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Investigation of Acetone–Butanol–Ethanol (ABE) Fermentation by Fluorescence

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Photophysical techniques have potential for the development of optical sensors in monitoring and controlling fermentors. In the particular case of acetone–butanol–ethanol (ABE) fermentation, carried out by bacteria of the species *Clostridium acetobutylicum*, we have developed two studies based on fluorescence spectroscopy. First, we measured the intrinsic fluorescence of NADH related to bacteria metabolism, leading to a linear relationship between the NADH specific fluorescence and the specific rate of butyric acid production. At the same time, we have correlated enzymatic activities (acetate kinase, butyrate kinase, acetoacetate decarboxylase) with NADH specific fluorescence. Second, we studied the fluorescence polarization of extrinsic DPH (1,6-diphenyl-1,3,5-hexatriene) related to membrane fluidity. A simultaneous increase in both DPH anisotropy (order parameter increase) and butanol production is observed. Even though these results seem contradictory, because of the well-known fluidizing effect of butanol on lipids, they can be explained by a homeoviscous response of *C. acetobutylicum* to the presence of butanol during fermentation. Thus the apparent changes in fluidity could be the result of the adaptative membrane alteration.

KEY WORDS: Fluorescence; fluorescence anisotropy; batch acetone–butanol–ethanol fermentation; NADH; 1,6-diphenyl-1,3,5-hexatriene; membrane fluidity.

INTRODUCTION

It is now obvious that improvement of the fermentation processes requires the previous development of sensors. Some fermentation parameters such as pH, mixing temperature, and partial pressure of gases are now well monitored by different sensors, but the control of microbial growth or metabolic activities appears much more complex [1].

For some years, we have been developing systems based on fluorescence techniques for determining fermentation parameters [2]. In fact, fluorescence techniques are well suited to following modifications of morphology and physiology of microorganisms induced by the culture conditions.

In the particular case of acetone–butanol–ethanol (ABE) fermentation, which has experienced a revival [3–5], we have developed two studies based on fluorescence spectroscopy. Especially to develop new process control, we searched to establish correlations between fluorescence measurements and kinetic values of ABE fermentation. First, we measured the intrinsic fluorescence of a metabolic intermediate NADH, and second, we investigated the use of fluorescence polarization of a membrane probe embedded in living cells, 1,6,-diphenyl-1,3,5-hexatriene (DPH).

EXPERIMENTAL

The microorganism used was *Clostridium acetobutylicum* strain ATCC 824. The conditions for stock

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cultures and fermentations for analysis processes were those previously described [6].

For the fluorescence analysis, because of the difficulties related to the on-line measurements, we decided to work off-line on a classical right-angle geometric arrangement with diluted samples of *Clostridium acetobutylicum* fermentation medium (an absorbance of the sample near 0.3 was generally used).

The fluorescence intensity of NADH was measured using a Jobin Yvon JY3D spectrofluorometer (λ_{ex} = 360 nm and λ_{fl} = 460 nm), equipped with a cell thermostated compartment. This is expressed as the dry weight of cells (g l^{-1}) in diluted samples and noted as specific fluorescence.

The fluorescence anisotropy $\langle r \rangle$ of DPH (steady-state excitation, λ_{ex} = 360 nm and λ_{fl} = 430 nm) in bacteria was measured using an automatic apparatus described previously [7], equipped with a cell thermostated compartment. For each measurement, 10 μl of DPH (from a 10^{-3} *M* stock solution in tetrahydrofuran) was added under stirring to 3 ml of a diluted culture medium sample. Equilibration of bacteria and DPH was complete after 10 min of incubation.

RESULTS AND DISCUSSION

Fermentations

For nonregulated pH fermentations, the two phases have the following characteristics.

(i) The acidogenic phase lasts about 20 h; it has a high maximum specific growth rate of the culture (μ = 0.35 h^{-1}), acetic and butyric acid production (2.1 and 2.7 g l^{-1} at 20 h, respectively), a decreasing pH (from 5.9 to 4.4), and an absence of solvent production until 17 h.

(ii) The solventogenic phase begins at about 20 h; it has a lower maximum specific growth rate (μ = 0.08 h^{-1}), acid consumption (from 2.1 to 0.6 g l^{-1} for acetic acid and from 2.7 to 0.2 g l^{-1} for butyric acid at 65 h, corresponding to the end of the fermentation), ABE production (at 65 h, 4.1, 6.9, and 0.7 g l^{-1}, respectively), and a slightly increased pH (from 4.4 to 4.7).

For regulated pH fermentations (4.8 and 5.1), the results are in good agreement with those reported previously [8].

Fluorescence Analysis

Two types of fluorescence analysis were investigated, NADH fluorescence (intrinsic probe) and fluorescence polarization of DPH (extrinsic probe).

Specific Fluorescence of NADH

This was correlated with the following parameters.

Fermentation Time (See Fig. 1). A decrease in fluorescence intensity during the first 20 h is observed; it corresponds to a high acidogenic metabolism, which is coupled with important NADH production. During the second phase, the metabolism's change induces a larger NADH consumption, corresponding to a low and roughly constant fluorescence intensity [4].

Specific Production Rate of Butyric Acid (See Fig. 2). The good linear correlation indicates that the metabolic state of the bacteria is oriented in the synthesis of the most characteristic product of the acidogenic phase.

Enzymatic Activities (Curves Not Shown). For acetate kinase (AK), which is essentially active in the first 10 h of fermentation, i.e., the acidogenic phase, a linear correlation is observed. For butyrate kinase (BK), which

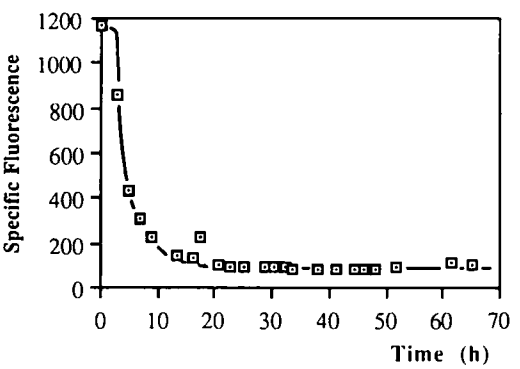

Fig. 1. Specific fluorescence of NADH versus time, for a nonregulated-pH ABE fermentation (diluted samples).

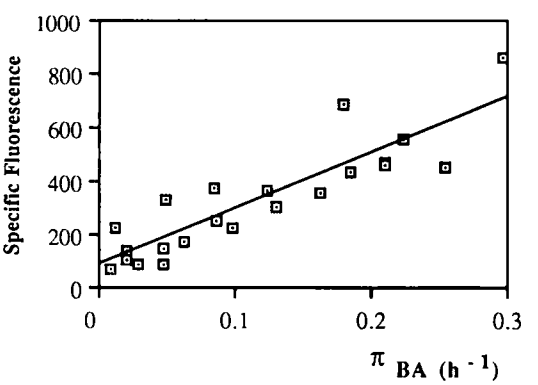

Fig. 2. Specific fluorescence of NADH versus specific production rate of butyric acid (results for three ABE fermentations, regulated pH or not).

is active not only in the acidogenic phase, but also in the solventogenic phase, the correlation with the specific fluorescence of NADH is not linear. For acetoacetate decarboxylase (AAD), which is essentially an enzyme of the solventogenic phase, no correlation can be drawn.

Fluorescence Polarization of DPH

This was correlated with the production of butanol (see Fig. 3). Similar results were obtained in the case of two other nonregulated pH fermentations. A priori, these results, which indicate an increase in both the DPH anisotropy (order parameter increase) and the butanol production, seem contradictory because of the well-known fluidizing effect of butanol on lipids, confirmed by our own experiments (effect of butanol on the thermotropic behavior of multilamellar vesicles of DPPC followed by fluorescence polarization of DPH: decrease in both the anisotropy and the T_M).

Recently, a study examined membrane tolerance to ethanol using fluorescence polarization of various extrinsic probes; the rat synaptic membrane becomes resistant to the disordering effect of ethanol [9].

According to previous publications, during fermentation, cells adapt their cellular acyl-chain composition to the presence of butanol [10], i.e., *Clostridium acetobutylicum* has a homeoviscous response to butanol. This leads to an increase in saturated chains, which could correspond to a net increase in cohesion. Also, in the presence of long-chain fatty acids (oleic acid and elaidic acid), the enrichment of the membrane increases butanol tolerance by up to twofold [11].

Elsewhere, using the same physical method as for the investigation of membrane cohesion and the same experimental conditions, we followed the influence of butanol added to the culture medium of *Clostridium acetobutylicum*, which are taken from the culture medium at two stages of fermentation (see Fig. 4). For each case, a decrease in DPH anisotropy is obtained, which is related to the fluidizing effect of added butanol. A larger effect of butanol on the cohesion of bacteria in the acidogenic phase compared to the solventogenic phase is observed. In fact, at each fermentation time, bacteria have a particular physiological state and butanol disturbs the molecular arrangement of the membranes in this particular situation.

These assumptions and observations explain why added butanol (external addition) and produced butanol (internal production) do not have the same effect on the state of membrane fluidity.

CONCLUSION

By the use of fluorescence, we are able to follow each phase of ABE fermentation. Especially for the acidogenic phase, the good relationship between the specific fluorescence of NADH and the specific production rate of butyric acid, which is related to the metabolic state of the culture, is an additive argument for the development of a fluorescence sensor based on NADH in fermentation processes. Especially, it now appears possible to adapt the measurement of NADH fluorescence to an on-line process using automatic sampling and diluting systems. Concerning an optical sensor based on the use of fluorescence polarization of DPH (or any other fluorescent probe), the development is too early. More fundamental studies must be done to understand the influence of chemical or physical constraints on membrane

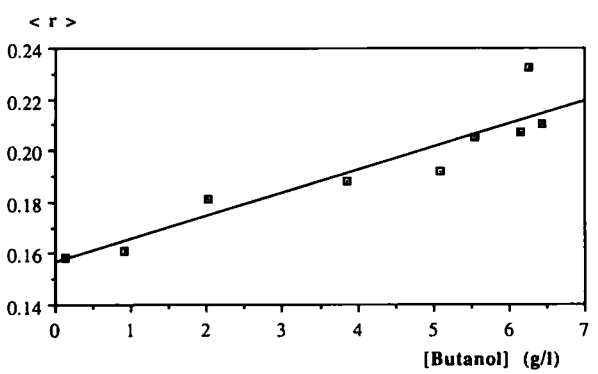

Fig. 3. Correlation between the fluorescence anisotropy $\langle r \rangle$ of DPH and the concentration of produced butanol, for a pH 4.8 ABE fermentation.

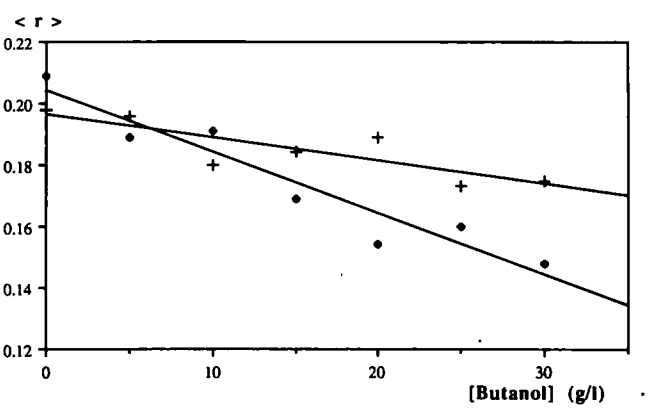

Fig. 4. Fluorescence anisotropy $\langle r \rangle$ of DPH versus added butanol, for the two phases of a pH 4.8 ABE fermentation. (♦) Acidogenic phase at 8 h; (+) solventogenic phase at 38 h.

fluidity and the response of microorganisms to microenvironmental changes.

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