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Continuous cultivation of *Clostridium thermobutyricum* in a rotary fermentor system

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The growth behavior of *Clostridium thermobutyricum* JW171K and its production of butyric acid were investigated under continuous cultivation in a recently developed rotary fermentor. Using low dilution rates (up to 40 times the shortest doubling time), the continuous culture conditions caused metabolic shifts from butyrate formation to the production of acetate. Using an 18-h volumetric retention time, no true steady state in butyrate formation was achieved after 22 days, although the optical density was stable. Acetate and butyrate were formed in an oscillatory mode with an alternating predominance between these two products, indicating an oscillation between the less exergonic acetate-forming but higher ATP (4ATP mol^{-1} glucose) forming mode, and the more exergonic butyrate and 3ATP mol^{-1} glucose forming mode. During the continuous culture drastic changes in cell morphology occurred and, at the lower dilution rates, long, granulose-containing, filamentous cells with rounded protuberances and swellings were observed. A maximal butyrate concentration of 18.4 g L^{-1} and a productivity of about 2.4 g L^{-1} per h (at 25–27 mM concentration in the broth) were obtained. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 7–13.

Keywords: butyric acid; rotary fermentor; continuous culture; thermophilic clostridia

Introduction

Butyric acid esters are presently used as additives in the food industry and as aromatic compounds for the production of perfumes. Butyrate is currently produced economically via petrochemicals, however there is presently an interest in using biologically produced food additives. The best-studied butyrate-producing microorganisms belong to the genus *Clostridium* but none of these organisms have been found suitable for present-day industrial applications.

Recent developments in the understanding of thermophilic microorganisms [3,4,21,22] have shown several benefits associated with the use of thermophilic fermentations for the production of fuels, organic acids and enzymes as well as for treatment of solid organic waste. In this regard, industrially useful applications have been frequently proposed for thermophilic clostridia, due to their wide substrate range, their metabolic potential, and the improbability of the presence or propagation of pathogenic organisms in the product or in the waste stream. Among thermophilic clostridia, appreciable amounts of butyric acid are produced under specific conditions by *Clostridium thermosaccharolyticum* [3,10] and *C. thermobutyricum* [3,23] but only the latter species shows ratios as high as 0.8–0.9 mol butyrate formed per mol glucose consumed. The wild-type strain of *C. thermobutyricum* (JW 171K) can easily be adapted to tolerate up to 350 mM (30 g L^{-1}) butyrate at 55°C in liquid cultures (Kuk *et al*, previously of Dept of Microbiology, University of Georgia, unpublished results). This organism might be a promising candidate for biological production of butyrate for the food industry.

The use of a rotary fermentation system for acetic acid production at low pH values has shown promising results for the thermophilic *C. thermoaceticum* and *C. thermoautotrophicum*, with high production rates of over 11 g L^{-1} h^{-1} [24]. The principal concept of the rotary fermentor (Figure 1) is to provide *C. thermobutyricum* an optimal surface for colonization, adequate exposure to fresh substrate and removal of products. Fresh medium is provided at the bottom of the system and the product-containing culture broth is removed from the top, creating inverse gradients of substrate and product. Subsequently cells at the bottom adapt to high glucose but low product concentrations and cells at the top adapt to lower (1–2 mM) glucose but high product concentrations.

The aim of the present study was to investigate the effect of continuous culture conditions and to study butyrate fermentation by *C. thermobutyricum* in this recently developed rotary fermentor system.

Materials and methods

Microorganism

Stock cultures of *C. thermobutyricum* strain JW 171K (ATCC 49875, DSM 4928), isolated in our laboratory from horse manure, were maintained at 4°C on agar slants using the mineral medium previously described [23].

Culture medium

The mineral medium for the fermentation experiment contained per liter of distilled water: 1.2 g KH_2PO_4; 3.2 g $Na_2HPO_4 \cdot 7H_2O$; 0.5 g NH_4Cl; 0.5 g $(NH_4)_2SO_4$; 0.10 g $MgCl_2 \cdot 6H_2O$; 0.05 g $CaCl_2$; 2.0 g $NaHCO_3$. The pH of the medium was approximately 7.2. The anaerobic techniques applied were described by Ljungdahl and Wiegel [13].

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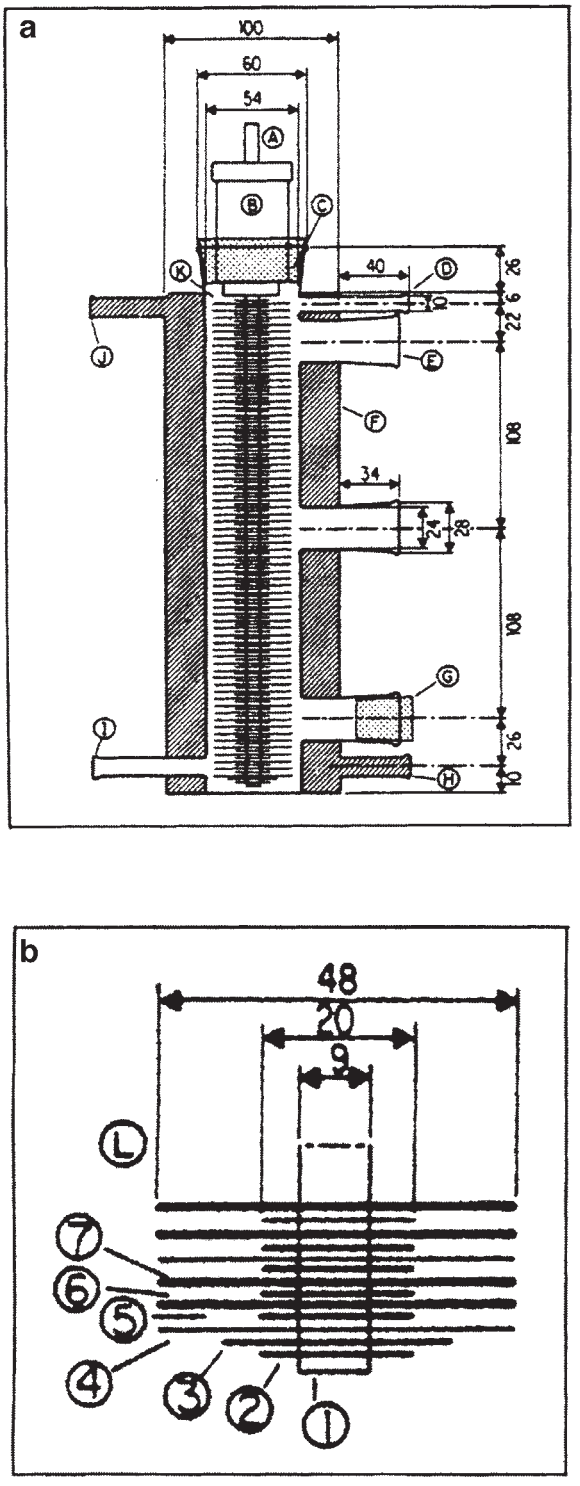

Figure 1 (a) Diagram of the rotary fermentor; (b) Close-up diagram of the rod with pads. A, stirring shaft (10-mm stainless steel rod) to be connected to stirring rotor; B, lipseal stirrer assembly (without coupling ring and motor studs); C, rubber stopper No. 11; D, culture outlet; E, sampling port; F, water jacket; G, rubber stopper No. 4; H, water jacket inlet; I, media/N2 inlet; J, water jacket outlet; K, stirring rod with pads; L, expanded section of stirring rod with pads; 1, stirring rod; 2, bottom pin to hold pads on; 3, bottom washer-stainless steel (2 mm × 30 mm); 4, Dupont Reemay 2033 pad (0.4 mm × 20 mm); 5, Teflon washer (0.8 mm × 48 mm); 6, 60:40 pad (2.5 mm × 48 mm); 7, 60:40 pad; (modified after Wiegel *et al* [24]).

After being autoclaved at 121°C for 120 min, the medium (16 liters in a 20-liter reservoir) was gassed with sterile O_2-free N_2 until cool. Glucose (50% w/v solution under O_2-free N_2 gas), yeast extract solution (20% w/v), vitamin solution (0.5 ml L^{-1} [6]), trace element solution (5 ml L^{-1} [7]) and the reducing solution (0.2 g $Na_2S \cdot 9(H_2O$, 0.2 g cysteine per liter) were autoclaved separately at 121°C for 40 min and were added to the cooled (below 60°C) medium as sterile solutions.

Fermentor design

The rotary fermentor (500 ml) was developed and described by Wiegel *et al* [24] and made in the glass shop of the University of Georgia, Athens, GA. A diagram of the rotary fermentor and pads is shown in Figure 1.

Fermentation modes

The medium in the fermentor was kept anaerobic by a continuous flow of sterile N_2. The central rod containing the filter discs rotated at 80 rpm using a stirring motor (Dayton, Model 22810, WW Grainger Co, Atlanta, GA, USA). The incubation temperature was 55°C. The pH was measured with an autoclavable glass electrode (Fermprobe®, Broadley James Corp, Santa Ana, CA, USA) and held at a constant pH value of 7.1 by automatic addition of 2 M NaOH. The inflow of the medium as well as of the glucose in the fermentor was regulated with peristaltic pumps (Model 1203, Harvard Apparatus, South Natick, MA, USA). Addition of glucose was adjusted to maintain a concentration between 5–10 mM in the outflow of the fermentor, ie, glucose never became limiting. The dilution rate of the continuous culture was determined by measuring the outflow periodically and the volumetric retention time (vol. RT) was expressed as total liquid fermentor volume (500 ml) divided by the dilution rate (h).

The rotary fermentor was initially inoculated with 10 ml of fresh culture of *C. thermobutyricum* JW171K. Before starting the continuous culture mode, two batch fermentation cycles (1.5 days each) were carried out to establish the initial culture and a more rapid colonization of the filter discs. The culture was checked for contamination weekly, by plating an aliquot on mineral medium plus 0.5% (w/v) glucose and also by VFA gas chromatographic analysis after anaerobic incubation in Hungate tubes. Morphological changes of the cells in the fermentation broth were investigated by phase contrast microscopy (Olympus Vanox, Deer Park, NY, USA) and electron microscopy (TEM).

Fermentation products

Volatile fatty acids (VFA) were analyzed by gas chromatography as previously described [23]; acetate was also determined using an enzymatic kit (Cat. No. 148261, Boehringer, Mannheim, Germany). Glucose and lactate were tested by the hexokinase/G6PD method (glucose diagnostic kit, Cat. No. 716260, Boehringer) and by the lactate dehydrogenase assay (lactate diagnostic kit, No. 826-UV, Sigma, St Louis, MO, USA), respectively. The presence of granulose was demonstrated by staining the cells with saturated KI solution and visual examination using light microscopy.

Cell protein content

At the end of the fermentation, the stirring rod with the filter discs was removed and the amount of cells trapped in and on the filters was analyzed by determining the total cell protein content. The removal of the stirring rod was carried out under aseptic anaerobic conditions so that the fermentor could be run for comparison without the filter pads. For protein analysis, filters were taken from the lower, middle and upper part of the fermentor. The protein assay was carried out as described by Schmidt *et al* [17] using BSA as a standard.

Electron microscopy

Samples for TEM investigations were fixed in 2% glutaraldehyde and 0.1 M cacodylate buffer, pH 7.0; for staining, uranyl acetate (20 min) and lead citrate (2 min) were used. Micrographs were taken with a JEOL 100C×II transmission electron microscope. Samples for SEM investigations were fixed in Parducz's fixative [15] and, after sputter coating, examined on a Philips 505 scanning electron microscope.

Growth studies

For studying the effect of the addition of acetate, lactate and yeast extract on growth and butyrate formation, *C. thermobutyricum* was cultivated in Hungate tubes using the mineral medium described above supplemented with 0.3% (w/v) yeast extract and 2% (w/v) glucose. The pH value was maintained between 6.8–7.3, and the metabolic products were periodically quantified as described above. Incubations of batch cultures were carried out in parallel, and growth was determined by following the optical density at 600 nm.

Results

A continuous fermentation of *C. thermobutyricum* growing on glucose in the rotary fermentor (Figure 1) was carried out for a total of 78 days. For the first 40 days the medium contained 0.3% (wt/vol) yeast extract followed for the remaining time with medium containing 2% yeast extract. In a control batch culture with 2% yeast extract using the same fermentation vessel but without the rotating filters, *C. thermobutyricum* showed a doubling time of 50 min, thus the volumetric retention times were varied between 1 and 120 h (Figure 2) to test for optimal butyrate production. Except for the lower retention times (1 and 4 h), the culture did not reach a true steady state for butyrate production although the optical density in the outflow was stable within about 30 volume changes and the pH was maintained at 7.1 ± 0.05. About a week after the continuous fermentation was started at a volumetric retention time of 38 h, a metabolic shift from the production of butyrate to acetate as the main fermentation product occurred. Subsequently an oscillatory mode with alternating predominance of butyrate and acetate formation was observed throughout the remainder of the experiment with large fluctuations above 100 mM in the production of butyrate and acetate. At 0.3% yeast extract, generally, acetate was the main product whereas at 2.0% yeast extract in the medium butyrate was usually the major product. However, at the shortest used volumetric retention time of 1 h, butyrate was the main product at both 0.3 and 2% (wt/v) yeast extract concentrations yielding 6 mM butyrate with 1 mM acetate, and 25 mM butyrate with 4–5 mM acetate, respectively. When the yeast extract concentration was raised to 2%, acetate was the main product for about a week at a retention time of 33 h, but after that butyrate became the main product and continued to be the main product even when the retention time was raised to 120 h or lowered stepwise to 1 h. At a retention time of 120 h, values of 210 mM butyrate (18.4 g L^{-1}) with acetate concentrations around 80–90 mM were obtained. However, after peaking, the butyrate concentration declined again while acetate increased, starting again the diasposed oscillatory mode of butyrate and acetate production. However, in contrast to the 0.3% at 2% yeast extract concentration, butyrate usually stayed at a higher concentration than acetate regardless of the oscillation. The production of lactate fluctuated between 0.5 and 18 mM (1.6 g L^{-1}), at both yeast extract concentrations, and such fluctuation approximately resembled the trend of the butyrate concentrations (values of lactate production omitted for clarity in Figure 2).

The highest production rate of 2.4 g L^{-1} per h was obtained using 2% yeast extract and 1 h retention time (over a time period of 10 h), with a butyrate concentration of about 25–28 mM. In order to compare the performance of *C. thermobutyricum* in the rotary fermentor with a conventional continuous culture, after 73 days of cultivation the stirring rod with the filter discs was removed and with it most of the biomass. The remaining culture was kept for 5 days at a retention time of 27 h. As expected, butyrate was the main product (about 60 mM butyrate and 40 mM acetate).

When the rotating pads were removed at the end of the continuous culture, individual filters were taken from the bottom, the middle and the top of the rod, and analyzed for cell protein content and cell morphology (see below). The highest biomass values were obtained from the middle and the upper filters (6 and 5.5 mg protein per filter respectively); the protein content of the bottom filters was only slightly lower (4.5 mg protein per filter).

Morphological changes of the cells during continuous culture

Significant morphological changes of the cells were observed by phase contrast microscopy after less than 1 week of continuous culture. Specifically, filamentous cells of more than 30 μm length appeared in the rotary fermentor, and cells tended to clump together and exhibited large protuberances (Figure 3b). These forms were usually not observed in stationary growth phases using batch cultures. However, when the abnormal cells were plated on agar, the normal colony type appeared. In liquid cultures the long cells converted to normal-sized rods (Figure 3a) which produced butyrate as the main fermentation product and only traces of acetate. The transmission electron micrographs of the filamentous cells with protuberances exhibited large amounts of intracellular structured granular material in both the rod-shaped and the swollen parts of the cells (Figure 4). Staining of the material with potassium iodine suggests that it is the polysaccharide storage material granulose. This has been previously described in *C. butyricum* and *C. botulinum*, and consists of a branched

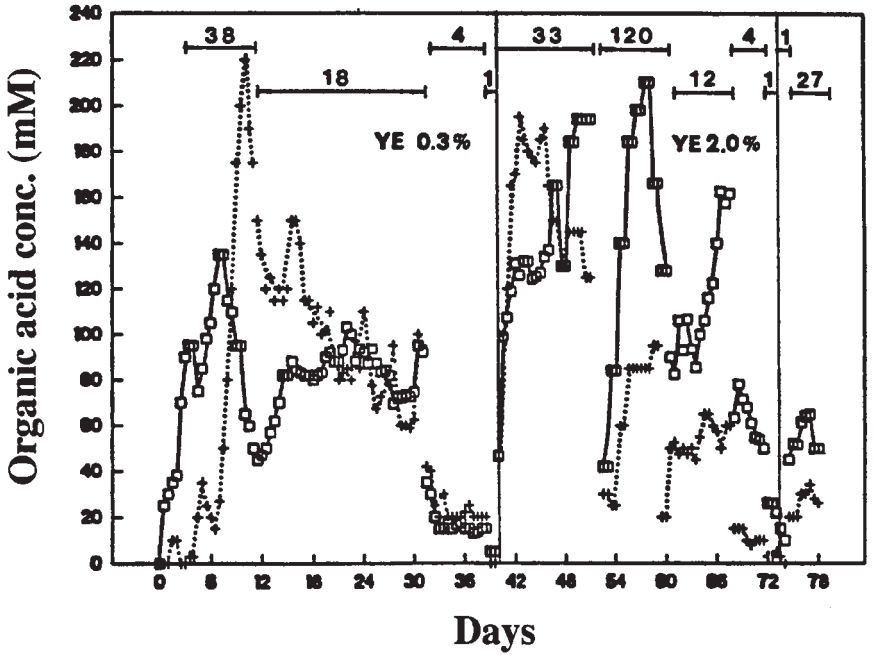

Figure 2 Production of butyrate and acetate in continuous culture of *C. thermobutyricum* JW-171K growing in rotary fermentor at 55°C and pH 7.1 ± 0.05. After 40 days, the concentration of yeast extract was increased to 2% (w/v). On day 51, a pH failure occurred so that the fermentor was drained, filled up and reinoculated. (□) butyrate; (+) acetate; (•——•) volumetric retention time in h.

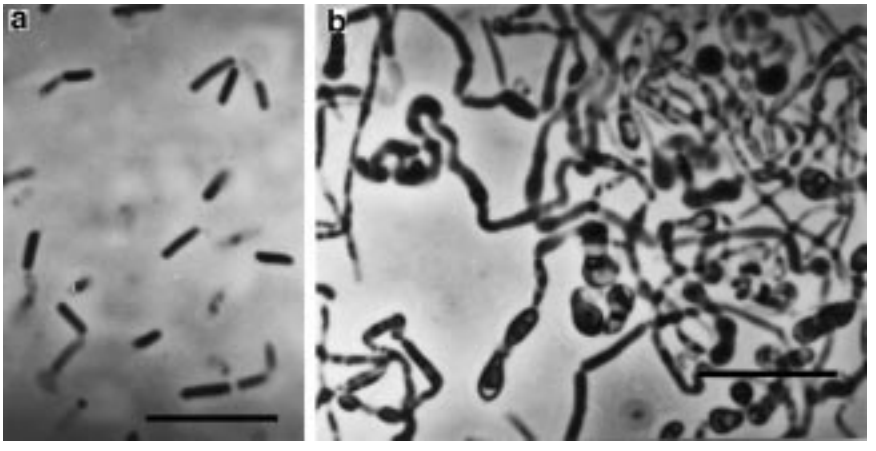

Figure 3 Phase contrast micrographs of *C. thermobutyricum* JW 171 showing regular cells (a) and filamentous swelling cells (b). Scale bar: 10 μm.

glycogen-like polymer usually composed of linear chains of $\alpha(1\rightarrow4)$-linked D-glucopyranose units [16].

Scanning electron micrographs of a sample taken from a filter disc showed that growth of the cells occurred both on and in between the fibers and, in the latter case, large agglomerates of filamentous cells could be detected (Figure 5a, b). The cells growing on the fibers (Figure 5b) were usually normal-sized, although long filaments were occasionally observed. The long filamentous cells were arranged in a helical structure (Figure 5c). Larger amounts of an exopolymer, not further characterized (Figure 5d), aiding cell adherence to the filter pad and forming the biofilm, were observed.

Discussion

One of the goals of this study was to evaluate the behavior of the anaerobic thermophile *C. thermobutyricum* when cultivated under continuous culture conditions in the rotary fermentor. In previous studies with *C. thermoaceticum* and *C. thermoautotrophicum* [24] grown in the rotary fermentor, a 10-times increased acetate production rate was found. For *C. thermobutyricum* the maximal concentration of 210 mM (18 g) butyrate was obtained in the presence of 2% yeast extract, a requirement which is similar to the one observed in *C. butyricum* for producing 19 g butyrate L^{-1} [2,5,19,20]. Compared to the results obtained with the

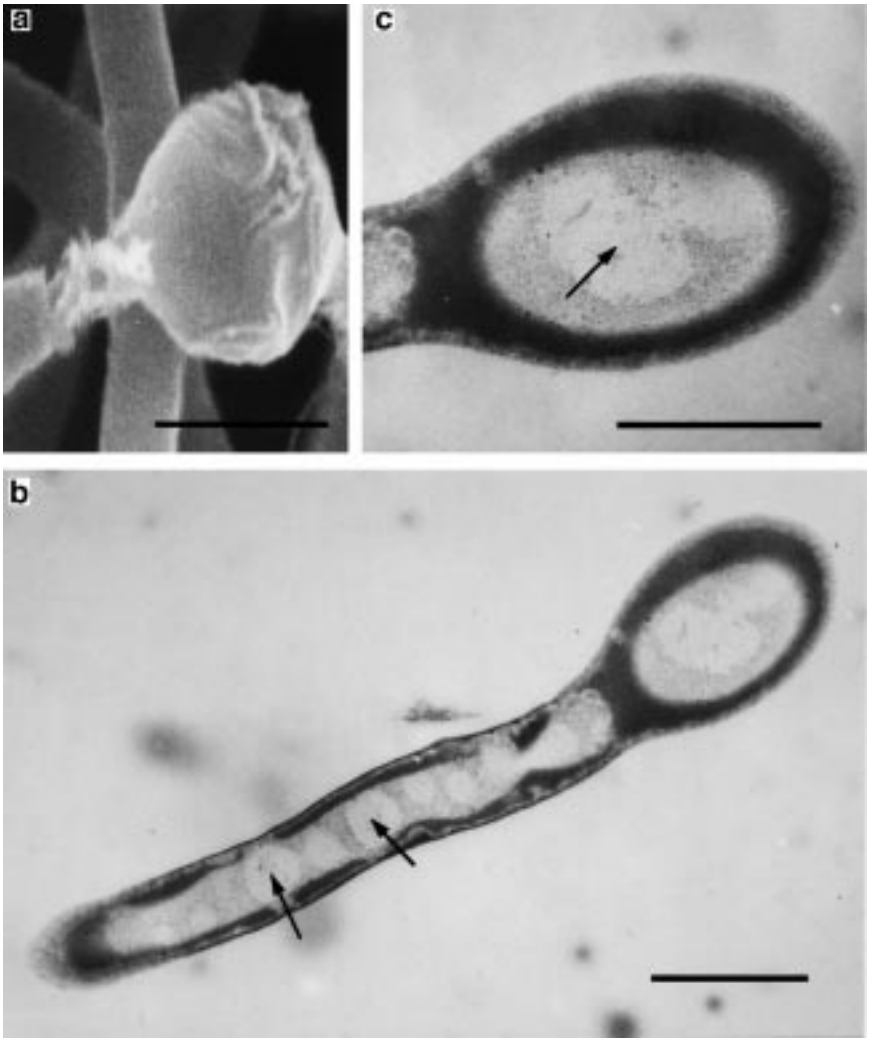

Figure 4 Electron micrographs of swollen cells of *C. thermobutyricum* JW 171. (a) Scanning electron micrographs (SEM) at 30 000 × magnification; (b) transmission electron micrograph (TEM) of a cell with swelling and containing granular material (arrows); (c) enlargement of a section of (b) with the swollen cell and revealing details of the granular material (arrows). Bars = 1 μm.

above acetogens, the less impressive results obtained with *C. thermobutyricum* are probably caused by the less-effective attachment of *C. thermobutyricum* to the fibers, leading to a lower biomass in the fermentor. Another reason might be the observed shift from forming predominantly butyrate to forming acetate. A similar shift has been described for *C. tyrobutyricum* strain CNRZ 596 [1,5]. *C. butyricum* strain LMD77–11 [9,14] also shifted to acetate when grown at increased growth rates in fed-batch and chemostat culture under a self-generated gas atmosphere. In this case, a high acetate formation rate was observed when *C. butyricum*, grown at low dilution rates, was sparged with N_2. A shift from acetate toward the formation of butanol and butyrate has been reported for *C. acetobutylicum* during cultivation on glycerol and pyruvate [8]. In this case the shift is presumed to be a consequence of the high NADH/NAD ratio and the low intracellular ATP concentration.

In our experiments, the shift to acetate was observed (mainly at lower yeast extract concentrations) at low dilution rates under sparging with N_2. The effect observed here was greater than that observed with *C. butyricum*. In fact the ratio of acetate: butyrate changed from 1 : 20 (or even higher in anaerobic batch cultures using Hungate tubes or serum bottles) to about 1 : 0.3, ie, acetate became the major fermentation product. The results reported here with the rotary fermentor are, however, only partially comparable with previous ones, since the ratio of the physiologically different cells (free submerged cells in the culture liquid and cells adsorbed to the fiber pads) changed during the fermentation. At low dilution rates, the influence of growing cells was not significant and, since the carbon source was in excess, the metabolically active resting cells, both free and adsorbed, became more dominant. Because the fermentor was sparged with O_2-free N_2-gas at the same rate, regardless of the dilution rate, the concentration of H_2 became very low at the low dilution rates, making the formation of acetate plus $2H_2$ thermodynamically more favorable [18]. Moreover, in batch cultures (Hungate tubes and serum bottles), the addition of H_2 gas to the headspace did not alter the ratio of acetate to butyrate [24]. This result may be explained by the self-generated H_2 which is already at a high enough concentration in the liquid culture in a

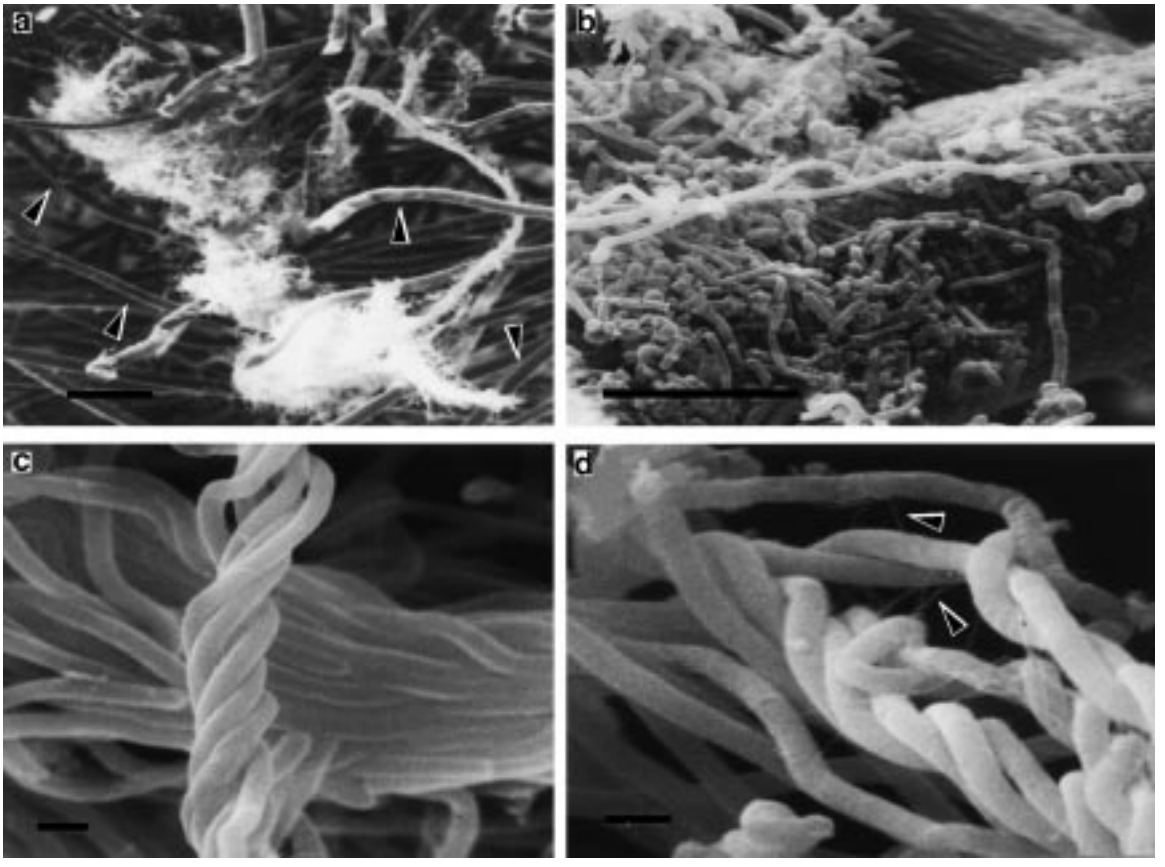

Figure 5 Scanning electron micrographs of bacterial growth on and in the fiber discs. (a) Cell agglomerate trapped between the fibers (120 × magnification, scale bar = 100 μm); some of the fibers are pointed out by arrows; (b) growing cells attached to a fiber presented at 2700 × magnification (scale bar = 10 μm); (c) enlargement of a section with cells from (a) at 6000 × magnification (scale bar = 1 μm); (d) exopolymer filaments (arrows) and fibers between the cells shown at 8500 × magnification (scale bar = 1 μm).

closed culture tube to cause the shift from the more energy-producing formation of acetate.
(glucose → 2 acetate + 4 H_2 + 2 CO_2 + 4 ATP, ΔG^0 = 206.3 kJ × mol^{-1})
to the less H_2-producing butyrate formation:
(glucose → butyrate + 2 H_2 + 2 CO_2 + 3 ATP, ΔG^0 = 254.0 kJ × mol^{-1}).
When H_2 was constantly removed by flushing or by growing the cells under shaking, with a large head-gas space (15 ml in a 2-liter serum bottle), the cells shifted as predicted to acetate formation without accumulating enough H_2 to cause the shift to butyrate (unpublished results). This suggests that the above conclusions are correct. A similar effect has been described for ethanol formation from cellulose by *C. thermocellum* [7].

The morphologically changed cells, which were observed in the rotary fermentor, but not in batch cultures and which reverted to 'normal' cells in subsequent batch subcultures indicate that the culture conditions in the rotary fermentor are indeed different to those in batch or conventional continuous cultures. In addition, many cells contained large amounts of granulose inclusions and exhibited cell wall degeneration. In *C. acetobutylicum* formation of granulose precedes sporulation [16] and polysaccharide formation is affected by acetate and butyrate addition [12]. *C. thermosaccharolyticum*, when arrested in the stage of early sporulation, forms elongated cells [11]. Thus, our assumption is that large cells are caused by an initiation of sporulation but then, because of the culture conditions, aborted.

The observed fluctuations of butyrate and acetate concentrations in the rotary fermentor were apparently not due to inhibitory concentrations of butyrate or acetate. In fact, the observed concentrations were far below the demonstrated inhibitory concentrations (350 mM, independently of a growth in pH between 8.0 and 5.5, and acetate concentrations above 500 mM; Canganella *et al*, unpublished results). However, the addition of 30 mM or more acetate under these conditions increased the formation of butyrate up to 2.5-fold or even up to 3- to 5-fold when the yeast extract concentration was increased to 1 or 2% (w/v). A re- utilization of formed acetate and its conversion to butyrate has been described for *C. tyrobutyricum* [14] and it is indicated for *C. thermobutyricum* by the conversion of ^{14}C-acetate to butyrate (Canganella *et al*, unpublished results).

Thus, it is hypothesized that the large fluctuation between acetate and butyrate formation observed during the cultivation of *C. thermobutyricum* in the rotary fermentor can—at least partially—be explained by the re-uptake of the acetate formed. The use of a medium with elevated concentrations of acetate (eg, using hydrolyzed hemicellulosic material containing acetylated xylan as added substrate) could lead to a better butyrate formation.

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