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Growth rate dependence of solventogenesis and solvents produced by *Clostridium beijerinckii*

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Summary. Acidogenesis and solventogenesis by *Clostridium beijerinckii* NRRL B-593 has been studied in batch growth, and in sucrose-limited chemostat and recycling fermentor growth. Cells grown in batch culture without pH control primarily produced either butyric and acetic acids, or these acids plus butanol, ethanol and isopropanol in ratios depending on the medium's content of reducing agent, calcium and iron. Cells in chemostat-culture at a mass doubling time (t_d) of 5.8 h produced primarily butyric and acetic acids at pH 6.8 and these acids plus butanol, ethanol and isopropanol at pH 4.8. Cells grown in a recycling fermentor (in which the t_d continuously increases) at pH 6.8 entered solventogenesis at a t_d of 43 h, producing primarily propanol, ethanol and butanol, along with butyric acid, but with greatly decreased production of acetic acid. Although "clostridial form" morphology, succeeded by sporulation, usually accompanied solventogenesis, the association was not invariant so that solventogenesis and sporogenesis can occur separately in this species.

Introduction

Some species of saccharolytic clostridia ferment sugars in two stages. In the first stage of the fermentation, butyric and acetic acids are the major products and smaller amounts of other organic acids, lactic in particular, may be produced, along with ethanol. The second stage develops as the culture ages and butanol, ethanol and acetone or isopropanol become the primary products.

The commercial value of the solvents makes understanding the physiological and metabolic basis of the shift from stage one to stage two fermentation of practical as well as theoretical interest. In *Clostridium acetobutylicum,* the shift occurs when the pH of the fermentation broth falls below five, and it has been shown (Terracciano & Kashket 1986) that a critical correlate of the pH factor is the presence in the cell of undissociated organic acids. When the intracellular concentration of acetic plus butyric acids reaches 40 to 45 mM, the shift from acidogenic to solventogenic metabolism begins. Reducing equivalents are used to convert both previously and newly formed butyric acid to butanol, and the metabolism necessary for the formation of acetone and its reduction product, isopropanol, occurs. However, George and Chen (1983) reported that *Clostridium beijerinckii* can shift from acidogenesis to solventogenesis when the pH of the broth in batch cultures is kept at 6.8. Therefore, in this species, an acid milieu is not necessary for the fermentation shift to occur and the internal level of undissociated acids may not be critical.

Inspection of the batch growth curves of *C. beijerinckii* published by these investigators shows that the shift to solventogenesis occured approximately at a mass doubling time between 20 and 25 h. That is, it resembled other batch-grown solventogenic clostridia in that the shift occurred at slow growth rates during the later stages of the culture.

It has been shown that *Bacillus polymyxa* (Arbige and Chesbro 1982), *Escherichia coli* (Chesbro et al. 1979; Van Verseveldt et al. 1984b) and *Paracoccus denitrificans* (Van Verseveldt et al. 1984b) show three distinct patterns of metabolic behavior

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(i.e. metabolic domains (Van Verseveldt et al. 1984a; 1984b). Each pattern is displayed within a defined growth rate range.

The first range is from the shortest mass doubling time of which the organism is capable to about 12 h. In *E. coli,* basal levels of the regulatory nucleotide guanosine 5′-diphosphate 3′-diphosphate (ppGpp) are present in the cell. The second range appears at about a mass doubling time of 20 h and the cellular level of ppGpp commences to rise until it reaches a maximum at 65 h. Restricted synthesis of ribosome components and slowed translation then occur (stringent regulation) in populations that are wild type in the genes whose products control the synthesis of ppGpp. This restriction of biosynthesis marks the beginning of the third range, which then extends to mass doubling times of at least 180 h and longer.

The mass doubling time of the acidogenic to solventogenic shift in batch cultures of *C. beijerinckii* grown at neutral pH thus corresponded to the mass doubling time at which the shift in metabolic behavior occurs in other bacteria going from the first to the second growth rate range. Consequently, we have sought to establish if the acidogenic-solventogenic shift was part of a growth-rate dependent, metabolic domain shift in *C. beijerinckii.*

Materials and methods

Bacterial strain, maintenance and bacteriological analyses. *Clostridium beijerinckii* strain NRRL B-593 was provided by Nakamura from the collection of the Northern Regional Research Laboratories. This strain was maintained as a spore stock in a sterile 1:1:1 mixture of garden soil, sand and $CaCO_3$. To produce active cultures, thioglycollate broth was seeded from the spore stock, heat-shocked for 10 min at 80° C and held at 30° C until growth was obtained. Transfers, culture on solid media and growth as batch cultures in 100—500 ml volumes were conducted in an anaerobic chamber (Coy Laboratory Products Inc, Ann Arbor, Mich, USA) containing a mixture of 85% N_2, 10% H_2 and 5% CO_2.

Except where noted otherwise in individual experiments, the medium was modified from that of George and Chen (1983), in g/l: Na_2SO_4, 0.18; K_2HPO_4, 3.48; biotin, 0.01; p-aminobenzoic acid, 0.01; sucrose, 60.0; tryptone, 1.0; yeast extract, 2.5; 1.0 ml of a mineral stock solution (George et al. 1983), and 1.5% agar when a solid medium was reguired.

Cell samples fixed with 1% formaldehyde were used in all analyses. Growth in liquid media was estimated optically using a Klett colorimeter (Klett Manufacturing Co, NY USA) with a #64 filter. Bacterial dry weights, used in yield calculations, were determined on polycarbonate membrane filters as described previously (Chesbro et al. 1979) except that the method of O'Toole (1983) was used to compensate for moisture absorbed during weighing operations. Microscopic examination of cultures for phase dark and phase-bright cell inclusions and for spores were made on wet mounts using an Olympus BH-2 (Macon Instruments Co., Norwood, Mass, USA) Differential counts of the foregoing and of vegetative cells were made by phase contrast examination of samples in a Petroff-Hauser chamber (Hausser Scientific, Blue Bell, Pa, USA).

Growth systems. For batch culture, 3 ml of an active culture were used to seed 100 ml of modified George and Chen medium (1983) as described, or altered, where noted, by addition of reducing agents (0.5 g/l sodium thioglycollate, 0.03% sodium or 0.5 g/l cysteine), by addition of a chelator (0.1 g/l nitrilotriacetic acid), or by omission of $CaCl_2$, $FeCl_3$ or both. All sample flasks were incubated at 30° C in the anaerobic chamber.

To seed the chemostat and recycling fermentor, 10 ml of an active culture was added to 300 ml of modified George and Chen medium which was incubated in a 30° C anaerobic chamber for 24—36 h and then used to charge the anaerobic fermentor (Chesbro et al. 1979).

The fermentor can be used as a chemostat or a biomass recycling fermentor (Chesbro et al. 1979) by switching from continuous withdrawal from the growth vessel (chemostat operation) to a recycle loop containing a 0.2 μM polycarbonate membrane (Nuclepore, Pleasanton, Calif, USA) which allows for the separation of spent medium from the cells, which are washed back into the fermentor's growth vessel. The medium used was modified George and Chen with sucrose limiting at 3.54 g/l. The pH was kept at 6.8 by the addition of 1 M NaOH. The fluid volume in the fermentor was 500 ml and the temperature was 35° C. The broth surface was continuously swept with 0_2-free N_2.

Chemical Analyses. For the analysis of solvents shown in Fig. 1, a Hewlett-Packard 5710A/30A gas chromatograph

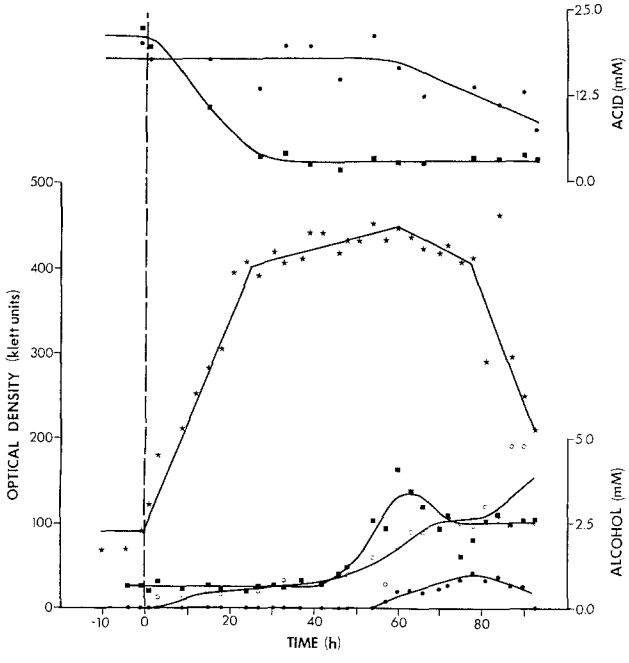

Fig. 1. *Clostridium beijerinckii* NRRL B-593 grown in a sucrose limiting medium at 35° C and a constant pH of 6.8 in chemostat mode ($D = 0.12\ h^{-1}$) and then switched to recycle mode at time indicated by dashed line: (★) optical density, (■) acetic acid and ethanol, (●) butyric acid and butanol, and (○) propanol. Isopropanol was not detected

(Hewlett Packard, Avondale, PA) equipped with a flame ionization detector and a stainless steel SP-1500 column packed with B/3% Carbopack on 80/120 mesh (Supelco Inc., Bellfonte, Pa, USA) was used. Two µl of the fermentation broth, clarified by centrifugation, was the amount injected. The column was operated at 70° C to 200° C at 8° C/min. The injection port and detector were held at 250° C and 200° C respectively and helium (carrier gas) flow rate was 20 ml/min. To identify and quantitate all other fermentation solvents and organic acids, a Perkin-Elmer 3920 instrument (Norwalk, Conn) was used. A glass column packed with 80/120 Carbopack B 3% SP-1500 (Supelco Inc.) with an oven temperature gradient starting at 70° C to 170° C at a rate of 8° C/min was used to identify solvents. The final holding time was 2 min and the sample volume was 2 µl. For organic acid analysis a glass column packed with 80/120 Carbopack B-DA 4% carbowax 20M (Supelco Inc.) was used. The oven was run isothermally at a temperature of 175° C and the injection volume was one µl. In all cases the injection port and detector were held at 200° C and nitrogen (carrier gas) flow rate was 24 ml/min. Retention times and standard curves for quantitation were determined using an alcohol mix and volatile organic acids standards (Supelco Inc.). To further identify propanol from the fermentation broth, 200 ml of supernate from a culture of strain NRRL B-593 was concentrated by differential freezing, microdistillation and reconcentration. The product was subjected to NMR analysis on a Varian EM 360A NMR spectrometer (Varian Inc, Sunnyvale, Calif, USA).

Results

Recycling fermentor

Clostridium beijerinckii strain NRRL B-593 was grown in the recycling fermentor at a constant pH of 6.8. This strain has been reported to produce butanol and isopropanol during solventogenesis in a 7.7:1.0 ratio (George et al. 1983) when cultured in batch at a constant pH of 6.8.

The growth curve and sequential shift from acidogenesis to solventogenesis is shown in Fig. 1. The system was first operated in chemostat mode for five residence times at a dilution value (D) of 0.12 h^{-1} ($t_d = 5.8$ h). In the chemostat mode, acidogenesis was evident and the culture produced butyric and acetic acids along with ethanol. After the change to recycling mode, the rate of acetic acid production immediately fell below that necessary to prevent washout from the fermentor. Butyric acid and ethanol continued to be produced at the rate produced in chemostat mode, but the culture now also produced small but measurable amounts of propanol. Examination of the microdistillation product by NMR confirmed that propanol was the product observed by gas chromatography. In the recycling mode, the typical pattern of linear mass increase appeared (Chesbro et al. 1979) producing a continuous increase in t_d. At 25 h after the switch to recycle, an abrupt inflection occurred in the growth curve at a t_d of 43 h producing a drop in the growth rate that increased the calculated t_d to 109 h. At 12 h after entry into this new growth-rate range, phase-dark inclusions were evident in the cells and the morphology of the cells became pleomorphic: swollen, "cigar-shaped" and irregular in outline. This is the "clostrial morphology" typical of sporogenesis and which has been associated with solventogenesis (Jones et al. 1982). At 59 h after the inflection in growth rate, phase-bright inclusions were observable, and 9 h after their appearance the culture was largely spores and the turbidity had decreased by 50%.

About 29 h after the culture entered this growth-rate range, solventogenesis increased. First, propanol and ethanol production rates increased, then 6 h later butanol began to be produced. The rate of propanol, ethanol and butanol production continued to increase until sporulation terminated the experiment.

The apparent molar growth yield from sucrose was 48 g/mol in the chemostat mode and 58 g/mol in the first growth interval following the change to recycling. The apparent molar yield dropped to 13 g/mol after the inflection. Thus, *C. beijerinckii*, unlike *Escherichia, Bacillus,* and *Paracoccus* species (Van Verseveldt et al. 1984a, 1984b) did not show two clearly marked decreases in growth yield as its t_d lengthened in the recycling fermentor, but only one whose magnitude and t_d value corresponded to those at which the other bacterial species had entered the growth rate range of stringent regulation.

At this point a change was made in the medium because the formulation produced a hazy, dark precipitate upon autoclaving. The precipitate seemed likely due to the formation of insoluble salts during autoclaving and to prevent it nitrilotriacetic acid (Jobses and Roels 1983), a chelating agent, was added to the medium before autoclaving.

The resultant growth pattern is shown in Fig. 2. Low levels of all solvents were produced throughout the experiment, but no significant production occured. Upon switching to recycle mode, acetic acid again washed out of the fermentor while butyric acid production remained essentially constant until sporulation terminated the experiment. By 14 h after switching to recycling, microscopic examination of the culture showed it to be sporulant.

The molar growth yield on sucrose was 67 g/mol in chemostat mode and 76 g/mol after shifting to recycling. Again, *C. beijerinckii* did not

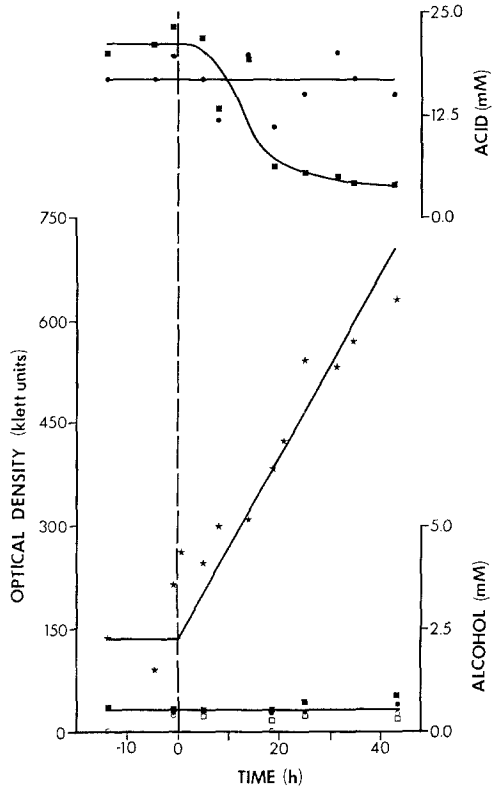

Fig. 2. *Clostridium beijerinckii* NRRL B-593 grown in sucrose limiting medium with 0.1 g/l nitrilotriacetic acid at 35° C and a constant pH of 6.8. The system began in chemostat mode ($D = 0.12\ h^{-1}$) and then was switched to recycle mode at the time indicated by the dashed line: (★) optical density, (■) acetic acid and ethanol, (●) butyric acid and butanol, (○) propanol, and (□) isopropanol

show the marked decrease in yield in this growth rate range shown by *Escherichia, Bacillus,* and *Paracoccus* (Van Verseveld et al. 1984a, 1984b).

Solventogenesis in chemostat culture under acid conditions

Strain NRRL B-593 was examined in chemostat culture at two pH levels to determine the effect of this factor on solventogenesis at a constant, rapid growth rate: $D = 0.12\ h^{-1}$ ($t_d = 5.8$ h). The pH was first held at 6.8. Examination of the broth showed that only butyric and acetic acids were being produced in quantity. When the pH was held at 4.8, the broth showed butanol, isopropanol, acetic and butyric acids to be present (Table 1). In both cases low levels of ethanol were present. The culture at pH 4.8 also showed the presence of "clostridial morphology" and phase-dark inclusions in the cells. The chemostat culture at pH 6.8 showed

Table 1. Product formation by *C. beijerinckii* NRRL B-593 in sucrose-limited chemostat growth at $D = 0.12\ h^{-1}$, 30° C, under neutral and acidic conditions

	Acids (mM)		Alcohols (mM)		
pH	Acetic	Butyric	Ethanol	Butanol	Isopropanol
4.8	13	8	0.4	2.5	0.6
6.8	28	8	0.7	0	0

neither of these manifestations, only the presence of regular bacilli-shaped cells.

Batch culture of Clostridium beijerinckii

C. beijerinckii was grown in batch culture without pH control. Table 2 shows the solvents produced in the sucrose-based medium and upon addition of reducing agents or deletion of calcium and iron from the medium. The addition of thioglycollate inhibited both sporulation and solventogenesis while addition of sodium sulfide, cysteine, nitrilotriacetic acid, or lowered $CaCl_2$ concentration permitted both sporulation and solventogenesis. Lowered levels of $FeCl_3$ or $FeCl_3$ and $CaCl_2$ prevented sporulation and permitted solvent production.

Discussion

Forsberg et al. (1987) reported that *C. beijerinckii* (synonym: *butylicum*) NRRL B-593 and NRC 33007, as well as one strain of *C. acetobutylicum,* produce propionic acid and 1,2 propanediol when grown on the methyl pentose sugar, rhamnose. Strain NRRL B-593 also produced propionic acid and propanol from certain substrate mixtures: arabinose plus 1,2 propanediol, pyruvate plus 1,2 propanediol and pyruvate plus lactate. Neither propionic acid nor propanol were detected when the strain was grown on glucose however, and they suggested that production of these compounds was repressed by glucose.

Our results and theirs are in accord when differences in experimental protocol are accounted for. Their experiments involved batch fermentation for four days in buffered, but not pH controlled media and employed the carbon/energy substrates at 1% (w/v) concentrations. The medium in some of our experiments contained a limiting sucrose level of 0.35%, and in the chemostat and recycling fermentor the extracellular concen-

Table 2. Fermentation products of *C. beijerinckii* NRRL B-593 from 6% sucrose after growth in batch culture at 30° C for 48 h in variously modified media

Medium modification	% Phase Dark spores	Acids (mM)		Alcohols (mM)			
		Acetic	Butyric	Ethanol	Butanol	Isopropanol	Acetone
None	34	9	6	0.6	78	11	0.4
0.5 g/l thioglycollate	0	17	27	0.1	9	0	0
0.03% sodium sulfide	53	6	5	1.5	102	16	0.5
0.5 g/l cysteine	23	9	9	0.5	79	16	1.5
0.1 g/l nitrilotriacetic acid	42	9	6	1.5	98	16	0.6
without added $CaCl_2$	69	9	11	0.6	69	16	1.5
without added $FeCl_3$	1	11	5	0.4	42	16	0.2
without added $FeCl_3$ and $CaCl_2$	0	5	3	0.6	62	26	0.4

tration of sucrose was effectively zero: conditions likely to minimize repressive effects of the carbon/energy source.

In our batch experiments where pH was not controlled, the final fermentation products of NRRL B-593 included butyric and acetic acids, ethanol, butanol and isopropanol, but not propanol. Butanol and isopropanol were in the 7:1 to 6:1 ratio reported by George and Chen (1983).

The chemostat experiments at a t_d of 5.8 h established that butanol and isopropanol were produced at pH 4.8, but not at pH 6.8. In the recycling fermentor, however, at pH 6.8 butanol and propanol were produced at increasing rates when the culture reached t_d longer than 109 h.

We conclude that *C. beijerinckii* produces butanol and isopropanol at more rapid growth rates in acid medium and butanol and propanol only at much slower growth rates in neutral medium. Ethanol was produced at all growth rates and at both pH levels.

"Clostridial morphology", a marker of sporogenesis (Jones et al. 1982), and phase-dark inclusion bodies were invariably observed in our studies whenever solventogenesis occured at pH 6.8. The relationship of sporogenesis to solventogenesis has received less attention in *C. beijerinckii* than in *C. acetobutylicum* (Holt et al. 1984; Jones et al. 1982; Long et al. 1984; Reysenbach et al. 1986; Roos et al. 1985). Meinecke et al. (1984) concluded that, "... the signals for the initiation of sporulation and solvent production are tightly connected." (in *C. acetobutylicum*). Jobses and Roels (1983) noted that spores were absent from their solventogenic culture of *C. beijerinckii* in continuous culture at pH 5.0.

In one recycling fermentor experiment in which a chelating agent was added to the medium, the culture entered sporulation while still acidogenic. This chance finding leads us to suggest that solventogenesis and sporogenesis are not necessarily connected in *C. beijerinckii,* although they occur sequentially at pH 6.8 and slow growth rates or at pH 4.8 and fast growth rates.

C. beijerinckii did not exhibit the two abrupt inflections in growth rate and molar growth yield from the energy substrate previously observed in other bacteria (Van Verseveldt et al. 1984a; 1984b) growing in the recycling fermentor. Those species exhibit an inflection in growth rate and molar growth yield between t_d values of 10 and 15 h that was not observed in *C. beijerinckii.* There is, however, an abrupt change in catabolic pathway in *C. beijerinckii* associated with the shift from chemostat to recycling fermentor at a t_d of 5.8 h. As the t_d increases after the shift, the rate of acetic acid production drops by nearly 90%, while a low rate of propanol synthesis commences.

After the single growth rate/molar growth yield inflection observed in *C. beijerinckii* at a t_d of 43 h, solventogenesis increased until truncated by sporulation. It has been shown in a taxonomically broad range of bacteria, including *Bacillus,* that cells at very slow growth rates are under global regulation by ppGpp (Arbige and Chesbro 1982; Van Verseveldt et al. 1984b). Our results argue that the expression of the solventogenic pathways are under the influence of this regulatory nucleotide and we are investigating this possibility now.

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