in non-pH-controlled and pH-controlled batch culture, i.e. 0.053 and 0.056 h$^{-1}$ respectively.

The metabolite production profiles were clearly different in the two fermentation modes (Fig. 2c and 2IIc). With pH regulation, higher final levels of acetate and ethanol were reached, whereas the lactate and extracellular...
pyruvate concentrations attained in the course of fermentation were higher in a non-pH-controlled run. Moreover, with the pH controlled at 7.2, pyruvate was reconsumed in the course of the fermentation more rapidly than without pH control. Thus once growth stopped, biocatabolic activity of cells ceased in the non-pH-controlled culture whereas in the pH-controlled condition the degraded cellulose was further metabolized by resting cells.

The kinetic profile for individual soluble glucide accumulation indicated that it was a non-growth-associated event (Figs 2Id and 2IId). A higher level of soluble cello-oligosaccharides was achieved in fermentation without pH control: up to 1-56 mM cellobiose was detected as well as 0-36 mM cellotriose. With pH control, the sugar level in the broth medium was very limited since only 0-16 mM cellobiose was found and no cellotriose could be detected. In both culture conditions, longer cello dex trins could not be detected either by HPLC or by TLC techniques. Inasmuch as cellulose was degraded to a greater extent in the pH-controlled culture, the difference in accumulation of sugars may reflect a difference in the cellulose substrate, e.g. its surface area (Weimer et al., 1990, 1991a; Fields & Russell, 2000), at the time cultures entered stationary phase, and/or differences in metabolism of stationary-phase cells.

**Effect of pH on the growth and metabolite production of C. cellulolyticum in cellulose continuous culture**

Growth parameters, notably end products measured at each steady state, as a function of the pH value are compiled in Table 1. *C. cellulolyticum* was grown in continuous culture under cellulose-limited conditions and at a constant dilution rate of 0.035 h⁻¹ with pH...
The cellulose input was 0.37% (w/v). Values are the means of samples at steady state and are shown ± standard deviation where appropriate. Where no standard deviation is given, individual values did not vary from the mean by more than 10%.

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**Table 1. Fermentation parameters from continuous steady-state cultures of C. cellulolyticum as a function of environmental pH at D = 0.053 h⁻¹**

The cellulose input was 0.37% (w/v). Values are the means of samples at steady state and are shown ± standard deviation where appropriate. Where no standard deviation is given, individual values did not vary from the mean by more than 10%.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results obtained at pH value of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.4</td>
</tr>
<tr>
<td>Biomass (g l⁻¹)</td>
<td>0.129±0.011</td>
</tr>
<tr>
<td>Cellulose consumed (mM hexose eq)</td>
<td>4.75±0.22</td>
</tr>
<tr>
<td>q&lt;sub&gt;cellulose&lt;/sub&gt; [mmol (g cells)⁻¹ h⁻¹]</td>
<td>1.95</td>
</tr>
<tr>
<td>q&lt;sub&gt;pyruvate&lt;/sub&gt; [mmol (g cells)⁻¹ h⁻¹]</td>
<td>2.76</td>
</tr>
<tr>
<td>Product yield (%)*</td>
<td></td>
</tr>
<tr>
<td>Acetate</td>
<td>70.3</td>
</tr>
<tr>
<td>Ethanol</td>
<td>29.3</td>
</tr>
<tr>
<td>Lactate</td>
<td>0.5</td>
</tr>
<tr>
<td>q&lt;sub&gt;extracellular pyruvate&lt;/sub&gt;</td>
<td>3.91</td>
</tr>
<tr>
<td>Glycogen [mg (g cells)⁻¹]</td>
<td>91.5±2.6</td>
</tr>
<tr>
<td>Extracellular proteins (mg l⁻¹)</td>
<td>11.3±0.7</td>
</tr>
<tr>
<td>Free amino acids (mg l⁻¹)</td>
<td>33.4±1.6</td>
</tr>
<tr>
<td>Carbon recovery (%)</td>
<td>94.7</td>
</tr>
</tbody>
</table>

* The product yields were expressed as percentages of q<sub>pyruvate</sub>.

---

**Fig. 3.** Dry weight of cells, percentage cellulose degradation, and molar and energetic growth yields during growth of C. cellulolyticum in continuous culture (D = 0.053 h⁻¹) under various pH conditions and with cellulose as sole carbon and energy source. ●, Biomass; ○, percentage cellulose degradation; □, Y<sub>X/S</sub>; ■, Y<sub>ATP</sub>.

values ranging from 7.4 to 6.4. The primary metabolic end products of the cellulose fermentation were acetate, ethanol, lactate, H₂ and CO₂. In addition to carbon conversion into biomass, amino acids and extracellular proteins were also detected in the supernatant (Table 1). Exopolysaccharides were readily observable by micro-

**Fig. 4.** Specific production rate (a) and product ratio (b) during growth of C. cellulolyticum in cellulose-fed continuous culture (D = 0.053 h⁻¹) under various pH conditions. □, q<sub>acetate</sub>; ▲, q<sub>ethanol</sub>; △, q<sub>lactate</sub>; ●, q<sub>H₂</sub>; ○, H₂/CO₂; ■, ethanol/acetate ratio.
The culture could not be established at pH 6; cells increased (Fig. 3); with a further pH decline, washout occurred. Whatever the pH conditions, *C. cellulolyticum* As the pH was lowered from 7 to 3 to 0, the dry weight of cells increased (Fig. 3). When the pH value was decreased from 7-4 to 7-0, the percentage of cellulose degradation increased from 20-6 to 34-2 % and then dropped to reach 7-3 % at pH 6-4. Whereas *C. cellulolyticum* showed depressed dry weight of cells at pH values higher than 7-0, the observed cell yields (Y<sub>X/S</sub>) did not decline, even at a pH value close to washout (Fig. 3); Y<sub>X/S</sub> increased from 27-1 to 31-3 g cells (mol ATP)<sup>-1</sup> between pH 7-4 and 6-4. The energetic yield of biomass (Y<sub>ATP</sub>) changed in the same way as Y<sub>X/S</sub>; such a result means that approximately the same amount of ATP was used for the process implicated in cell growth regardless of the pH value.

In all runs, acetate was the main fermentative end product, showing depressed X/S observed cell yields (Y<sub>X/S</sub>), due to the erroneous estimation of their concentration. Taking into account amino acids, proteins, fermentative end products and biomass concentration, the carbon balance ranged between 91-4 and 99-3 % (Table 1).

As the pH was lowered from 7-4 to 7-0, the dry weight of cells increased (Fig. 3); with a further pH decline, however, the cell density decreased and a steady state of the culture could not be established at pH 6-2 since washout occurred. Whatever the pH conditions, *C. cellulolyticum* always left some cellulose undigested.

### Table 2. Redox and energetic balance of *C. cellulolyticum* cells at steady state

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results obtained at pH value of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7-4</td>
</tr>
<tr>
<td>NADH [μmol (g cells)&lt;sup&gt;-1&lt;/sup&gt;]</td>
<td>475 ± 0-85</td>
</tr>
<tr>
<td>NAD&lt;sup&gt;+&lt;/sup&gt; [μmol (g cells)&lt;sup&gt;-1&lt;/sup&gt;]</td>
<td>11-31 ± 2-22</td>
</tr>
<tr>
<td>NADH/NAD&lt;sup&gt;+&lt;/sup&gt; ratio</td>
<td>0-42</td>
</tr>
<tr>
<td>q&lt;sub&gt;NADH produced&lt;/sub&gt; [mmol (g cells)&lt;sup&gt;-1&lt;/sup&gt; h&lt;sup&gt;-1&lt;/sup&gt;]</td>
<td>2-76</td>
</tr>
<tr>
<td>q&lt;sub&gt;NADH used&lt;/sub&gt; [mmol (g cells)&lt;sup&gt;-1&lt;/sup&gt; h&lt;sup&gt;-1&lt;/sup&gt;]</td>
<td>1-63</td>
</tr>
<tr>
<td>q&lt;sub&gt;NADH produced&lt;/sub&gt;/q&lt;sub&gt;NADH used&lt;/sub&gt; ratio</td>
<td>1-69</td>
</tr>
<tr>
<td>Pool ATP + ADP [μmol (g cells)&lt;sup&gt;-1&lt;/sup&gt;]</td>
<td>5-13</td>
</tr>
<tr>
<td>ATP/ADP ratio</td>
<td>0-50</td>
</tr>
<tr>
<td>q&lt;sub&gt;ATP&lt;/sub&gt; [mmol (g cells)&lt;sup&gt;-1&lt;/sup&gt; h&lt;sup&gt;-1&lt;/sup&gt;]</td>
<td>4-53</td>
</tr>
<tr>
<td>ATP-Eff&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2-32</td>
</tr>
</tbody>
</table>

* ATP-Eff is the ATP generation efficiency.

### Table 3. Estimation of carbon flow based on the steady-state values from a cellulose chemostat of *C. cellulolyticum* at D = 0-053 h<sup>-1</sup> as a function of environmental pH

Carbon flow was calculated as specified in Methods. See Fig. 1 for the metabolic pathways. The values are expressed both as meq C (g cells)<sup>-1</sup> h<sup>-1</sup> and, in parentheses, as a percentage of the specific rate of cellulose consumed (q<sub>cellulose</sub>).
product: it represented between 70-3 and 47-7% of the carbon directed towards catabolites (Table 1). With lowering of pH, the specific production rate of ethanol increased while that of acetate declined, the latter always remaining the higher (Fig. 4a). The ethanol-to-acetate ratio increased with decreasing pH and reached 0.94 at pH 6.4 (Fig. 4b). In contrast, the \( \text{H}_2/\text{CO}_2 \) ratio followed a downward trend with decreasing pH (Fig. 4b). Lactate was also produced at all pH values, but whereas the specific production rate \( (q_{\text{lactate}}) \) was low at pH 7.4, i.e. 0.01 mmol (g cells\(^{-1}\) h\(^{-1}\)), it increased as the pH decreased, reaching 0.16 mmol (g cells\(^{-1}\) h\(^{-1}\)) at pH 6.4 (Fig. 4a). As the pH was lowered, the specific rate of acid production \( (q_{\text{H}^+}) \), calculated from the spectrum of acid end products formed in cellulose chemostat culture grown at the different pH values, continuously decreased from 1.95 to 1.23 mmol (g cells\(^{-1}\) h\(^{-1}\)) (Fig. 4a).

**Redox and energetic balance in a cellulose-fed chemostat at various pH values**

The NADH balance calculated from the known metabolic pathways producing and consuming reducing equivalents, i.e. \( q_{\text{NADH \_prod}}/q_{\text{NADH \_used}} \), continuously decreased with pH from its value of 1.69 at pH 7.4 and was approximately 1 at pH 6.4 (Table 2). Whereas NAD\(^+\) molecules are reduced by glyceraldehyde 3-phosphate dehydrogenase during ethanol, lactate or acetate formation, only the ethanol and lactate production pathways allow the regeneration of the NAD\(^+\) pool via dehydrogenase activities. Despite the apparent imbalance at pH values higher than 6.4, the intracellular NADH/NAD\(^+\) ratio was always lower than 1 (Table 2). At pH values higher than 6.4, \( q_{\text{lactate}} \) and \( q_{\text{ethanol}} \) were then not sufficient to regenerate NADH to NAD\(^+\). This result correlated with the \( \text{H}_2/\text{CO}_2 \) ratios, which were always higher than 1 (Fig. 4b), suggesting that low lactate and ethanol production was balanced by \( \text{H}_2 \) gas formation via NADH-Fd reductase and hydrogenase activities.

The stoichiometry of ATP generated over hexose equivalents (ATP-Eff) decreased from 2.32 to 1.82 as the pH was lowered from 7.4 to 6.4; \( q_{\text{ATP}} \) also declined, from 4.53 to 3.08 mmol (g cells\(^{-1}\) h\(^{-1}\)) (Table 2). The ATP + ADP pool fluctuated between 4.79 and 6.11 mmol (g cells\(^{-1}\) h\(^{-1}\)), while the ATP/ADP ratio decreased from 0.50 to 0.34 with environmental acidification (Table 2). This variation was probably correlated with the change of the end products, namely the decrease of acetate biosynthesis.

**Metabolic flux analysis of cells grown on cellulose in an acid environment**

The metabolism of *C. cellulolyticum* when grown on cellulose is depicted in Fig. 1. With acidification of the growth medium from pH 7.4 to 6.4, the rate of cellulose consumption declined from 11.74 to 10.13 meq C (g cells\(^{-1}\) h\(^{-1}\)) (Table 3). The proportion of the cellulose converted into biomass, free amino acids and extracellular proteins, i.e. \( q_{\text{biosynthesis}} \), varied from 24.2 to 27.4% with this pH decline (Table 3); considering each, the biomass formation increased from 17.9 to 20.7% of the original carbon while cellulose conversion into amino acid and extracellular protein accounted for around 5.3% and 1.6% of the carbon uptake respectively. Another part of the carbon flow was directed towards metabolite fermentation, i.e. acetate, ethanol, CO\(_2\), extracellular pyruvate and lactate, which as a whole decreased from 70.7 to 65.2% of the cellulose consumed (Table 3). Metabolism distributed carbon differently over known catabolic routes as the pH declined. Carbon flow through the CO\(_2\) and acetate formation pathways declined from 23.4 to 19.8% and from 33.1 to 20.4% respectively. However, ethanol formation increased from 13.8 to 19.2% of the carbon consumed; lactate
formation also increased, from only 0.3% at pH 7.4 to 4.8% at pH 6.4. At the same time, the proportion of carbon flow towards extracellular pyruvate rose from 0.1% to 1% with lower environmental pH.

As the pH decreased, the G6P pool slightly increased (Fig. 5a); in terms of turn-over, this pool varied from 116(4) to 86.5 h−1 from pH 7.4 to 6.4. This result was correlated with the concomitant decrease of the carbon flow through glycolysis on the one hand and the increase of the carbon flow through the biosynthesis pathway on the other, as the environmental pH fell. The qG6P decreased from 11.15 to 9.38 meq C (g cells)−1 h−1 with acidification, which represented 94.9–92.6% of the original carbon metabolized (Table 3). As for the GIP pool, it increased with decreasing pH (Fig. 5a) which was expressed as a decrease of the turn-over of this pool from 91.3 to 42.7 h−1 from pH 7.4 to 6.4. As a result the G6P/GIP ratio ranged from 1.9 to 0.73 with decreasing environmental pH (Fig. 5b). GIP is an important node in cellulose metabolism since carbon could either flow down glycolysis via phosphoglucomutase or stored as glycogen, or be converted into exopolysaccharide. $q_{\text{phosphoglucomutase}}$, decreased from 6.80 to 5.63 meq C (g cells)−1 h−1 with lowering of pH; when expressed as a percentage of $q_{\text{cellulose}}$, this flux ranged from 57.9 to 55.5% (Table 3). The proportion of carbon through $q_{\text{glycogen}}$ varied from 1.2 to 1.9%, while $q_{\text{exopolysaccharide}}$ represented 3.5% of the carbon uptake at pH 7.4 to reach 6.2% at pH 6.4 (Table 3).

The CoA pool was fuelled by phosphotransacetylase and acetaldehyde dehydrogenase, whereas acetyl-CoA was formed via pyruvate-Fd oxidoreductase activity (Fig. 1). With lowering of the pH, both pools increased (Fig. 5c), however the acetyl-CoA/CoA ratio decreased from 4.46 to 3.06 (Fig. 5d). Such results were correlated with (i) the rerouting of carbon flow towards biosynthesis, which increased from 24.2 to 27.4% at the expense of $q_{\text{pyruvate}}$ which decreased from 70.7 to 65.2 meq C (g cells)−1 h−1 (Table 3) and (ii) the decrease of carbon flow through acetate production from 33.1 to 20.4% whereas it increased only from 13.8 to 19.2% through the ethanol pathway as pH declined (Table 3).

### Enzymic activities as a function of environmental pH

The influence of environmental pH on the specific activities of the enzymes studied is compiled in Table 4. In vitro, PFO, PTA, AK and AADH activities were higher under conditions giving higher in vivo specific production rates (Table 4). When the carbon flow was expressed as µmol (mg protein)−1 h−1 from previously calculated values (Table 3), R (the ratio of the specific enzyme activity to metabolic rate) could be calculated (Holms, 1996). In the metabolic branch leading to acetate production through PTA and AK, R fluctuated between 18.8 and 24.0, and between 20.6 and 12.9, respectively, as pH decreased (Table 4). R for the enzymes of the ethanol pathway varied between 6.3 and 12.4, and between 15.7 and 12.3 for AADH and ADH, respectively (Table 4). At each step in the central metabolic pathways, the intracellular concentration of substrates, products, cofactors or effector molecules as well as intracellular ionic strength, redox potential or pH can influence the partition and regulation of the carbon flux (Holms, 1986). Nevertheless, the fact that fluxes were much less than the available enzyme activity indicated that the carbon flows were determined by the concentration of substrate available rather than the enzyme activity (Holms, 1996). Despite the variation of enzyme biosynthesis, the amount of these enzymes was always sufficient to catabolize the flowing carbon since R was much higher than 1. At pH 7.4, for the lactate formation pathway, R was very high, i.e. 529.5. This indicated that although LDH was readily available, little carbon was catabolized by this metabolic pathway. As

### Table 4. Specific enzymic activity and flux relative to available enzyme activity in C. cellulolyticum cell extract at steady state

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Results obtained at pH value of</th>
<th>7.4</th>
<th>7.2</th>
<th>7.0</th>
<th>6.8</th>
<th>6.4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>SEA</td>
<td>R</td>
<td>SEA</td>
<td>R</td>
<td>SEA</td>
</tr>
<tr>
<td>PTA</td>
<td></td>
<td>0.83±0.08</td>
<td>19.1</td>
<td>0.97±0.07</td>
<td>24.0</td>
<td>0.71±0.08</td>
</tr>
<tr>
<td>AK</td>
<td></td>
<td>0.88±0.09</td>
<td>20.6</td>
<td>0.83±0.10</td>
<td>20.1</td>
<td>0.64±0.06</td>
</tr>
<tr>
<td>AADH</td>
<td></td>
<td>0.12±0.01</td>
<td>63.1</td>
<td>0.18±0.02</td>
<td>10.1</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td>ADH</td>
<td></td>
<td>0.29±0.02</td>
<td>15.7</td>
<td>0.28±0.03</td>
<td>15.6</td>
<td>0.25±0.02</td>
</tr>
<tr>
<td>LDH</td>
<td></td>
<td>0.15±0.01</td>
<td>529.5</td>
<td>0.19±0.03</td>
<td>215.3</td>
<td>0.18±0.01</td>
</tr>
<tr>
<td>PFO</td>
<td></td>
<td>0.61±0.05</td>
<td>9.8</td>
<td>0.66±0.03</td>
<td>11.2</td>
<td>0.34±0.04</td>
</tr>
</tbody>
</table>

*PTA, phosphotransacetylase; AK, acetate kinase; AADH, acetaldehyde dehydrogenase; ADH, alcohol dehydrogenase; LDH, l-lactate dehydrogenase; PFO, pyruvate-Fd oxidoreductase.*

† SEA is the specific activity of enzyme expressed in µmol min−1 (mg protein)−1.

‡ R is the ratio of specific enzymic activity to metabolic flux through the considered metabolic pathway. Flux was previously expressed as µmol (mg protein)−1 min−1.
the pH decreased, however, R declined to 46.1, indicating that the LDH was more and more implicated in carbon conversion (Table 4). As for the metabolic route through PFO, R was in the same range as PTA, AK, AADH or ADH for environmental pH values between 7-4 and 7-0, i.e. R between 6-3 and 11-2 (Table 4), indicating that the fluxes were much less than the available enzyme activity. Yet for a pH lower than 7-0, R markedly decreased and reached 1.8 at pH 6.4 (Table 4). Then both biosynthesis and catalytic efficiency of PFO declined with pH since the specific enzyme activity and \( q_{\text{acetyl-CoA}} \) decreased (Tables 3 and 4).

**DISCUSSION**

Contrary to what was first observed by Giallo et al. (1983), pH control during batch culture fermentation of *C. cellulolyticum* greatly influenced cell growth and metabolism. In fact, maintaining the pH at 7.2, increased cell density, enhanced ethanol and acetate production and raised the extent of cellulose hydrolysis, but did not increase the amount of soluble glucides formed, contrary to what was observed in non-pH-controlled fermentation. As previously observed with increasing the concentration of cellulose (Desvaux et al., 2000), the maximum rate of cellulose degradation observed in pH-controlled cultivation reflects the higher cell mass compared to a non-pH-controlled culture. Cellulolysis continued after the cessation of growth and a high level of soluble glucide accumulation was only observed in a non-pH-controlled culture. The gain in cellulose degradation achieved under pH control was offset by catabolite production rather than soluble sugar accumulation.

In continuous culture, maximum cell density was obtained at pH 7.0, but as the pH declined from 7.0 to 6.4 at constant D, biomass was lowered more than fourfold. At the same time, the specific rate of cellulose consumption, however, decreased only from 18.4 to 1.69 meq C (g cells)^{-1} h^{-1}. Thus environmental acidification influenced chiefly the biomass formation rather than cellulose degradation and assimilation. *C. cellulolyticum* did not show depressed yields and the transition to wash-out appeared abrupt. This result would be more consistent with a direct effect on a cellular constituent, such as the negative effect of acid on an enzyme or transport protein (Russell & Dombrowski, 1980; Russell & Diez-Gonzalez, 1998).

During cellulose catabolism by *C. cellulolyticum*, soluble β-glucans are first converted into G1P and G6P (Desvaux et al., 2000). With environmental acidification, the G1P pool increased, since the proportion of carbon flowing via phosphoglucomutase varied between 57.9 and 55.5%. The remaining G1P was directed towards exopolysaccharides (up to 9.9% of the G1P) rather than glycogen synthesis (3.0% maximum of the G1P), both allowing dissipation of carbon surplus (Guedon et al., 2000b). As the culture pH was lowered, the flow through glycolysis decreased while carbon directed to biosynthesis increased; as a result, the G6P pool was between 15-9 and 18-1 μmol (g cells)^{-1}. Compared with conditions of uncoupling between catabolism and anabolism encountered during ammonium-limited chemostat performed with cellobiose (Guedon et al., 2000a), the excess of carbon at the G1P–G6P branch point was here limited; in fact, exopolysaccharides and glycogen could represent up to 16.0 and 21.4% respectively of the cellobiose consumed and cellotriose was detected extracellularly (Guedon et al., 2000a).

The increase of the acetyl-CoA pool was corroborated by the analysis of carbon flux; the proportion of cellulose consumed flowing through PFO diminished as the pH declined, as did the ratio of specific enzymic activity to metabolic flux (R), and the flux was rerouted away from acetate production. The acetyl-CoA/CoA ratio decrease was paralleled by decreases in H₂/CO₂ and \( q_{\text{NADH produced}}/q_{\text{NADH consumed}} \). Despite the variation of the NADH balance, calculated from catabolic pathways producing and consuming reducing equivalents, the intracellular NADH/NAD⁺ ratio was well regulated. Such a result is in good agreement with the model of Decker et al. (1976), where the NADH-Fd reductase is activated by the acetyl-CoA and inhibited by CoA and which underlines that the fates of NADH and acetyl-CoA regulation are intertwined. From acetyl-CoA, acetate was mainly formed but the flux split differently as the environment was acidified, favouring ethanol production. In addition, as pH declined, the level of lactate production rose and coincided with the pyruvate leak, indicating that PFO could no longer support carbon flow from glycolysis, R decreasing to 1.8 at pH 6.4. Whatever the pH, LDH was always biosynthesized. This enzyme operated mainly as the pH declined but always remained in excess since even at pH 6.4, R was 46.1. In these experimental conditions, LDH allowed draining off part of the pyruvate surplus. At high pH values, H₂/CO₂ ratios higher than 1 suggested that H₂ was produced via NADH-Fd reductase and hydrogenase activities in addition to pyruvate-Fd oxoreductase and hydrogenase activities. With lower pH values, this ratio decreased and was compensated by the increase of ethanol production until washout occurred.

Reinvestigation of cellulose degradation by *C. cellulolyticum* (Desvaux et al., 2000) showed marked differences in the catabolism of this bacterium as compared with the first investigations carried out (Giallo et al., 1985). The present paper demonstrates that the inhibition of growth first observed with batch culture performed in penicillin flasks sealed with butyl rubber stoppers and without shaking of the medium (Giallo et al., 1983, 1985) is mainly the result of low pH due to acid production in the course of fermentation. The range of pH allowing maximum cell density is restricted; strict control of pH is therefore necessary to obtain the optimum cellulolytic performance in biotechnological processes using *C. cellulolyticum*. Cellulolytic bacteria so far investigated cannot grow at pH values significantly less than 6.0 (Stewart, 1977; Russell & Dombrowski, 1980; Russell & Diez-Gonzalez, 1998). However, it is well established that in anaerobic habitats, particularly in the natural environment, high
fermentation acid concentrations and, as a result, low pH values are often encountered (Ljungdahl & Eriksson, 1985; Goodwin & Zeikus, 1987). Since these bacteria have not developed resistance to low pH environments, this implies that they have evolved in an ecological niche where competition for efficient metabolism in acidic conditions is not crucially important. In the same way that growth of *C. cellulolyticum* under an excess of nutrients (Guedon *et al*., 1999b) or with an easily available carbon source, such as soluble glcides, appeared as aberrations considering the natural bacterial ecosystem (Desvaux *et al*., 2000, 2001), cultures without pH control have been shown to be detrimental for optimum growth of this bacterium. These data from monospecies laboratory culture must be extrapolated to microbial ecosystems to explain the maintenance of *C. cellulolyticum* in natural environments. Clearly much remains to be learned about the complex interactions in which this bacterium takes part in microbiota (Kuznetsov *et al*., 1979; Ljungdahl & Eriksson, 1985; Leschine, 1995; Costerton *et al*., 1995).

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