

The Cause of “Acid Crash” and “Acidogenic Fermentations” During the Batch Acetone-Butanol-Ethanol (ABE-) Fermentation Process

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Abstract

Experiments were performed to determine the cause of “acid crash”, a phenomenon which occasionally occurs in pH-uncontrolled batch fermentations resulting in premature cessation of ABE (acetone – butanol) production. The results indicate that “acid crash” occurs when the concentration of undissociated acids in the broth exceeds 57 – 60 mmol/l. Prevention can be achieved by introducing some limited pH control to minimize the concentration of undissociated acids or by slowing the metabolic rate, and thus the rate of acid production, by, for example, lowering the fermentation temperature. “Acidogenic fermentations”, which occur when batch fermentations are performed at pH values close to neutrality, are due to rapid production of acids followed by inhibition of solventogenesis when the total acid concentration reaches 240 – 250 mmol/l. Solventogenesis can be achieved at these pH values by lowering the glucose uptake rate / acid production rate by use of e.g. elevated glucose or lowered yeast extract concentrations in the growth medium.

Introduction

The phenomenon known as “acid crash” is an occasional feature of batch fermentations which are performed without any pH control. In a typical batch acetone- butanol (ABE-) fermentation using *Clostridium acetobutylicum* or *Clostridium beijerinckii*, the process is characterized by two phases. In the first, known as the acidogenic phase, sugars are converted to acetic and butyric acids accompanied by a decrease in culture pH value. In the second, known as the solventogenic phase, which is possibly triggered by a minimum concentration of undissociated acids, sugars and some of the acids are converted to acetone, butanol and

ethanol, accompanied by a pH increase. When an “acid crash” occurs, excess of acid production takes place without a significant switch to the solventogenic phase. The term “acid crash” was used in industry to depict this phenomenon, and should be introduced into the scientific literature to clarify the terminology when low-solvent producing ABE fermentations are described: The phenomenon should not be confused with that of “culture degeneration”, which is a feature of the strain rather than of a particular batch fermentation and which takes place over a longer time period, particularly in continuous culture, and is normally associated with genetic change. In this case there is a slow “drift” from a solventogenic to an acidogenic culture (due to accumulation of mutant organisms), rather than an apparent failure of a “switch” from an acidogenic to a solventogenic culture. In detailed studies of degeneration in continuous cultures it was observed that a population of non-solventogenic organisms appears, which coexists for a while with the solventogenic ones, but takes over gradually (Woolley and Morris, 1990). Nor should the “acid crash” be confused with those batch fermentations which are performed at pH values close to neutrality, e.g. at pH 6, where acids are the predominant products. These processes may be referred to as “acidogenic fermentations”. Table 1 gives an overview of the terminology and some references in which the three different phenomena were described. However, the term “degeneration” is mostly used in connection with all non-solventogenic cultures.

The purpose of the present work was, primarily, to determine the cause of the “acid crash” in batch cultures, and so devise ways to prevent its occurrence. Secondly, the reasons why solvents are not normally produced during “acidogenic fermentations” performed at pH 6 were sought.

Results and Discussion

Fermentations of *Clostridium beijerinckii* NRRL B592 were performed in a laboratory scale stirred tank bioreactor in a setup described earlier (Schuster *et al.*, 1998 b). A series of five fermentations was performed at 34°C, without any control of the pH value of the culture and under conditions which were believed to be identical for all experiments. The results, after cessation of each fermentation, are summarised in Table 2. Experiment No. 1 represents the desired situation, *i.e.* glucose was initially converted to acids, causing a decline in pH value, but subsequently the “switch” to solventogenesis occurred and a high concentration of ABE was produced. Glucose was almost fully utilized and the final concentration of acids (dissociated + undissociated, acetic + butyric) was very low. The maximum observed concentration of undissociated acids was 53 mmol/l and occurred just after the onset of solventogenesis, after which it decreased. The acid

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Table 1. Clarification of Terms for the Phenomena of Low-Solvent Producing ABE Fermentations (Extended from Schuster *et al.*, 1998a)

Type of low-solvent phenomenon	Degeneration	Acid Crash	Acidogenic fermentation
Occurrence	Continuous cultures (2,5,6,7) Repeated batch cultures (vegetative) (1,8) Cultures on solid media (1,4,6)	Batch cultures without pH control (8) Continuous cultures with control problems	Batch (9) and Continuous (3) cultures at high pH
"Speed" (time factor)	Very slow	Fast	Instant
Feature of	Strain	Particular fermentation run	Particular fermentation run
Proposed background	Genetic loss / change Population selection	Fermentation kinetics Genetic properties	Metabolic regulation

Examples for the phenomenon in the literature, mostly termed "degeneration" for both acid crash and degeneration:

- (1) Kutzenok and Aschner, 1952
- (2) Finn and Nowrey, 1958
- (3) Bahl *et al.*, 1982
- (4) Adler and Crow, 1987
- (5) Clarke *et al.*, 1988
- (6) Woolley and Morris, 1990
- (7) Reardon and Bailey, 1992
- (8) Kashket and Cao, 1995
- (9) this work

(dissociated + undissociated, acetic + butyric) production rate was measured during the period of rapid bacterial growth prior to the onset of solventogenesis.

In contrast to Experiment No. 1, the four other experiments suffered from an "acid crash". In each, solventogenesis was initiated but within a relatively short time all metabolic activity, including glucose uptake, acid production and ABE production ceased (Figure 1b). Noteworthy is the fact that in each of these experiments the concentration of undissociated acids at the time of cessation (the maximum observed) was 59 mmol/l or higher, while the acid production rate prior to solventogenesis was noticeably higher than in Experiment No.1. Interestingly, in Experiment No.5, about 60 h after cessation, metabolic activity recommenced (Figure 1c). Subsequently, all of the glucose was utilized and the ABE concentration reached 280 mmol/l. This phenomenon of "recommencement" after an "acid crash" is well known to researchers in the field. Close examination of the data revealed that during the period of apparent metabolic inactivity the concentration of undissociated acids slowly decreased from 65 mmol/l to less than 55 mmol/l. It was at this point that solventogenesis recommenced.

Two further experiments were carried out under identical conditions but at 30°C. The results are summarised in Table 3. Experiment No. 6 showed a

solventogenic fermentation but it was terminated, unfortunately, while glucose was still being utilized. The maximum observed concentration of undissociated acids was 57 mmol/l and occurred just after the start of solventogenesis, after which it decreased. In contrast, Experiment No. 7 showed an "acid crash", and all metabolic activity ceased shortly after the commencement of solventogenesis. At this time the undissociated acids concentration was 80 mmol/l. As in Experiment No. 5, however, after a further period of about 60 h, and when the concentration of undissociated acids had decreased from 80 mmol/l to 44 mmol/l, solventogenesis and glucose uptake recommenced. The final ABE concentration was 270 mmol/l.

Examination of the data in Table 2 and Table 3 shows that a common feature and perhaps a probable cause of the "acid crash" is the high concentration of undissociated acids which accumulates in the culture broth. The time course of undissociated acid concentration in relation to the outcome of the fermentation experiments 1 – 7 are summarized in Figure 2, showing the suggested threshold level of undissociated acids above which solventogenesis ceases. These acids are known to be toxic and, indeed, solventogenesis is believed to be a detoxification process (Rogers *et al.*, 1993). This high concentration, in turn, may be related to the acid production rate during the early stage

Table 2. Summary of Fermentations Performed at 34°C, with No Culture pH Control

Experiment No.	Glucose utilized mmol/l	ABE final concentration mmol/l	Acids, final concentration mmol/l	pH, minimum observed	Undissociated acids maximum concentration mmol/l	Acid production rate (*) mmol/l.h
1	330	270	28	4.4	53	3.9
2	150	50	93	4.2	75	6.0
3	147	87	67	4.5	59	6.4
4	145	68	88	4.4	70	8.3
5	120	80	80	4.4	65	7.1

(*) determined in the phase of rapid growth, before onset of solventogenesis

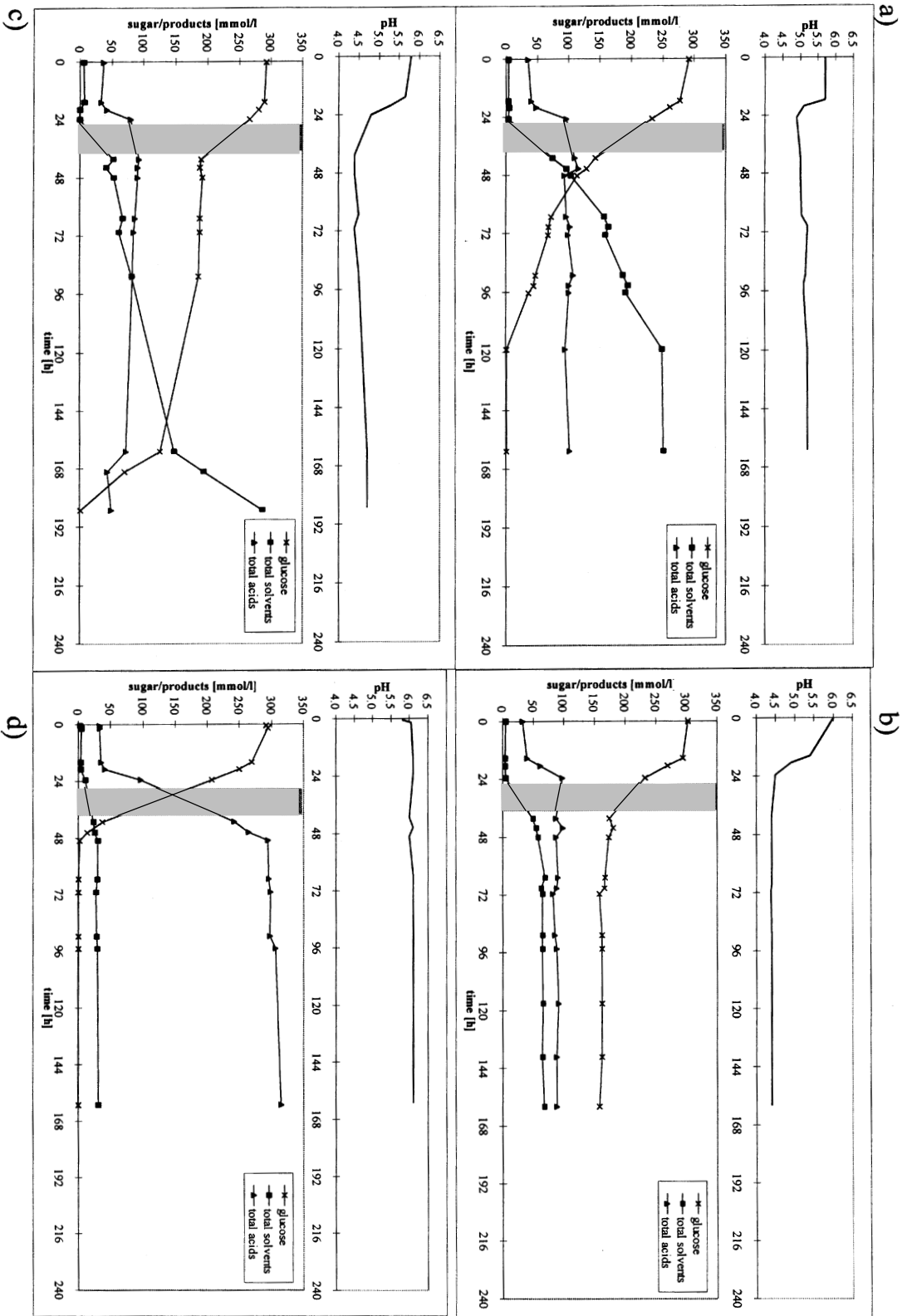


Figure 1. Examples for four different outcomes of batch ABE fermentation runs. All runs were performed at 34 deg C. Symbols: X: glucose, ■: total solvents, ▲: total acids. The grey band marks the solvent shift (butanol > 0.1 g/l). (a) Good solventogenic fermentation: 60 g/l glucose, 5 g/l yeast extract, pH minimum set to 5.0 (Experiment 8); (b) Acid crash: Batch fermentation: 60 g/l glucose, 5 g/l yeast extract, no pH-control (Experiment 4); (c) Acid crash and recommencement: 60 g/l glucose, 5 g/l yeast extract, no pH-control (Experiment 5); (d) Acidogenic fermentation: 60 g/l glucose, 5.0 g/l yeast extract, pH 6 (Experiment 15).

Table 3. Summary of Fermentations Performed at 30°C, with No Culture pH Control

Experiment No.	Glucose utilized mmol/l	ABE final concentration mmol/l	Acids, final concentration mmol/l	pH, minimum observed	Undissociated acids maximum concentration mmol/l	Acid production rate (*) mmol/l.h
6	288	159	25	4.4	57	2.2
7	144	81	100	4.2	80	3.7

(*) determined in the phase of rapid growth, before onset of solventogenesis

of the fermentation. Thus, if acids are produced too quickly they accumulate to toxic levels before they can be removed by solventogenesis. To investigate this and to seek ways to prevent the “acid crash”, three further fermentations were performed (Table 4).

Experiment No. 8 (Fig. 1a) was performed at 34°C and the culture pH value was not allowed to decrease below pH 5.0, thus minimizing the concentration of undissociated acids. The rate of acid production was high, reflecting the higher operational pH value, but the concentration of undissociated acids did not exceed 50 mmol/l, and the ABE concentration was high. In Experiment No. 9, the process was maintained at the lower temperature of 28°C without any pH control. The acid production rate was low (reflecting the lower operational temperature), the concentration of undissociated acids reached a peak of 56 mmol/l after which it decreased, and the fermentation was solventogenic. Finally, in Experiment No.10, the initial glucose concentration was 90 g/l (rather than 60 g/l) and the process was performed without pH control. However, the initial temperature of 28°C was maintained only until the initiation of solventogenesis, after which it was increased to 34°C. This fermentation was highly solventogenic showing a maximum undissociated acids concentration of only 53 mmol/l shortly after the initiation of solventogenesis. The ABE concentration reached 292 mmol/l.

As a result of these experiments it is proposed that the “acid crash” occurs in those batch fermentations where

the combined concentration of undissociated acetic and butyric acids exceeds a critical threshold value. Above this value metabolic activity, including glucose uptake and ABE production, ceases. A similar conclusion has been reached by Evans *et al.* (1998) using mutants that displayed reduced growth rates. The exact value of the threshold is difficult to determine, but it is in the range 57 – 60 mmol/l. This may vary, however, depending on the relative concentrations of acetic and butyric acids, since the latter is known to be the more toxic. The main factors which contribute to the concentration of undissociated acids are the acid production rate and the culture pH value. The former will be influenced by the glucose uptake rate (*i.e.* the growth rate), the rate of decrease of acid production as solventogenesis commences and the subsequent rate of acid uptake. Given that the initiation of solventogenesis requires a minimum concentration of undissociated acids (say, 20 mmol/l), it is proposed that the “acid crash” occurs when the concentration continues to rise until the maximum concentration is exceeded, causing cessation of glucose uptake and, thus, ABE production. On this basis, then, the “acid crash” is not due to the failure of a switch from an acidogenic to a solventogenic culture. Rather, it is due to rapid termination of solventogenesis after the switch has occurred.

Several techniques are available to prevent the “acid crash” and they all involve minimizing the undissociated acid production rate and, thus, the undissociated acids concentration. First, the process can be operated with pH

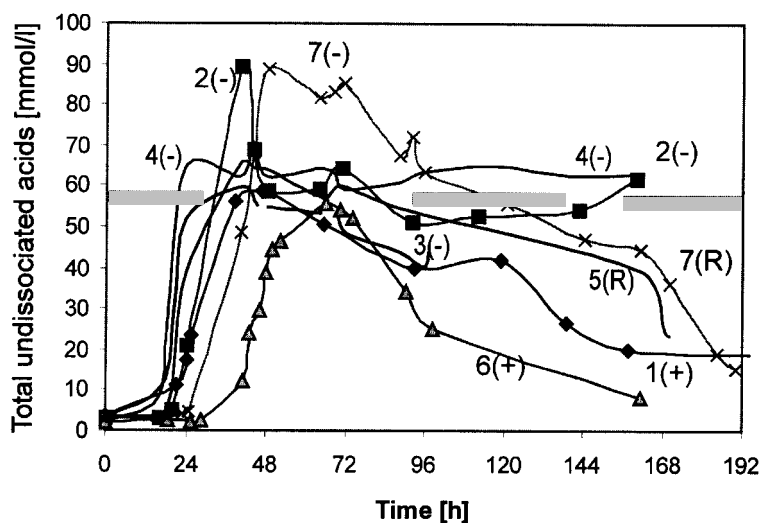


Figure 2. Time course of the concentration of total undissociated acids in experiments 1 – 7. Highly solventogenic fermentations are marked “(+)”, predominantly acidogenic ones with “(-)”. The two experiments were solvent production recommenced are marked “R”. The suggested threshold level above which solventogenesis is inhibited and the metabolism ceases is marked with the grey band.

Table 4. Summary of Fermentations Performed to Prevent an "Acid Crash"

Experiment No.	Condition	Glucose utilized mmol/l	ABE final concentration mmol/l	Acids final concentration mmol/l	pH, minimum observed	Undissociated acids maximum concentration mmol/l	Acid production rate mmol/l.h
8	34°C	291	251	101	5.0	49	8.5
9	pH 5.0 28°C	194	157	83	4.4	56	2.1
10	no pH control 28°C/34°C no pH control glucose 90 g/l	494	292	20	4.4	53	4.0

control at, for example, pH 5.0. (Figure 1a). At this pH value the majority of the acids will be in the dissociated form. However, this approach may leave an unacceptably high acid concentration in the broth (refer to Experiment 8). Secondly, the fermentation can be performed at a relatively low temperature, for example 28°C as has been suggested previously (Kashket and Cao, 1995). However, although this is a successful approach the overall metabolic rate, and thus the fermenter productivity, may be too low. Finally, it is possible to commence the fermentation at a relatively low temperature and then, after solventogenesis has been initiated, increase the temperature to a more productive value. Although Experiment No. 10 was operated at a high initial glucose concentration (90 g/l) it is believed that the same result would have been achieved at the more usual glucose concentration of 60 g/l (unpublished observations).

The second problem to be addressed in this paper is the reason why ABE production does not normally occur in cultures which are operated at pH values approaching neutrality (Long *et al.*, 1984; Jones and Woods, 1986). In this case the fermentation is almost entirely acidogenic, although occasional instances have been recorded of solventogenesis at these pH values (George and Chen, 1983; Holt *et al.*, 1984; Ennis and Maddox, 1987). A series of fermentations was performed at 34°C and with pH control at pH 6.0, in which the initial glucose and yeast extract concentrations in the medium were varied. The results are summarized in Table 5. An example of a fermentation time course (Experiment 15) is shown in Figure 1d. When glucose was initially at 60 g/l and yeast extract at 5 g/l the fermentation was acidogenic, and the glucose utilization rate and acid production rate, expressed per unit biomass, were relatively high. However, examination of the data reveals that solventogenesis did commence, at an undissociated acids concentration of 6.9 mmol/l, although production soon ceased. In the next experiment, where the initial yeast extract concentration was decreased to 1 g/l, similar results were obtained although the glucose utilization and acid production rates were lower. At an initial yeast extract concentration of 0.05 g/l, however, a marked

difference was seen. The glucose utilization and acid production rates were markedly lower while the ABE concentration was markedly higher.

Similar results were observed when the initial glucose concentration was at 140 g/l, with yeast extract at 1 g/l (Experiment 14).

Several points arise from these results. First, solventogenesis was initiated in all of the experiments, and this occurred at an undissociated acids concentration lower than 10 mmol/l. Subsequently, acids and ABE production proceeded simultaneously. The question whether these two pathways are performed by a mixed population of solventogenic and acidogenic cells, or by the individual cells simultaneously, remains open. Secondly, in the strongly acidogenic fermentations, ABE production ceased when the total acid concentration (acetic + butyric, dissociated + undissociated) reached 240 – 250 mmol/l (time course data not shown). Thirdly, in the strongly acidogenic fermentations, the glucose utilization and acid production rates were considerably higher than in those fermentations where high ABE concentrations were observed.

At one time it was postulated that solventogenesis did not occur in batch fermentations at pH 6.0 because the concentration of undissociated acids never became sufficiently high to warrant their detoxification to ABE (Rogers *et al.*, 1993). However, the present experiments show that this is not necessarily true and that provided that the glucose uptake and acid production rates are sufficiently low, relatively high concentrations of ABE can be produced, albeit in conjunction with acids. When the acid production rate is high, however, as it usually is at pH values approaching neutrality, there is an inhibitory effect on ABE production and the latter ceases when the total acids concentration reaches 240 – 250 mmol/l. It is proposed, therefore, that in batch fermentations performed at pH values approaching neutrality, the acid production rate is normally so high that toxic concentrations of acids are formed very rapidly, thus inhibiting solventogenesis and restricting the ABE concentration to low levels. When the

Table 5. Summary of Fermentations Performed at 34°C and pH6.0

Experiment No.	Glucose initial concentration g/l	Yeast extract initial concentration g/l	Glucose Utilization mmol/l	Glucose utilization rate mmol/g.h	ABE concentration mmol/l	Total acids mmol/l mmol/g.h	Acid production rate at initiation of	Undissociated acid concentration solventogenesis mmol/l
11	60	5	320	1.68	21	244	1.46	6.9
12	60	1	320	1.22	45	306	1.28	8.7
13	60	0.05	320	0.76	134	221	0.59	6.9
14	140	1	470	0.65	122	225	0.44	8.8
15	60	5	292	n.d.	31	315	n.d.	4.0

acid production rate is sufficiently low, as in the presence of high glucose (*i.e.* low water activity) or low yeast extract concentrations, solventogenesis can persist for a longer time period giving higher ABE concentrations. Since, at pH 6.0, the concentration of undissociated acids never reaches the threshold value of 57 – 60 mmol/l which is crucial for the “acid crash”, some other factor must be responsible for inhibition of solventogenesis at this higher pH value. Based on the information available, this factor may be the concentration of dissociated rather than undissociated acids, although the mechanism of such inhibition is unclear. Alternatively, since in those experiments where the “acid crash” occurred (*i.e.* the pH value was less than 5.0), the concentration of butyrate remained equal to or less than that of acetate, while in those experiments performed at pH6 the butyrate concentration was always higher than that of acetate (data not shown), it is possible that the inhibitory value is lower in the latter case since butyric acid is the more toxic species. It should also be noted, however, that in the “acidogenic fermentations” the inhibitory effect was on ABE production alone, while in the “acid crash”, glucose uptake also ceased. Whatever the case, prevention of both phenomena can be achieved, to a greater or lesser extent, by a lowering of the acid production rate, *i.e.* by imposing less than optimum growth conditions on the organism.

Experimental Procedure

Organism

Clostridium beijerinckii NRRL B592 was obtained from Richard Gapes, Vienna University of Technology, Vienna, Austria. The organism was maintained as a spore suspension in sterile distilled water at 4°C.

Cultivation

Spore suspension (2 ml) was added to 18 ml of sterile peptone water (5 g/l), heated for 90 s in an 85°C waterbath, immediately cooled under cold tap water and left at 20°C for 3-4 h for germination. This culture was used to inoculate 1.5 l of medium contained in a 2-l glass bioreactor of the stirred tank type (New Brunswick Scientific Co., USA) equipped with a pH probe and an optional pH controller. The equipment and its operation were as described by Schuster *et al.* (1998b) while the medium contained (g/l of distilled water): glucose, 60; yeast extract, 5; K₂HPO₄, 0.8; KH₂PO₄, 1.0; MgSO₄·7H₂O, 1.0; FeSO₄·7H₂O, 0.5; ammonium acetate, 3.0; *p*-aminobenzoic acid, 0.1.

Calculations

The acid formation rates were calculated from the time course of the acid concentrations in the phase of rapid growth before onset of solventogenesis as the slope of the linear regression curve.

The free acid concentrations were calculated according to the Henderson-Hasselbalch- buffer equation:

$$\text{pH} = \text{pK}_a + \log \frac{[\text{A}^-]}{[\text{HA}]}$$

$$[\text{HA}] = \frac{10^{-\text{pH}} \times c_{\text{total acid}}}{10^{-\text{pK}_a} + 10^{-\text{pH}}}$$

[HA]...	concentration of undissociated acid (mol/l),
[A ⁻] ...	concentration of dissociated acid (mol/l),
c _{total acid} ...	concentration of total acid (mol/l)
pK _a ...	negative decadic logarithm of the acid dissociation constant

This was done separately on a molar basis for both butyric and acetic acid, then the numbers were added to give the total undissociated acids.

Analyses

Solvents and acids were determined by gas chromatography (Ennis *et al.* 1986) while glucose was determined using a Yellow Springs Glucose

Analyzer. Cell counts were measured both microscopically and by reading the optical density of the culture at 615nm. Dry biomass was then estimated using a calibration curve.

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