Investigation of acetone-butanol-ethanol fermentation by fluorescence

Abstract Acetone-butanol-ethanol fermentation by *Clostridium acetobutylicum* was followed by two variations of fluorescence: the intrinsic fluorescence of NADH, related to bacterial metabolism, and the fluorescence polarization of extrinsic 1, 6-diphenyl-1, 3, 5-hexatriene (DPH) related to membrane fluidity. First, NADH fluorescence was correlated to the specific rate production of butyric acid (linear relationship) and to enzymatic activities (acetate kinase, butyrate kinase and aceto-acetate decarboxylase). Second, a simultaneous increase in both DPH anisotropy (order parameter increase) and butanol production was observed. Even though these results seem contradictory, because of the well-known fluidizing effect of butanol on lipids, the apparent changes in fluidity can be the result of adaptive membrane alteration.

Introduction

Improvement in fermentation processes requires the previous development of sensors. Some fermentation parameters such as pH, mixing, temperature, partial pressure of gases are now well monitored by different sensors (Wolfbeis 1991), but the control of microbial growth or metabolic activities still appears much more complex. For some years, we have been evolving sensors based on fluorescence spectroscopy (Baut et al. 1988). In this work, in order to develop new process control, we have sought to establish correlations between fluorescence parameters and kinetic values of the acetone-butanol-ethanol (ABE) fermentation (Ennis et al. 1986; Rao and Mutharasan 1989; Srivastava and Volesky 1991).

First, we measured the intrinsic fluorescence of NADH, a metabolic intermediate. NADH is the most important cellular fluorophoric component, because of its central position in the pathways of the energy metabolism of all living cells (bacteria, yeast, fungi, plant and animal cells). When cells are irradiated at 360 nm, NADH emits characteristic fluorescent light at 460 nm. The oxidized form (NAD) is not fluorescent. This photophysical property was previously used to establish a relationship between the fluorescence intensity of the culture and the cell concentration, together with environmental factors. Correlations with the rate of butyric acid production and with some enzymatic activities were sought.

Secondly, we investigated the use of fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH), a membrane probe embedded in living bacteria. DPH is often used to obtain information on the relationship between diffusion and local cohesion in membranes: the fluorescence anisotropy of DPH, embedded in membranes (artificial, as liposomes, or natural) is concerned with the rotational motion of the probe (André et al. 1987). The steady-state anisotropy ($\langle r \rangle$) can be correlated with the order parameter of the membrane and to its cohesion. A decrease in the local order will induce a decrease in $\langle r \rangle$. Even though, this method can be subjected to criticism, because of an unrigorous relationship between diffusion and viscosity on the molecular scale, studies based on the use of fluorescence anisotropy are useful for monitoring a system under chemical (exogeneous additives, pH changes) or physical (temperature, pressure) constraints. An example is the effect of alcohols on the fluidity of the lipidic membrane, which is well-known and can be studied by this method. Thus it seemed possible a priori to develop a system based on the use of fluorescence anisotropy of DPH embedded in *Clostridium acetobutylicum* cells under alcohol production during fermentation. In fact, because of modifications of the
bacteria occurring during the fermentation process, a surprisingly complex behaviour of the fluorophore DPH was observed.

### Materials and methods

*C. acetobutylicum* ATCC 824 was maintained at 4°C in Reinforced Clostridial Medium (Oxoid, Basingstoke, UK). A growth medium containing (NH₄)₂SO₄, KH₂PO₄, K₂HPO₄, MgSO₄, 7H₂O, FeSO₄·7H₂O, glucose, p-aminobenzoic acid and biotin at 2.2, 0.5, 0.5, 0.2, 0.01, 60, 10⁻³ and 10⁻⁵ g l⁻¹, respectively, in distilled water was used.

A Chemap fermentor (71) was used; the stirring rate was maintained at about 200 rpm and the broth temperature at 35°C. If necessary, the pH of the medium was controlled by automatic addition of 2 M NaOH. The propagation of inoculum and the fermentation were carried out as described by Fick (1986). The fermentor was inoculated with a growing culture taken at the end of the growth phase. The medium was initially kept anaerobic, by bubbling through it pure N₂ until a sufficient fermentation activity had been reached. Absence of contamination was regularly checked by microscopic observation.

Cell concentration was estimated by dry weight and absorbance at 660 nm. Other analyses were made on samples previously filtered on 0.2-μm membranes. Residual glucose was determined on a Technicon autoanalyzer with a hexokinase reagent. Concentrations of acids (acetic and butyric) and solvents (acetone, butanol and ethanol) were determined by injection of acidified supernatants into a Intersmat ICG 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. N₂ was used as the carrier gas. The injector and detector temperatures were 220°C and the column temperature was 190°C. Analyses of the chromatographic data were carried out on an Intersmat ICR 1B integrator.

Acetate kinase (AK) and butyrate kinase (BK) activities were determined via their effect on the reaction between ATP and potassium acetate or butyrate, in the presence of MnSO₄ to produce ADP and acetyl or butyryl phosphate. These latter compounds reacted with hydroxylamine to give a coloured complex in the presence of Fe³⁺. Acetate kinase (AK) and butyrate kinase (BK) activities were determined (Viriot et al. 1982). The final concentration of DPPC (Sigma) was 10⁻⁴ M. DPH (final concentration of 10⁻⁶ M) was introduced in chloroform before evaporation of the solvent. Butanol was introduced in the multimodal phase.

Rates were obtained by (i) the determination of mass balance (r_B = dX/dt) where r_B is the production rate of biomass (X) (g dry weight produced 1⁻¹ h⁻¹), using the batch reactor and (ii) the measurement of the production of butyric acid (r_BA = d[BA]/dt), where r_BA is the production rate of butyric acid (g 1⁻¹ h⁻¹) during the acidogenic phase. Then, the specific rates for, respectively, growth of biomass (μ; h⁻¹) and butyric acid production (n_BA; h⁻¹) are: μ = r_B/X and n_BA = r_BA/X.

### Results

#### Fermentations

Several ABE fermentations were carried out under various conditions. Three discontinuous fermentations were retained as examples: one without pH regulation and two with the pH regulated at 4.8 and 5.1 respectively.

A typical time course of a non-regulated-pH fermentation characteristics is shown in Fig. 1 for pH, biomass concentrations and residual glucose concentration. The procedure used for the preparation of multimodal vesicles of dipalmitoylphosphatidylcholine (DPPC) was as previously described (Viriot et al. 1982). The final concentration of DPPC (Sigma) was 10⁻⁴ M. DPH (final concentration of 10⁻⁶ M) was introduced in chloroform before evaporation of the solvent. Butanol was introduced in the multimodal phase.

For fluorescence analysis, according to the difficulties related to the on-line measurements, we decided to work off-line on a classical right-angled geometric arrangement with diluted samples of *C. acetobutylicum* fermentation medium. The fluorescence intensity of NADH was measured using a Jobin Yvon JY3D spectrofluorometer, equipped with a cell thermostatted compartment. The excitation wavelength was 360 nm and the emission wavelength was 460 nm. An absorbance of the sample near 0.3 at 360 nm was obtained by adjustment with distilled water. The spectrofluorometer was calibrated with a fluorescein solution. The fluctuations of the excitation light intensity of the spectrofluorometer were about 5% during all the experiments and corrected. Samples were treated as quickly as possible and in all cases, no more than 1 h after sampling. This allowed the cessation of metabolism without disturbing the fluorescence measurements. Fluorescence units were relative to the spectrophotometer used; then the fluorescence intensity of NADH was expressed on the basis of cell dry weight (g 1⁻¹) in diluted samples and the noted specific fluorescence.

For the fluorescence anisotropy of DPH, absorbance of the sample near 0.3 at 360 nm was obtained by adjustment with distilled water. For each measurement, 10 μl of DPH, from a 10⁻³ M stock solution in tetrahydrofuran (THF), was added under stirring to 3 ml diluted culture medium sample. Equilibration of bacteria and DPH was complete after 10 min incubation time. The fluorescence anisotropy (r) of DPH (steady-state excitation) in the bacteria was measured using an automatic apparatus, previously described (Donner et al. 1983), equipped with a cell thermostatted compartment. The excitation wavelength was 360 nm and the emission wavelength was 430 nm.

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![Fig. 1](image-url)