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ABSTRACT

Clostridium cellulolyticum sporulation was investigated during growth on cellulose fibers in a mineral-salt based medium which corresponds to conditions linked to its natural ecological niche. At steady state of the continuous cultures under limitation and with an excess of cellulose and/or ammonium, bacterial cells mainly sporulated at low dilution rates (*D*), at least 10% sporulation being observed at the lowest *D* tested. Increasing the cellulose concentration in the feed-medium reservoir increased the percentage of spores in the bioreactor. It appeared that the remaining undigested cellulose could serve as an exogenous carbon source supply at a continuous but limited rate throughout the sporulation process. In addition to the proportion of carbon and nitrogen, the influence of the environmental pH on spore formation was studied. In cellulose-fed continuous cultures at a constant *D* and a pH decreasing from 7.2 to 6.4, the percentage of spores increased to 14% at the lowest pH tested. When *C. cellulolyticum* was grown in batch culture, the level of sporulation was dramatically higher in unregulated-pH fermentation compared to pH-controlled growth conditions at pH 7.2 since in the former it reached 45% within 5 days of cultivation. It then appeared that a low specific growth rate and a low environmental pH in the presence of an insoluble carbon substrate were the major factors inducing sporulation in *C. cellulolyticum*. Furthermore, since the spores adhere to the carbon substrate (the cellulose) the bacteria gain advantages when the environment allows germination thanks to the recovery of suitable growth conditions. By allowing the maintenance and the integrity of the bacteria in the microbiota, spore formation could then explain the successful survival of *C. cellulolyticum* in cellulosic anaerobic habitats where low environmental pH conditions are often found.

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Introduction

The ecology of cellulose degradation in anaerobic environments is closely linked to global carbon cycling in the biosphere [29]. Anaerobic environments rich in decaying plant material are prevalent and the final products of cellulose fermentation are methane and carbon dioxide [2, 43]. Bacteria are the major cellulose hydrolyzers in anaerobic cellulosic microbiota [30, 39] where cellulolytic clostridia play a key role [29].

Clostridium cellulolyticum, a nonruminal cellulolytic mesophilic bacterium isolated from decayed grass [35], enables digestion of cellulosic materials via extracellular multienzymatic complexes containing various cellulases organized around a scaffolding protein called CipC [4, 33]. The cellulosomes allow both bacterial adhesion and efficient degradative activity against crystalline cellulose [3, 6], For the cellulose degradation process by *C. cellulolyticum,* which must be considered as a microbial process rather than a purely enzymatic event, an adhesion–colonization–release–readhesion model has been proposed [21, 22].

In this latter model, the long-term survival of the cellulolytic bacterium is assured by sporogenesis and germination following recovery of substrates [19]. Most of the cellulolytic clostridia isolated from various biotopes form spores [5, 40], but admittedly considerable variation in the environmental conditions triggering sporulation in clostridia has been found [37, 44]. Recently it was shown that *C. cellulolyticum* sporulation was mainly related to the dilution rate and followed a down–up–down sporulation pattern as specific growth rates increased [34].

Most of the first investigations carried out with *C. cellulolyticum* were performed on complex media with cellobiose, a soluble cellodextrin, which countered the continuous cultivation of the bacterium on cellulose as sole carbon and energy source. Later investigations demonstrated that growth of *C. cellulolyticum* in a complex medium [25] and/or with an easily available soluble carbon source [12, 14, 15] resulted in metabolic deregulations, pointing out that these growth conditions did not properly represent the natural ecosystem of the bacterium.

Continuous culture is a method of choice to analyze bacterial metabolism, and in the past decade, efficient continuous culture devices for growth on insoluble compounds have been developed [41]. In natural environments, growth conditions most likely resemble those of an open continuous-culture system or at least somewhere between an open system and a closed batch culture [28]. By using both types of culture system, the aim of the present study was to investigate the sporulation of *C. cellulolyticum* when grown in a mineral-salt based medium with cellulose, which are growth conditions closely linked to its natural environment.

Methods

Organism and Medium

Clostridium cellulolyticum ATCC 35319 was isolated from decayed grass [35]. Stocks of spores, stored at 4°C, were transferred to cellulose medium and heat shocked at 80°C for 10 min [12]. Anaerobic cell cultures were subcultured once on cellulose before inoculation and growth in the bioreactor [12]. The defined medium used in all experiments was a modified CM3 medium [24] containing various amounts of cellulose MN301 (Macherey-Nagel, Düren, Germany) and ammonium as specified in the Results section.

Growth Conditions

Clostridium cellulolyticum was grown on cellulose as sole carbon and energy source and ammonium as nitrogen source using a mineral salt based medium. All experiments were performed in a 1.5 L working volume fermentor (LSL Biolafitte, St. Germain en Laye, France). The temperature was maintained at 34°C and the pH was controlled by automatic addition of 3 M NaOH or 1 M HCl. The inoculum was 10% by volume from an exponentially growing culture.

Batch culture with cellulose was performed as previously described [12, 13], and the cellulose-fed continued-culture was a segmented gas–liquid continuous-culture device as described by Weimer et al. [41] with some modifications [14]. Each continuous-culture run was independent and performed as previously described [13–16]. Once steady state of the continuous culture was reached, culture samples were removed at 6 to 30-h intervals and for each condition the data were the average from at least 3 samples collected over 2- to 8-day periods [14].

Analytical Procedures

Biomass was estimated by bacterial protein measurement [12].

The percentage of spores versus total cells was estimated by counting using an epifluorescence microscope as previously described [34].

Culture supernatants (10,000 × *g*, 15 min, 4°C) were stored at −80°C until analysis.

Ammonium was assayed by the method of Chancy and Marbach [8].

Cellulose concentration was determined as described [12].

Results

Clostridium cellulolyticum Sporulation with Increasing Concentrations of Ammonium and Cellulose

With increasing concentration of cellulose in the feed medium from 1.9 to 27.0 g L^{-1} and 15.13 mM of ammonium, continuous cultures were performed at a dilution rate of 0.048 h^{-1} in independent runs (Fig. 1a). Above 7.6 g L^{-1} of cellulose, the biomass at steady state leveled off at 296 mg L^{-1}. In contrast, the percentage of cellulose degradation declined from 32.3 to 8.3% as the amount of cellulose input was increased (Fig. 1a). Cellulose digestion was not complete at any of the cellulose concentrations tested but the production of biomass paralleled the amount of consumed cellulose [12, 15]. As for the sporulation efficiency, it increased as the cellulose concentration was increased to reach 8% at the highest cellulose concentration tested (Fig. 1a). At a dilution rate of 0.048 h^{-1} with 18.1 g L^{-1} of cellulose MN301 and with increasing concentrations of ammonium from 2.27 to 30.27 mM, biomass leveled off at 305 mg L^{-1} above 8.10 mM of ammonium input (Fig. 1b). Thus the stagnation of biomass formation could not be related to starvation for either cellulose or ammonium, or for other nutrient(s) since cell density was not modified when the amount of all other components of the medium was increased [16]. As

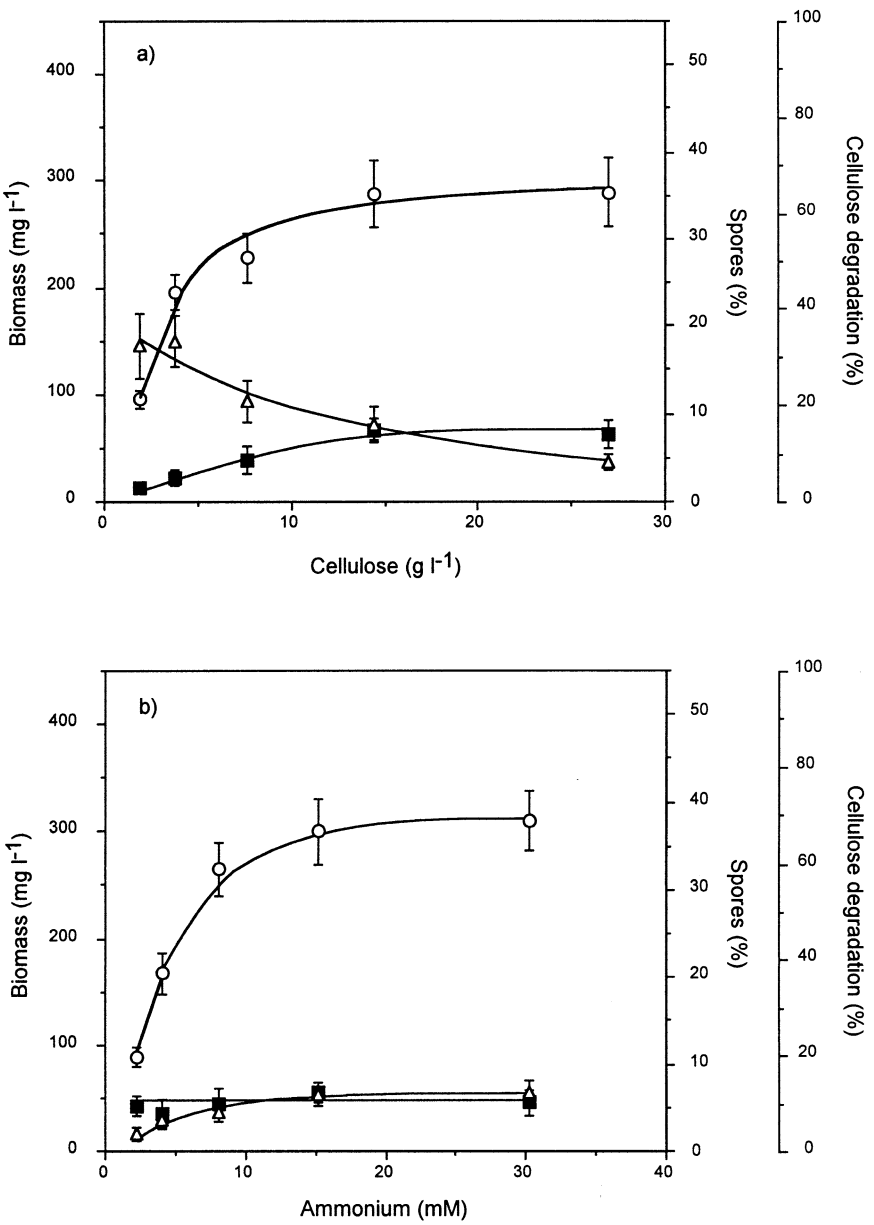

Fig. 1. Influence of increasing concentrations of carbon and nitrogen source on sporulation of *C. cellulolyticum*. Bacteria were grown in continuous culture at $D = 0.048$ h^{-1} with (a) various amounts of cellulose while ammonium was in excess, i.e., 15.13 mM, or (b) ammonium while cellulose was in excess, i.e., 18.1 g L^{-1} (b). ○, biomass; Δ, percentage of cellulose degradation; ■, percentage of sporulation.

the ammonium concentration in the feed reservoir was increased, the percentage of cellulose degradation increased as well to reach 12.1% with 30.27 mM of ammonium and was correlated with the higher generated biomass (Fig. 1b). The percentage of sporulation, however, was not changed by the amount of ammonium input and remained around 5%.

Influence of Dilution Rate on Clostridium cellulolyticum Sporulation

Cellulose-limited continuous cultures were carried out with 3.7 g L^{-1} of cellulose in the feed reservoir and dilution rates ranging from 0.027 to 0.083 h^{-1} (Fig. 2a) [14]. Maximum cell density was obtained at $D = 0.027$ h^{-1}, i.e., 212 mg L^{-1}, and slowly declined as D rose to reach 154 mg L^{-1} at the highest D tested. The highest percentage of cellulose degradation was obtained with the lowest D tested, i.e., 50.4%, and decreased with increasing growth rate to reach 20.9% at 0.083 h^{-1}. Under these experimental conditions, cultures of *C. cellulolyticum* were observed to sporulate at low specific growth rates; a maximum percentage of spores of 11% was found at $D = 0.027$ h^{-1}.

Nitrogen-limited chemostats with 4.00 mM ammonium and with cellulose at saturated concentration (18.1 g L^{-1}) were performed with dilution rates from 0.027 to 0.085 h^{-1} (Fig. 2b) [16]. The residual ammonium concentration was barely detectable at low specific growth rates (<0.10 mM), but was 3.02 mM with the highest μ tested. Such data are typical of continuous culture performed under limitation of a selected nutrient [47]. Under these experimental conditions, biomass at steady state ranged from 187 to 62 mg L^{-1} as D rose while at the same time, the percentage of cellulose degradation ranged only from 9.5 to 1.8%. As under cellulose limitation, the same sporulation pattern as a function of D was observed; continuous cultures of *C. cellulolyticum* were observed to sporulate mainly at low dilution rates with a maximum percentage of sporulation of 10% found at $D = 0.027$ h^{-1}.

With saturated concentrations of all nutrient including both ammonium (15.13 mM) and cellulose (18.1 g L^{-1}) in the feed reservoir, *C. cellulolyticum* was cultivated at various D values (Fig. 2c) [15]. Cell density was higher than in cellulose- or ammonium-limited chemostats since it ranged from 367 to 161 mg L^{-1}. As observed under limitation of ammonium or cellulose, the biomass concentration declined with D but more sharply than in the previously tested culture conditions. With cellulose and ammonium saturation the proportion of digested cellulose was lower than in cellulose-limited condition since it ranged from 21.4 to 5.3% with increasing D. As previously observed, spore formation mainly occurred at low D values; the percentage of sporulation was, however, consistently higher than in limited conditions since it ranged from 3 to 15% as D declined.

Influence of pH on Clostridium cellulolyticum Sporulation in Batch and Continuous Cultures

Batch cellulose cultivations of *C. cellulolyticum* were performed with 6.7 g L^{-1} of cellulose under unregulated-pH and pH-stat conditions (Fig. 3) [12, 13]. Within 5 days, the maximum cell density was reached in batch cultures controlled at pH 7.2 with 495 mg L^{-1} compared with 281 mg L^{-1} in unregulated pH fermentation, as was the percentage of cellulose digestion, which was 80.3% and 61.2% with and without pH regulation, respectively (Figs. 3a and 3b). Sporulation of *C. cellulolyticum* began after 50–60 h of culture once bacterial cells had entered the stationary phase. Contrary to the maximum dry weight of cells or the maximum percentage of cellulose degradation, the percentage of sporulation was dramatically higher in non-pH-controlled cultures, i.e., 45% (Fig. 3b), than in pH-controlled batch cultivation, i.e., 7% (Fig. 3a).

C. cellulolyticum was grown in continuous cultures under cellulose-limited conditions at $D = 0.053$ h^{-1} and pH values decreasing from 7.2 to 6.4 in independent runs (Fig. 4) [13]. At and below pH 6.2, a steady state could not be established since washout occurred. As the pH was lowered, the cell density at steady-state declined to 56 mg L^{-1} at pH 6.4. Similarly the percentage of cellulose digestion decreased with lower pH values to reach 7.3% at pH 6.4 (Fig. 4). Contrary to the downward trend observed for the biomass and cellulose degradation for more acidic cultures, the percentage of sporulation increased to reach 14% at the lowest pH tested.

Discussion

In contrast to the genus *Bacillus*, where the regulation of sporulation has been investigated on a molecular basis [18, 46], in clostridial-type bacteria spore formation has so far not attracted much interest, especially in cellulolytic *Clostridia* [26, 31, 37, 38, 42, 44, 45]. As already shown with *Bacillus* [10, 11], in continuous culture the fraction of

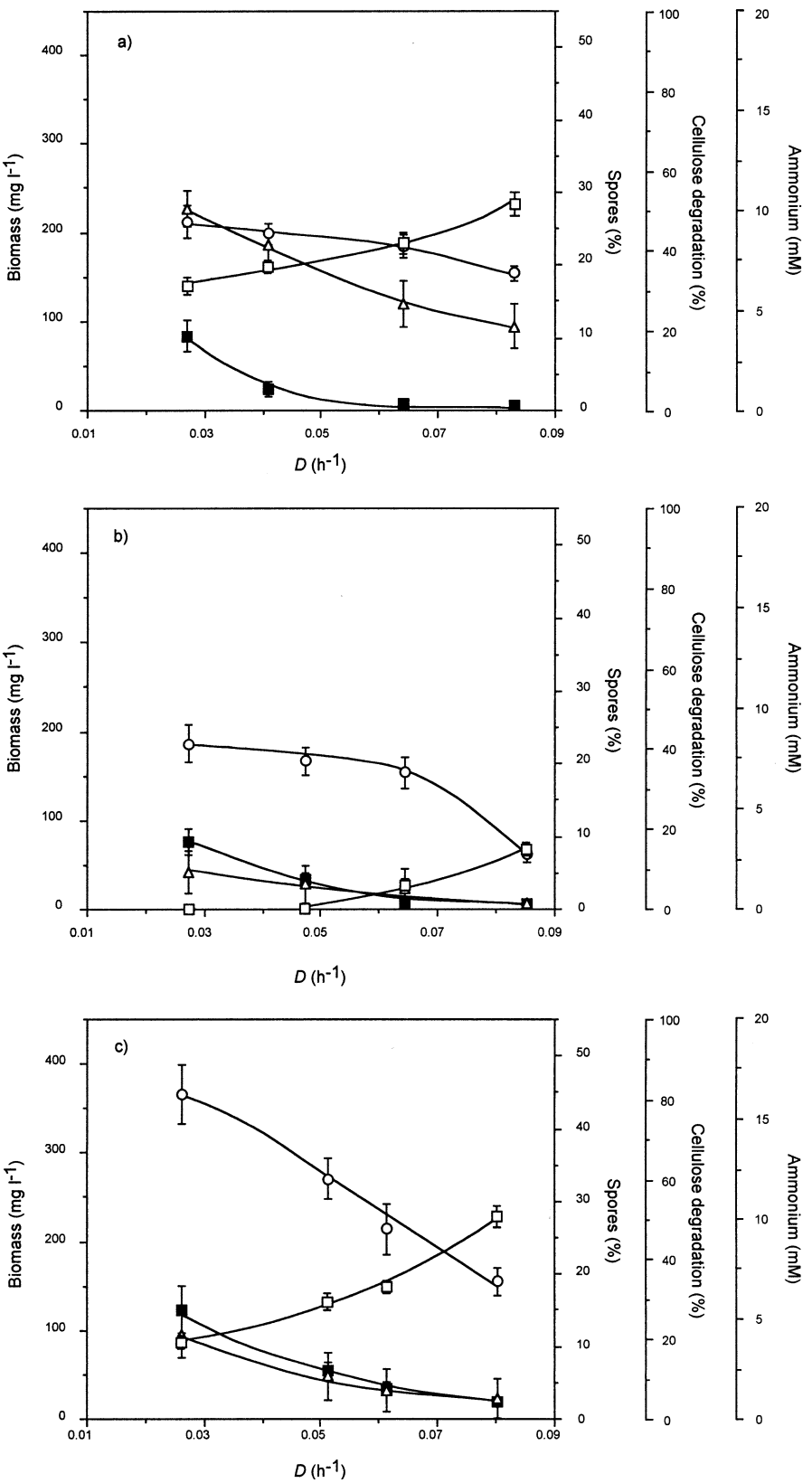

Fig. 2. Effect of dilution rate on spore formation by *C. cellulolyticum*. Cellulose-fed continuous cultures of *C. cellulolyticum* were performed (a) under cellulose limitation, i.e., 3.7 g L^{-1}, (b) under ammonium limitation, i.e., 4.00 mM, and (c) under saturation of both cellulose and ammonium, i.e., 18.1 g L^{-1} and 15.13 mM, respectively. ○, biomass; Δ, percentage of cellulose degradation; ■, percentage of sporulation; □, ammonium.

sporulated cells increases gradually with decreasing dilution rate. In natural environments the proportion of carbon and nitrogen can strongly influence cell growth and sporulation since these two compounds are major elements of microbial cell composition [1]. Furthermore, while cellulose accumulates in the environment because of

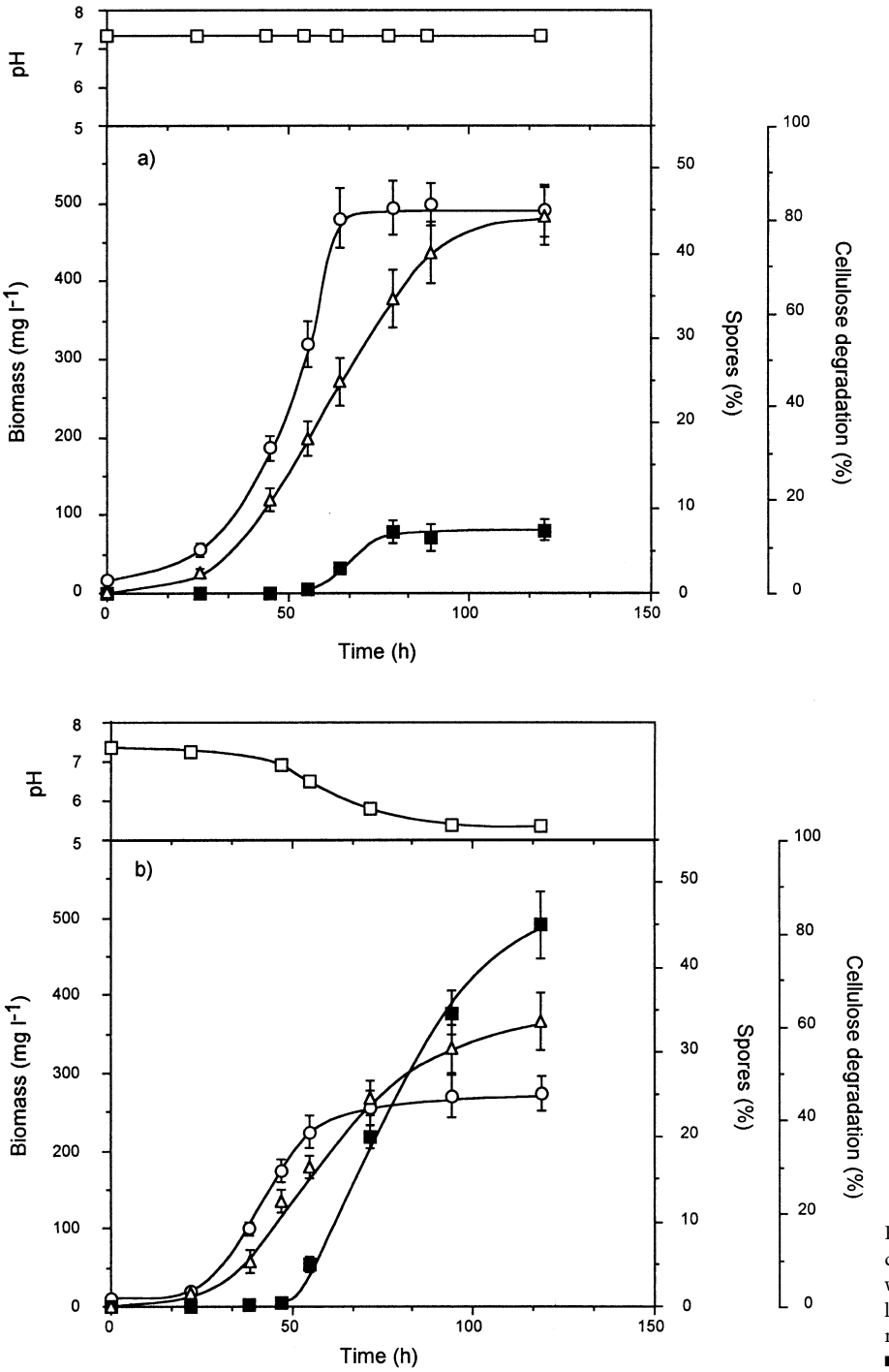

Fig. 3. *C. cellulolyticum* sporulation in cellulose-batch cultures. Bacteria were grown with (a) pH regulated at 7.2 and in (b) unregulated-pH condition for 5 days □, pH; ○, biomass; Δ, percentage of cellulose degradation; ■ percentage of sporulation.

its recalcitrant, durable nature [2], lignocellulosic compounds usually contain a low level of nitrogen [16, 23]. In both cases of limitation and excess of ammonium and/or cellulose, sporulation efficiency was dependent upon growth rate.

From previous investigations with cellobiose limitation [34], *C. cellulolyticum* sporulation was maximal (12%) at $D = 0.035\ h^{-1}$ and decreased at lower and higher D values, i.e., 4% at $D = 0.016\ h^{-1}$ and 3% at $D = 0.053\ h^{-1}$ [34]. However, in the present study with insoluble cellulose, a similar down–up–down sporulation pattern as D increased was not observed. Moreover, contrary to what was observed on cellobiose where an excess of soluble carbon substrate repressed spore formation [34], increasing cellulose concentrations promoted sporulation. These differences could be related to the physical nature of the

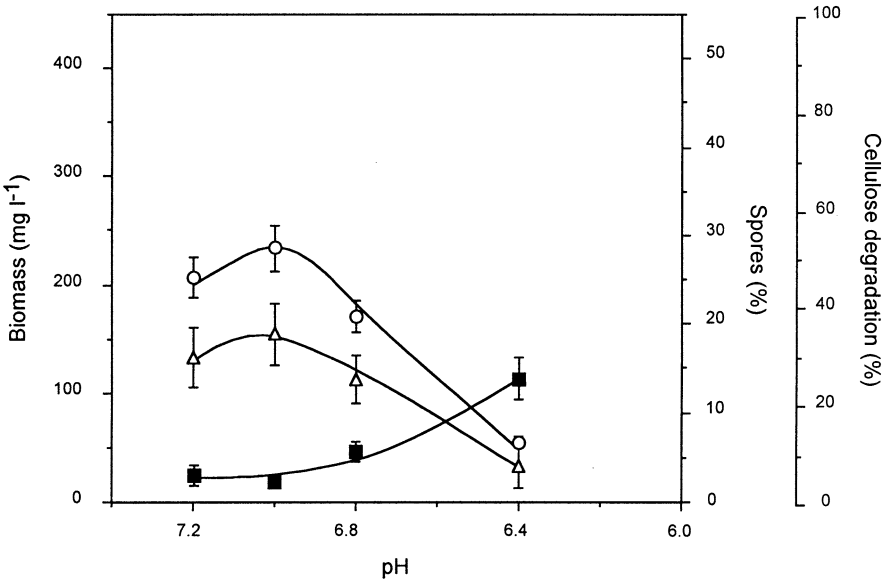

Fig. 4. Effect of pH on spore formation by *C. cellulolyticum*. Bacteria were cultivated in cellulose-fed continuous cultures at $D = 0.053\ h^{-1}$ with 3.7 g L^{-1} of cellulose and 15.13 mM of ammonium. ○, biomass; Δ, percentage of cellulose degradation; ■, percentage of sporulation.

cellulose. Cell attachment could potentially modulate and trigger a particular physiological regulation [27] and the remaining cellulose could serve as an exogeneous energy source for sporulation by feeding bacterial cells continuously at a limited rate. This could be comparable to culture of *C. thermosaccharolyticum* where continuous and limited feeding of glucose, once the original carbon source was completely exhausted from the batch culture, allowed cell sporulation [26].

Quorum sensing could not be excluded as an explanation of some variations of the percentage of sporulation observed in different growth conditions [17]. In fact, at steady state with an excess cellulose concentration, cell density was lower in ammonium limitation than in ammonium excess, resulting in different percentages of sporulation at the same *D*. Similarly, when growth of *C. cellulolyticum* was studied as a function of *D* under cellobiose limitation, the biomass concentration decreased as *D* was lowered from 0.085 to 0.016 h^{-1} [26] while, in the same conditions on cellulose, the cell density at steady state increased [14], resulting in different percentages of sporulation, especially at low *D* values where the difference of biomass concentration was greater between these two carbon substrates [34].

The growth of *C. cellulolyticum* on a complex medium and on a mineral salt-based medium resulted in different metabolic regulation and sporulation rates [24, 25, 34]. Also, the use of an easily available carbon source, such as cellobiose, compared to substrates more closely related to natural lignocellulosic compounds, such as cellulose, was shown to induce deregulation of *C. cellulolyticum* metabolism [14, 15, 25] and discrepencies in the percentage of sporulation attributed to growth conditions far from the natural ecosystem of the bacteria. It should thus be noticed that the use of nitrogen sources different from ammonium could induce a particular metabolic regulation and a variation of sporulation efficiency [16]. In *C. cellulolyticum*, no correlation between glycogen accumulation and endospore formation was detected [34]. Glycogen is of key importance in *C. cellulolyticum* metabolism since it actively participates in the regulation of the carbon flow [16, 23]. With *C. acetobutylicum*, Meinecke et al. obtained a stable asporogeneous mutant strain only after 35 days of chemostat culture at $D = 0.125\ h^{-1}$ [31]. Yet sporulation was not directly assayed since a correlation between the presence of spores and the presence of glycogen in colonies was used. Therefore, the asporogeneous strain was in the first instant aglycogenic. Such results point out that the influence of the carbon flow on sporulation and the importance of glycogen seem different in *C. cellulolyticum* and *C. acetobutylicum*.

Previous investigations [34] missed the possible influence of pH on sporogenesis in *Clostridia* [44, 45]. Anaerobic habitats are characterised by low pH conditions resulting from high concentrations of fermentation acids [30]. Clostridial-type bacteria are often considered to be sensitive to a low pH and restricted to less acidic ecological niches [36]. Culture of *C. cellulolyticum* under unregulated pH conditions was shown to be detrimental for optimum cellulose fermentation [13] which then affected spore formation. In continuous culture, as the pH declined, the cells sporulated and steady-state conditions

could not be attained with a pH lower than 6.2. In unregulated-pH batch culture, sporulation of *C. cellulolyticum* was high and associated with the entry into the stationary phase. Spore formation can partially explain the arrest of metabolite production, previously observed in unregulated-pH conditions [13]. Spore formation then appeared as a process allowing the maintenance and the integrity of the cell mainly in response to an environmental pH decline rather than exhaustion of carbon substrate as previously assumed [20–22, 34, 42].

Adhesion and detachment to cellulose fibers are important factors in the behavior and physiology of *C. cellulolyticum*, which could also have regulatory function(s) [2, 20]. While vegetative bacteria adhered to cellulose at specific sites [21], the adhesion of spores occurred by hydrophobic interactions and was thus a nonspecific process [19, 42]. Sporulation of *C. cellulolyticum* while attached to lignocellulosic compounds allows successful survival and competition with other microorganisms since, as soon as environmental conditions permit it, cellulolytic bacteria can start a new vegetative cycle on a directly available carbon substrate, the cellulose fibers. For *C. cellulolyticum*, a low specific growth rate and mainly a low environmental pH in the presence of an insoluble carbon substrate were the major factors inducing sporulation. This study is a first step providing grounding for further investigation of the physiology and molecular biology of regulation of sporulation in *C. cellulolyticum*. Furthermore, understanding of the phenomena which allow favorable conditions for spore germination and thus cellulose degradation in the natural ecological niche requires that data from monospecies laboratory cultures must be extrapolated to microbiota where, as observed with the rumen microflora, complex interactions between cellulolytic and noncellulolytic microorganisms and environmental conditions take place [7, 9, 32].

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