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*Short communication*

# Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *Clostridium acetobutylicum*

**Fréderic Monot[1], Jean-Marc Engasser[2], and Henri Petitdemange[2]**

[1] Laboratoire des Sciences du Génie Chimique, CNRS-ENSIC Institut National Polytechnique de Lorraine, 1, rue Grandville, F-54042 Nancy Cedex
[2] Laboratoire de Chimie Biologique, Université de Nancy, B.P. 239, F-54506 Vandoeuvre les Nancy, France

**Summary.** The kinetics of growth and acid and solvent production are examined in batch fermentation of Clostridium acetobutylicum at pH between 4.5 and 6.0. At the lower pH, growth occurs in two consecutive phases and solvents are the main excreted metabolites. At the higher pH, there is a single growth phase with only acid formation. The influence of the pH can be correlated with a critical role of the concentration of undissociated butyric acid in the medium : cellular growth is inhibited above 0.5 g/l and solvent production starts at an undissociated acid level of 1.5 g/l. Reducing the intracellular acid dissociation by lowering the intracellular pH also favours the production of acetone and butanol.

## INTRODUCTION

The growth and metabolism of Clostridium acetobutylicum strongly depends on the composition of the medium and the age of the culture (Gottschal and Morris 1981 ; Monot et al. 1982 ; Monot and Engasser 1983). An essential regulatory parameter on cellular metabolism is the pH of the medium. With several strains of C. acetobutylicum an acidic pH below 5.0 was found optimal for acetone and butanol production. At higher pH only acids are generally excreted (Bahadur and Saraj 1960 ; Bahl et al. 1982). The butyric acid concentration in the medium has also been reported to be an important fermentation parameter, as the addition of butyric acid at a sufficiently low pH was observed to result in solvent formation (Gottschal and Morris 1981 ; Bahl et al. 1982 ; Martin et al. 1983)

We here present a more detailed analysis of the effect of pH on the kinetics of batch cultures of C. acetobutylicum, which suggests that both the influence of pH and butyric acid can be related to an essential role of the undissociated butyric acid on cellular growth and solvent production.

## MATERIALS AND METHODS

**Microorganism.** The organism used was Clostridium acetobutylicum strain ATCC 824. Spores of the culture were stored at 4°C in RCM ("Reinforced Clostridial Medium" -Oxoïd).

**Medium.** The culture medium was a synthetic one (Monot et al, 1982) containing the following components per liter of distilled water : 55 g glucose; 2.2 g ammonium acetate; 0.5 g $KH_2PO_4$ ; 0.5 g $K_2HPO_4.3H_2O$ ; 0.2 g $MgSO_4.7H_2O$ ; 0.01 g $FeSO_4.7H_2O$ ; 1.0 mg p-aminobenzoïc acid ; 0.01 mg biotin.

**Batch fermentation.** A 2-l Biolafitte fermentor was used. The agitation speed was maintained at 200 rpm and the temperature at 35°C. When regulated, the pH of the medium was controlled by automatic addition of 5 N NaOH. A 10% growing culture taken at the end of the growth phase was used as inoculum. Precultures were also made on the described medium. The fermentor medium was kept anaerobic by a flow of purified nitrogen before and after inoculation. The nitrogen flow was stopped once the culture was observed to be growing, since bacteria provided sufficient quantities of hydrogen and carbon dioxyde to realize their own anaerobiosis.

**Methods of analysis.** Cell concentration was estimated by optical density and by cell dry weight measurement using a predetermined correlation between optical density at 660 nm and cell dry weight.
Other analyses were made on supernatants of samples previously centrifuged at 12 000 rpm during 10 minutes. Residual glucose was deter-

*Offprint requests to:* J. M. Engasser

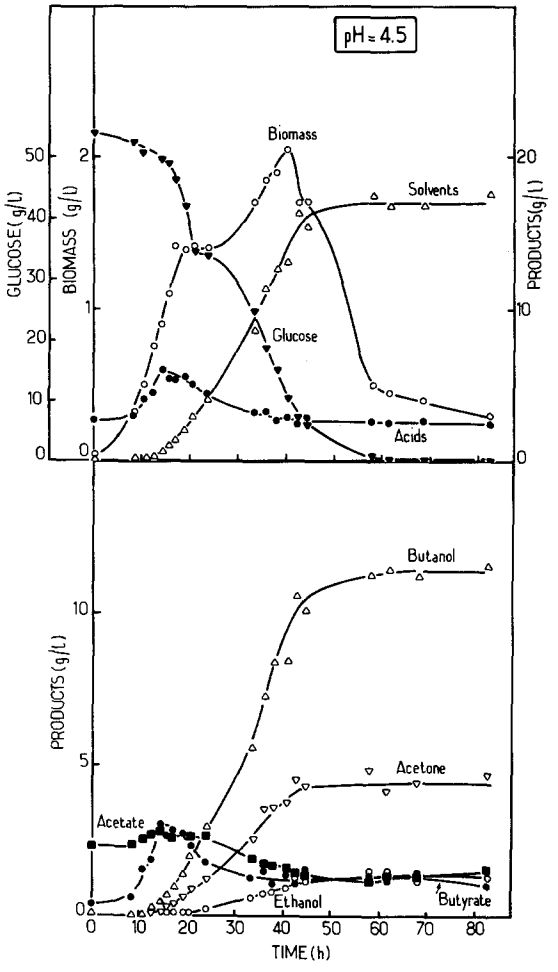


**Fig. 1a** Kinetics of a batch fermentation of Clostridium acetobutylicum at pH 4.5

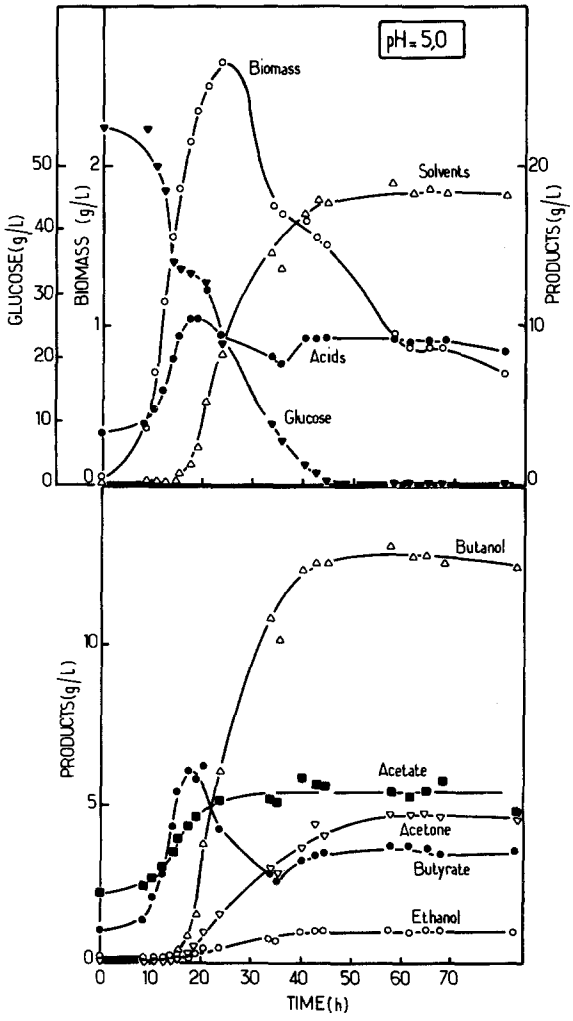


**Fig. 1b** Kinetics of a batch fermentation of Clostridium acetobutylicum at pH 5.0

mined on a Technicon autoanalyser with an hexokinase reagent. Ammoniacal nitrogen determination was based on the production of a coloured complex between ammonium, sodium salicylate and chlorine in an alcaline medium. This analysis was also made on a Technicon autoanalyser. Concentrations of solvents (ethanol, acetone, butanol) and acids (acetic and butyric) were determined by injecting acidified supernatants into a Intersmat IGC 121 FL gas chromatograph equipped with a flame ionization detector. Separation took place in a glass column, 2 m long by 2 mm in diameter, and packed with PORAPAK Q, 80/100 mesh. $N_2$ was used as carrier gas. Injector and detector temperatures were 220°C and column temperature was programmed from 160°C to 200° C. The analysis of chromatographic data were carried out by a Intersmat ICR 1B integrator.

## RESULTS AND DISCUSSION

### C. acetobutylicum fermentation kinetics at different regulated pH values

In order to establish the precise influence of pH en C. acetobutylicum metabolism, the fermentation kinetics was studied at four different controlled pH values : 4.5, 5.0, 5.5, 6.0. The results, reported in Fig. 1a to 1d, clearly demonstrate that both cellular growth and solvent production are strongly pH dependent.

One of the effects of pH relates to the growth pattern of C. acetobutylicum. At pH 4.5 growth takes place in two phases : a first phase with glucose utilization, then a second with consumption of glucose and acids. At a pH from 5.0 to 6.0, however, a single growth phase is observed with only glucose assimilation. The initial

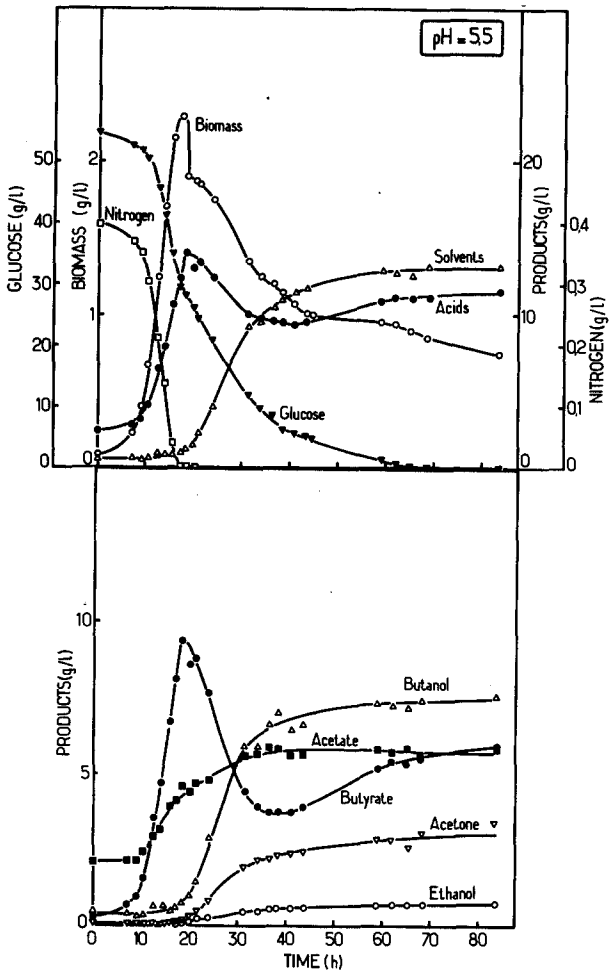


**Fig. 1c** Kinetics of a batch fermentation of Clostridium acetobutylicum at pH 5.5

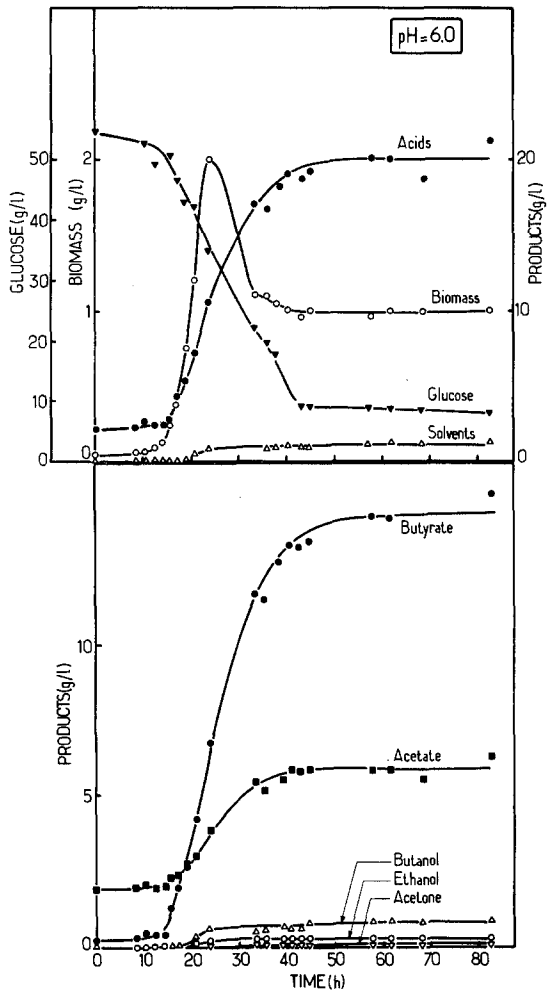


**Fig. 1d** Kinetics of a batch fermentation of Clostridium acetobutylicum at pH 6.0

specific growth rate is always close to 0.35 $hr^{-1}$, and the maximal biomass concentration near 2 g/l. Fig. 1c, which also records the nitrogen concentration in the medium, indicates that under our experimental conditions cellular growth is limited by nitrogen, as the maximal biomass concentration coincides with the point of nitrogen depletion in the medium.

The strongest effect of pH is on the production of acids and solvents. At pH 4.5 solvents are the dominant metabolites, and both acetic and butyric acid, produced during the first growth phase, are partly reassimilated for solvent production. At pH 6.0, a single growth-related acetic and butyric production phase with negligible solvent production is observed.

## Influence of undissociated butyric acid concentration on growth inhibition and solvent production

Butyric acid has previously been reported to inhibit cellular growth and induce solvent production. The comparison of the kinetic results at different values of fermentation pH does not reveal a simple correlation between the butyric acid concentration and growth inhibition or solvent formation. When taking into account the additional influence of pH on the acid dissociation, we found, however, a close relationship between growth inhibition and solvent production on the one hand, and the concentration of undissociated butyric acid in the medium on the other.

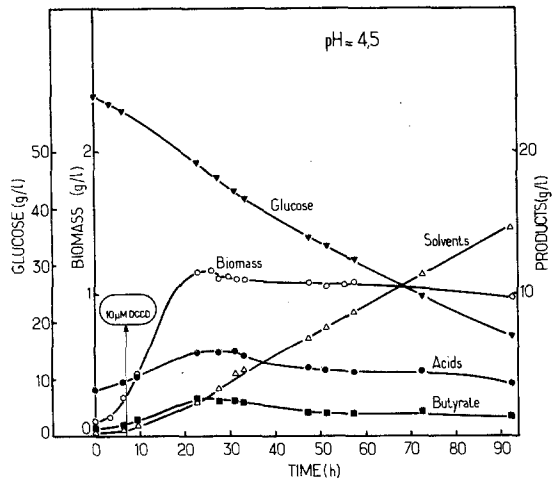


**Fig. 2a** Influence of the addition of N-N' dicyclohexylcarbodiimide (DCCD) on the kinetics of a batch fermentation of Clostridium acetobutylicum at pH 4.5.

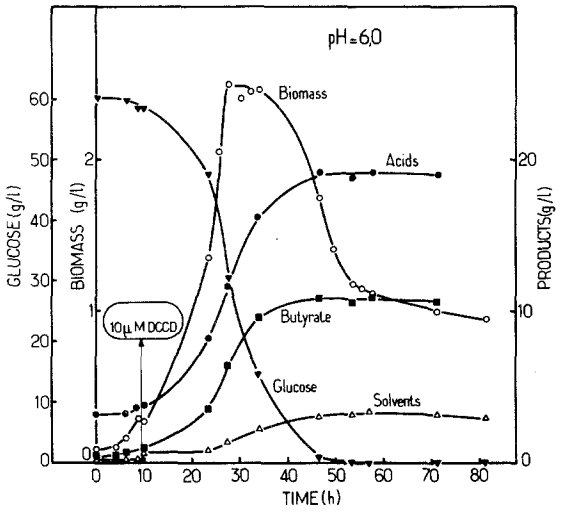


**Fig. 2b** Influence of the addition of N-N'dicyclohexylcarbodiimide (DCCD) on the kinetics of a batch fermentation of Clostridium acetobutylicum at pH 6.0.

From the previous kinetic results, we first evaluated at different pH the concentration of butyric acid at the end of the first exponential growth phase, i.e. when the initial specific growth rate starts to decrease. We calculated the corresponding concentration of undissociated butyric acid, assuming a dissociation constant of 4.8 for the acid. Whereas the inhibiting total butyric acid concentration varies from 1 to 5 g/l with increasing pH, the corresponding concentration of undissociated butyric acid, always remains close to 0.5 g/l.

Secondly, from the experimental results we evaluated the total butyric acid concentration at the beginning of solvent production. This critical concentration rises from 2.5 to 9.5 g/l when pH increases from 4.5 to 5.5. However, when calculating the corresponding concentration of undissociated butyric acid, it is very interesting to observe that between pH 4.0 and 5.5 solvent formation always starts at an undissociated butyric acid concentration between 1.6 and 1.9 g/l. At pH 6.0, despite a high level of excreted acids, the undissociated butyric acid does not exceed 0.8 g/l, and no significant amounts of solvents are produced.

Undissociated butyric acid thus appears to be a critical component of the medium with respect to the growth and metabolism of C. acetobutylicum. It clearly accounts for the very different end ratios of solvents to acids when changing the pH from 4.5 to 6.0. It also explains the different growth patterns observed at pH values above and below 5.0. At the lower pH, which favours the undissociated form of the acid, cellular growth is first totally inhibited by butyric acid before depletion of the nitrogen source. After a lag phase for cellular adaptation to the new acid environment, there is a second growth phase with acid consumption until the nitrogen source has been completely utilized. At the higher pH, on the contrary, the undissociated butyric acid does not reach a sufficiently high level to stop growth before depletion of nitrogen. As a result a single growth phase is obtained.

### Influence of changes in intracellular pH on solvent production

As the cellular metabolism is regulated by intracellular concentrations, from a physiological point of view it would be preferable to relate the metabolic activity of C. acetobutilicum to the intracellular concentration of undissociated butyric acid rather than to its concentration in the medium.

The internal acid concentration is likely to be different from the measured concentration in the culture medium. Since acids are produced and excreted by the cells, there will certainly be a concentration gradient across the cell membrane. Moreover, with growing Clostridia, the internal pH is higher than the external pH due to a proton translocation process catalysed by a membrane bound ATPase. (Booth and Morris 1975 ; Riebeling and Jugermann 1976 ; Herrero 1983). This results in a modified dissociation equilibrium inside the cell.

Because of the difficulty in determining the intracellular acid concentration, it is at present not possible to establish a precise correlation between cell activity and the intracellular concentration of undissociated butyric acid. Our study was thus aimed at examining the influence on the cellular metabolism of changes in intracellular undissociated acid induced by modifications of the internal pH. At a controlled external pH, the intracellular pH can indeed be decreased by inhibiting the proton translocation ATPase.

A known specific inhibitor of the membrane bound ATPase, N-N' dicyclohexylcarbodiimide (DCCD) was added to the culture medium (Riebeling et al. 1975). At a $10^{-4}$ M concentration DCCD completely inhibits the growth of C. acetobutylicum. A smaller $10^{-5}$ M concentration added at the beginning of the growth phase still allows cellular growth but modifies the metabolic activity of the cell.

Figure 2a shows the effect of the addition, after 8 h of culture, of $10^{-5}$M DCCD on the fermentation kinetics at pH 4.5. Comparing this with the results in Fig. 1a, the maximal biomass concentration is reduced in the presence of the ATPase inhibitor, but the formation of solvents starts earlier, after 10 h instead of 15 h. Similar results are obtained in Fig. 2b for the addition of DCCD to a fermentation controlled at pH 6.0. Whereas without inhibitor the final level of solvent does not exceed 1 g/l, with DCCD 3 g/l solvents are obtained.

Consequently, lowering the intracellular pH by inhibition of the membrane-bound ATPase, and thereby increasing the concentration of undissociated acid inside the cell, has a favorable influence on the production of acetone and butanol. This result tends to confirm our previous hypothesis on the essential role of undissociated butyric acid on the induction of solvent production in C. acetobutylicum.

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