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# On-line fluorescence measurements in assessing culture metabolic activities

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**Summary.** NADH fluorescence aided by a stoichiometric metabolic pathway model and culture dynamics was used to elucidate the unobservable intracellular physiological state in two metabolically different phases during culture of *Clostridium acetobutylicum*. The validity of the theoretical model was examined over a range of culture pH regimes and initial sugar concentrations. The $H_2/CO_2$ gas concentration ratio was found to be an important process parameter. NADH fluorescence detection was compared with simultaneous enzymatic measurements. The specific fluorescence (fluorescence per biomass, $F/X$) provided a distinction between oxidative and reductive culture metabolism independent of the pH or substrate concentration changes. A good indicator of the type of culture activity proved to be the $dF/dt$ parameter. The net fluorescence measurements correlated with butanol accumulation under all growth conditions suggesting the possible use of the fluorescence probe as a butanol probe in this fermentation.

## Introduction

Batch production, particularly of butanol and acetone by the solvent-producing bacterium *Clostridium acetobutylicum*, proceeds in two stages. The initial growth-associated stage involves accumulation of butyric and acetic acid intermediates. The acids are eventually converted into solvents during the second stage of fermentation, which features a low growth rate. The batch culture eventually ceases due to butanol toxicity and substrate depletion. Efficient solvent production by this process would require a well optimized culture system based on a highly instrumented computer-controlled bioreactor. For guiding such a process, detection of the culture physiological state is crucial to predict the culture behaviour and performance under different culture conditions.

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Due to the lack of on-line sensors for estimation of the intracellular state of a growing cell, indirect methods have been proposed. The validity of the stoichiometric "fermentation equation" for prediction of physiological parameters for cultures of *C. acetobutylicum* has been demonstrated (Meyer et al. 1986). Reardon et al. (1987) recently proposed the use of on-line fluorescence measurement and pseudosteady state mass balance calculations only on carbon-containing intermediates to elucidate the NADH-related physiological state of the culture. However, the validity of the fluorescence-probe-coupled theoretical approach for NADH related metabolic state estimation under different culture conditions still remains to be experimentally confirmed.

Although some NADH fluorescence investigations of batch (Reardon et al. 1986, 1987; Srinivas and Mutharasan 1987) and transient (Rao and Mutharasan 1989) *Clostridium* cultures have been carried out, almost all are based on cumulative fluorescence observations that are invariably distorted by variable culture conditions (pH changes, external perturbations). Variable culture environment can have a pronounced effect on NADH/NAD levels and the fluorescence property of the fermentation broth. Particularly in batch cultures, everything is in a state of flux. Reasonably constant environmental conditions are mandatory for meaningful interpretation of culture fluorescence data.

The present investigation uses the methodology of Reardon et al. (1987) to elucidate the mode of reduced ferredoxin ($Fd_{red}$) regeneration and flow of electrons under different pH-stat growth conditions in order to establish the limits of applicability of the above theoretical model. The use of the ratio $H_2/CO_2$ can assist in characterisation of the culture metabolism and associated electron flow under controversial culture growth conditions where the theoretical apparatus becomes unreliable. An attempt is also made to develop fluorescence-based process parameters that are desirable for appropriate characterisation of the metabolic state of the culture under different growth conditions.

## Materials and methods

*Organism and culture conditions.* A series of anaerobic batch culture experiments using *C. acetobutylicum* ATCC 824 were conducted in a modified 14-l Microferm laboratory fermentor (New Brunswick Scientific, Edison, N. J., USA) with a working volume of 8 l. The basic culture medium composition (Votruba et al. 1986) contained 50 g/l glucose. When the sugar concentration was varied, the amounts of ammonium sulphate and yeast extract were adjusted correspondingly (Srivastava 1990). Standard inoculum development procedures for the acetone-butanol fermentation were followed (Votruba et al. 1986; Srivastava 1990). Because of the fast growth of the culture, it was necessary to limit the pH fluctuations in the shake-flask inoculum: it was not allowed to fall below 4.6 by the manual addition of 0.1 N NaOH. The concentrations of metabolites in the culture and off gases were monitored by gas chromatography (Votruba et al. 1986). The glucose concentration in the fermentation broth was estimated by the hexokinase method (Votruba et al. 1986) and the biomass concentration was determined by optical density at 610 nm (Votruba et al. 1986).

*Culture fluorescence.* The computer-coupled optical device measuring culture broth fluorescence used in this work was obtained from Biochem Technology (Malvern, Pa., USA). It employs a low pressure mercury lamp which provides 360 nm excitation light and NADH fluorescence at 460 nm is detected by a photomultiplier. The output of the fluorescence measuring probe was quantitatively standardized using a thioflavin solution, resulting in a linear correlation between the fluorophore concentration and the fluorescence reading in normalised fluorescence units (NFU), at least in the range of fluorescence of interest (up to 1200 NFU).

*Background culture fluorescence.* Although Reardon et al. (1987) reported that the clean fermentation broth background fluorescence remains constant in *C. acetobutylicum* fermentations carried out at pH 4.6 and pH 6.0, during the course of this work and elsewhere (Srinivas and Mutharasan 1987), it was observed that immediately after the introduction of the culture inoculum there was a sharp increase in the monitored fluorescence value followed by a decrease, which invariably extended significantly below the cell free culture broth background value ($NFU_0$). In the present investigation, the fluorescence of the cell-free broth from a batch fermentation [initial glucose concentration $(S_0) = 50$ g/l, pH 4.6], examined in custom-made fluorescence measuring device designed to accommodate a 1 $cm^2$ cuvette, remained constant except for a small decrease in the lag phase and at the beginning of the log phase of the culture growth (up to 3-4 h). This suggests a rapid consumption of some strongly fluorescent growth factor (e.g., some amino acid) for initiation of exponential culture growth. The lowest detected fluorescence value was adopted as a baseline for the fluorescence measurements reported in the present work. It was always subtracted from observed and recorded total fluorescence signals (NFU).

*Cellular biochemistry and representation of the physiological state:* The biochemical pathway illustrating carbon and electron flow for acetone-butanol fermentation has been summarized by Gottschalk (1986). Two moles of NADH are generated in the primary culture metabolism of glucose by the Emden-Meyerhof-Panas pathway. The resulting pyruvate is decarboxylated by the pyruvate ferredoxin oxidoreductase enzyme system to produce acetyl CoA, $CO_2$ and $Fd_{red}$. The latter is invariably regenerated by a hydrogenase enzyme with the liberation of $H_2$ gas during both phases of culture metabolism. The two primary NADH reducing equivalents can be regenerated in the formation of butyric acid. The net production of acetic acid effectively creates excess NADH since it siphons off acetyl CoA that could have been used to reductively form butyric acid. The regeneration of excess NADH is done by the reaction of NADH and FD to form $Fd_{red}$ catalysed by the key enzyme NADH ferredoxin oxidoreductase. The accumulated $Fd_{red}$ is eventually regenerated by the action of hydrogenase to liberate $H_2$ and free oxidized coenzyme ($Fd_{ox}$) required for the progress of the acidogenic-phase metabolism. The solventogenic culture metabolism features a decreased activity of the hydrogenase due to reduced availability of its substrate $Fd_{red}$ caused by the lower growth rate of the culture (Kim and Zeikus 1985). The culture eventually finds an alternative mode of $Fd_{red}$ regeneration occurring in the solventogenic phase by the reduction of NAD to produce NADH, a reaction sequence catalysed by the reversible activity of NADH: ferrodoxin oxidoreductase. The solventogenic culture regenerates its NADH by the production of solvents. It has been indicated that an increase in electron transfer from $Fd_{red}$ to NAD is feasible only under the conditions of rapid NADH regeneration by solvent producing pathways (Petitdemange et al. 1976).

A key reaction represented by Eq. (1) reflects the flow of electrons from glucose to different electron sinks resulting in the accumulation of different metabolic products:

$$Fd_{red} + NAD \rightleftharpoons Fd_{ox} + NADH \quad (1)$$

Reardon et al. (1987) outlined the methodology for obtaining the direction of flow of electrons and the rate of the above reaction under dynamic batch conditions by using the stoichiometric relationships linking steps in the metabolic pathway of *C. acetobutylicum*.

The stoichiometric change in the intracellular concentration of NADH is given by:

$$\frac{d(NAD(P)H)}{dt} = v_1 + v_{13} - 0.125 v_G - 2v_4 - 2v_8 - 2v_{11} - v_{12} \quad (2)$$

where $NAD(P)H$ represents the total concentration of reduced NAD and NADP, $t$ represents time and $v$ represents the rates of individual metabolic reactions (Reardon et al. 1987). All the rates in the above equation could be calculated from the known concentrations of extracellular products except for rate $v_{13}$ (Reardon et al. 1987). This rate corresponds to the NADH: ferredoxin oxidoreductase reaction and cannot be determined since neither NADH nor ferredoxin are considered to be constant with time. However, the qualitative estimation of rate, $v_{13}$, and NADH changes with time could be derived by comparing specific fluorescence vs time curves of various fermentations with the following integrated form of the above equation which assumes $v_{13}$ to be zero. Equation 3 defines the stoichiometric change in the NADH concentration ($NADH$) as:

$$(NADH)_t - (NADH)_0 = \int_0^t (v_1 - 0.125 v_G - 2v_4 - 2v_8 - 2v_{11} - v_{12})\, dt \quad (3)$$

If the time profiles of stoichiometric $NADH/X$ and Fluorescence/$X$ could be compared directly, the difference between areas under the two curves at a given time $\Delta$NADH ($= F/X$-NADH) would be the qualitative amount of NADH produced (or consumed) by the reaction in Eq. 1 through a given time in the batch fermentation. In other words, the difference of ordinate values of the two curves at a given time reflects the amount of NADH produced (or consumed) during that time interval. Symbol $X$ designates the biomass concentration in the broth and $F$ the fluorescence. To obtain the sign and magnitude of this important rate of reaction ($v_{13}$) the slope of curves $F/X$ vs $t$ and $NADH$ vs $t$ could be compared. It must be noted that negative values of rate $v_{13}$ and the decreasing trend of $\Delta NADH$ reflect the acid-forming phase of the fermentation when reduced ferredoxin is oxidised by the hydrogenase reaction. Positive values of $v_{13}$ indicate net accumulation of NADH by the reaction in Eq. 1 in the solventogenic culture metabolism. The above methodology can be adopted to interpret the flow of electrons under different batch *Clostridium* culture conditions.

## Results and discussion

### *Batch experiments with controlled pH*

The acetone-butanol-ethanol (A-B-E) fermentation experiments were conducted at four different controlled

pH levels of 4.6, 5.0, 5.5 and 6.0. The dynamics of the fermentations, and stoichiometric *NADH* and *ΔNADH* are plotted in Figs. 1 and 2. The mode of $Fd_{red}$ regeneration is indicated by the values of the *ΔNADH* parameter and the $H_2/CO_2$ ratio.

*Batch fermentation controlled at pH 4.6.* A decrease in ΔNADH indicated the regeneration of $Fd_{red}$ by $H_2$ production from hour 4 to hour 8 in the acidogenic phase of the culture. This is reflected to some extent in the relatively high $H_2/CO_2$ ratio and low *F/X* during that time period. At the onset of the solvent production by the culture (hour 8) the $H_2/CO_2$ ratio attained a low and constant value indicating the flow of electrons towards NAD leading eventually to increasing NADH levels. During the solventogenic phase of the culture, there was a large NADH pool, as characterised by high *F/X* values, due to increased transfer of electrons from $Fd_{red}$ to NAD. The transfer of electrons from $Fd_{red}$ to NAD after hour 8 was further confirmed by the increasing calculated ΔNADH and increased solvent production.

*Batch fermentation at controlled pH 5.0.* From hour 2–6 the acidogenic culture was characterised by decreasing *ΔNADH* values. However, from hour 6 onwards, increased ΔNADH reflected an altogether different metabolic activity of the culture whereby NAD obtained electrons from $Fd_{red}$ increasing the NADH pool required for higher solvent production. The extracellular culture dynamics also featured solvent accumulation and confirmed the model-predicted hypothesis of electron transfer.

*Batch fermentation at controlled pH 5.5.* The culture featured higher acid than solvent accumulation, suggesting the regeneration of $Fd_{red}$ by $H_2$ production reflected in decreasing calculated values of parameter *ΔNADH* from hour 3 to hour 6. The solvent production commenced at hour 9 but the calculated parameter

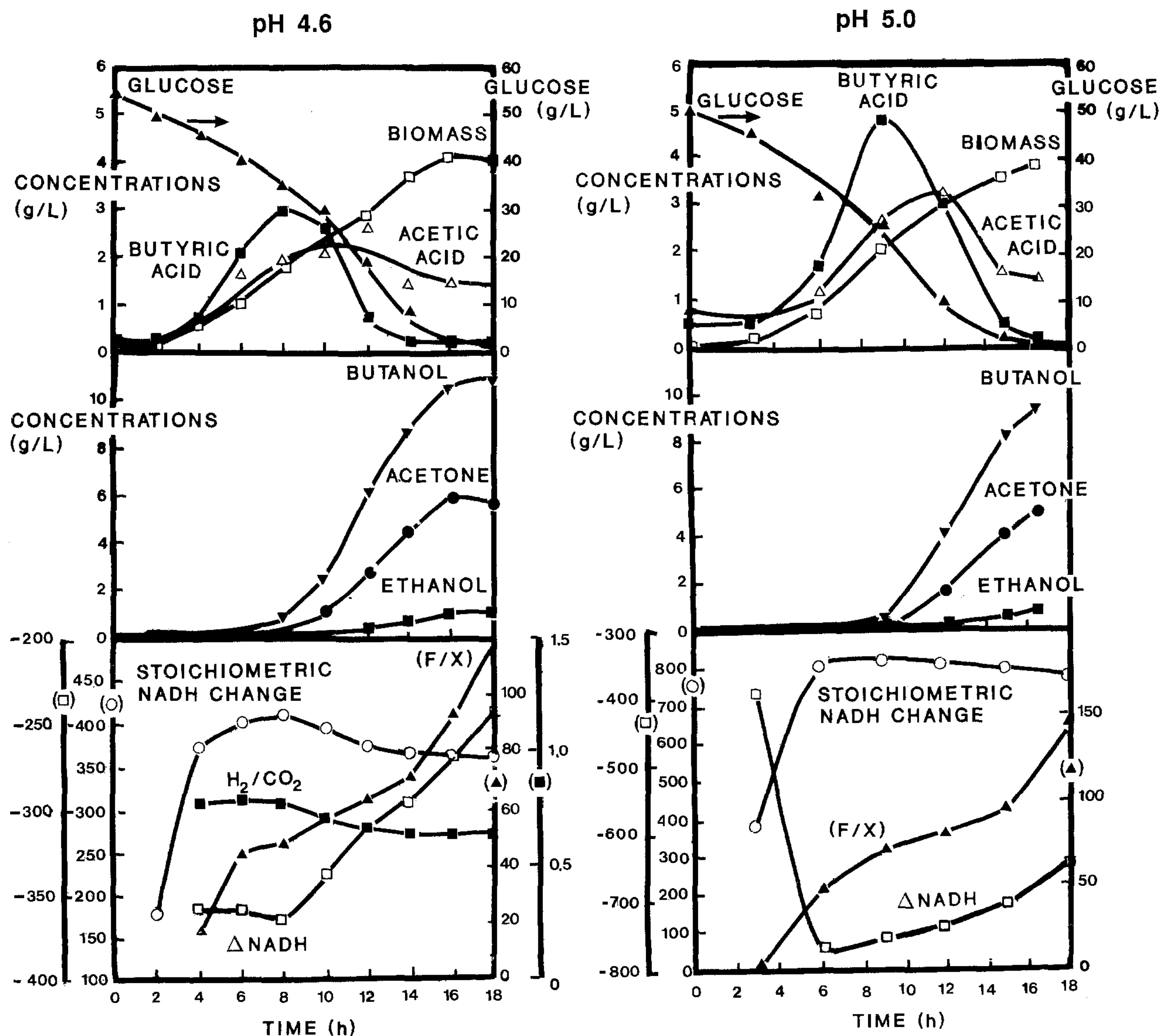


**Fig. 1.** Kinetic and fluorometric properties of the batch acetone-butanol-ethanol (A-B-E) fermentation at the initial sugar concentration $(S_0)=50$ g/l and pH = 4.6 or 5.0: [$\Delta NADH = (F/X - NADH)$]. *F/X*, specific fluorescence (fluorescende/biomass concentration); *NADH*, concentration of NADH

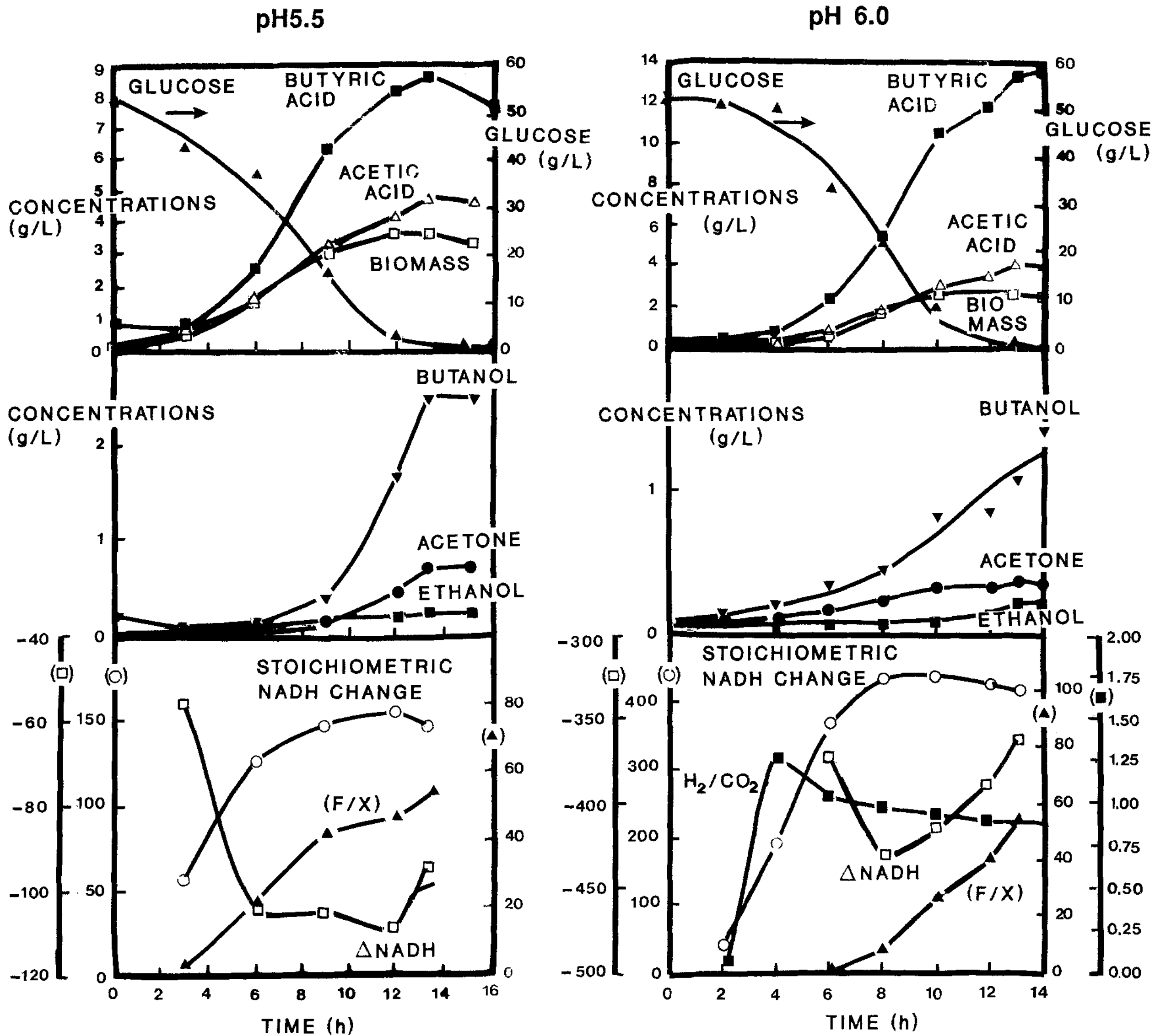


Fig. 2. Kinetic and fluorometric properties of the batch A-B-E fermentation at $S_0 = 50$ g/l and pH = 5.5 or 6.0

(*ΔNADH*) still indicated acidogenic metabolism by showing decreasing values during hours 9-12. The increase in *ΔNADH* after hour 12, with reduced acid accumulation, signifies the transfer of electrons from $Fd_{red}$ to NAD which promoted the observed solvent accumulation.

*Batch fermentation at controlled pH 6.0.* The calculated parameter ΔNADH predicted the transfer of electrons from $Fd_{red}$ to hydrogen by decreasing values of *NADH* change between hours 6-8. The increased values of calculated NADH change (ΔNADH) for the rest of the fermentation suggested the transfer of electrons from $Fd_{red}$ to NAD and a solventogenic metabolism. This is in contradiction to the experimentally observed acidogenic culture behaviour under those conditions. The inability of the metabolic pathway rate concept of Reardon et al. (1987) to predict the mode of $Fd_{red}$ regeneration for the exclusively acidogenic culture (pH 6.0) was also acknowledged by Reardon et al. (1987) themselves. This suggests that the pathway leading to acid accumulation is not well elucidated and/or electrons are transferred by alternative mechanisms. Niranjan and San (1989) also pointed out the importance of accurate knowledge of intermediate metabolic pathways for constructing relevant stoichiometric metabolic models. Under the present circumstances, the experimental $H_2/CO_2$ ratio can assist in elucidation of the electron flow direction. As shown in Fig. 2, the higher values of $H_2/CO_2$ observed during almost the entire pH 6.0 fermentation indicate high $H_2$ evolution typically characterising an acidogenic culture regenerating $Fd_{red}$ through the evolution of $H_2$.

*Batch fermentations at pH 4.6 with varying initial sugar concentrations*

The kinetic and fluorometric properties of the culture at pH 4.6 and different initial sugar concentrations are represented in Figs. 3 and 4.

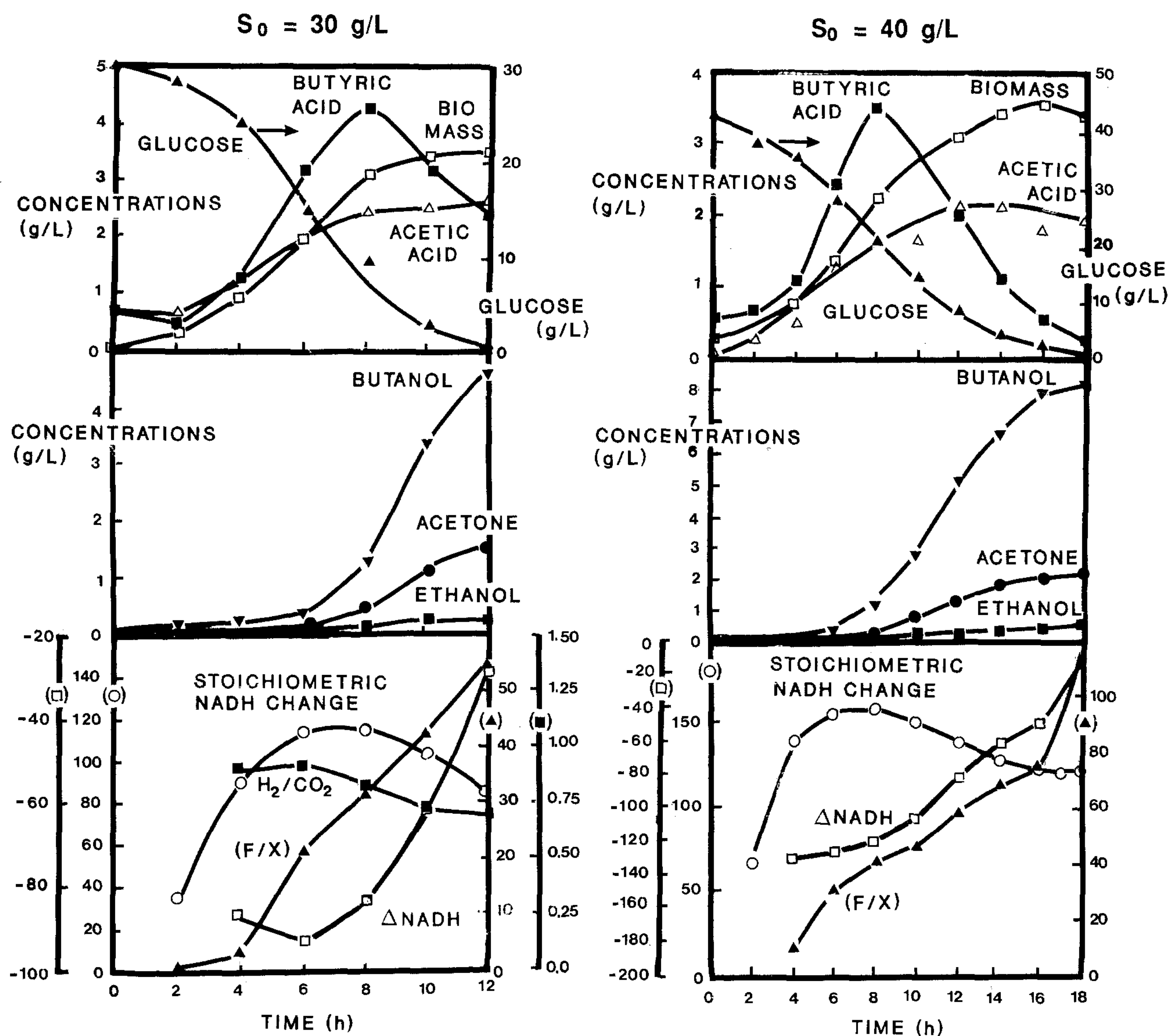


Fig. 3. Kinetic and fluorometric properties of the batch A-B-E fermentation at pH 4.6 and $S_0 = 30$ g/l or 40 g/l

*Initial glucose concentration 30 g/l.* The calculated values of $\Delta NADH$ and the $H_2/CO_2$ ratio provided a reasonable indication of the direction of the electron flow. During hours 4–6, a decreasing $\Delta NADH$ and a high constant $H_2/CO_2$ ratio reflected acid production and accumulation in the culture characterized by $Fd_{red}$ regeneration by $H_2$ evolution. This trend reversed itself resulting in increasing values of $\Delta NADH$ after hour 6 indicating a flow of electrons from $Fd_{red}$ to NAD accompanied by solvent accumulation. The switchover of the metabolic activity could also be located by lowering the $H_2/CO_2$ ratio at hour 6 as shown in Fig. 3.

*Initial glucose concentration 40 g/l.* The $\Delta NADH$ parameter remained relatively constant during the acid accumulation phase from hours 4–6 and increased gradually in the solvent accumulation phase after hour 6 indicating the transfer of electrons from $Fd_{red}$ to NAD. It is relatively difficult to characterise the culture metabolic activity during hours 4–6 due to constant $\Delta NADH$.

*Initial glucose concentration 60 g/l.* The calculated $\Delta NADH$ demonstrated regeneration of $Fd_{red}$ by $H_2$ production and acid accumulation from hours 4–12 by decreasing values of $\Delta NADH$. The increased value of $\Delta NADH$ after hour 12, however, indicated NADH production from $Fd_{red}$ and a solventogenic culture activity as confirmed by increasing butanol accumulation. The $H_2/CO_2$ ratio showed a sharp expected decrease at hour 8 when the culture switched to the solventogenesis. However, the distinct change in the trend of the $\Delta NADH$ parameter at the initiation of solventogenesis cannot be easily detected.

*Initial glucose concentration 75 g/l.* The calculated $\Delta NADH$ featured a decrease between hours 4–8 reflecting predominantly acidogenic culture metabolism with transfer of electrons from $Fd_{red}$ to $H_2$. From hour 8 onward, the solvent production was active, reflected also in the increased values of $\Delta NADH$. During solvent production from hour 8, parameter $\Delta NADH$ showed an expected increase reflecting transfer of electrons from

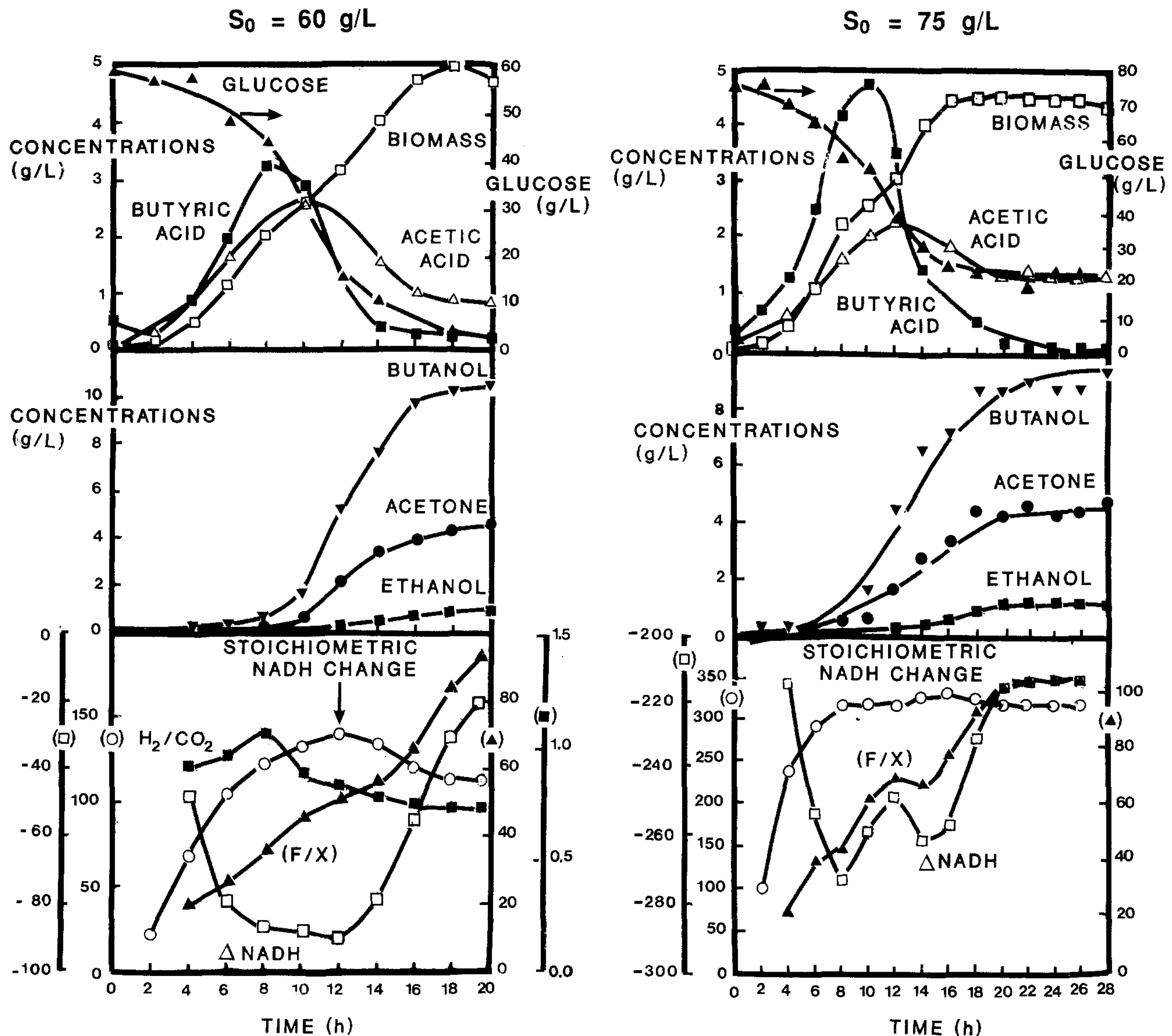


**Fig. 4.** Kinetic and fluorometric properties of the batch A-B-E fermentation at pH 4.6 and $S_0 = 60$ g/l or 75 g/l

$Fd_{red}$ to NAD. However, an unexpected dip in $\Delta NADH$ between hours 12–14 resists interpretation even though disturbances of smooth patterns could be seen in other culture parameters (e.g. biomass concentration).

*The use of fluorescence data.* A question is always asked as to just how close is the fluorescent NADH determination to the values indicated by standard wet analytical methods. Figure 5 shows the comparison of NADH fluorescence observations with simultaneous enzymatic measurements during a fermentation conducted at pH 4.6 and $S_0 = 50$ g/l to establish the reliability of the fluorescence measurements. Lower levels of the initial NADH content in the fermentation system could not be detected by the enzymatic procedure up to hour 4. From hour 4 to hour 16 a parallel increase in $F$ with enzymatically determined NADH neglected the contribution of the inner filter effect or the presence of other interfering fluorophores in the fermentation broth.

*Clostridium* metabolism is relatively complex, particularly with respect to the accumulation and disappearance of NADH, which limits the application of the NADH probe for biomass measurements. The culture fluorescence in the *Clostridium* fermentation is a function of both NADH and biomass concentration. If the biomass concentration is determined independently, the fluorescence probe can provide unique information on the oxidative or reductive state of the culture. From Figs. 1–4 it can be generally concluded that $F/X > 65$ characterised solventogenic culture activity and $F/X < 20$ indicated acidogenic culture metabolism. In the range $F/X = (20–65)$ both acids and solvents accumulated in the culture. A high $F/X$ ratio for solventogenic batch culture was also reported (Srinivas and Mutharasan 1987) for pH-corrected batch cultures of *C. acetobutylicum*. Different values of $F/X$ for oxidative and reductive phases of the culture metabolism have also been reported by Scheper et al. (1987) for *Saccharomyces cerevisiae*. The present study of *Clostridium* metabolism suggests that the $F/X$ ratio may eventually

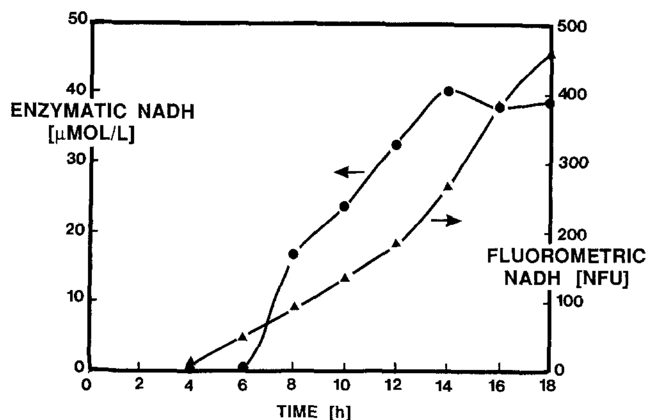


**Fig. 5.** Comparison of fluorometric and enzymatic *NADH* values at $S_0=50$ g/l and pH 4.6. NFU, normalised fluorescence units

serve as a suitable marker of the culture physiological state under varied culture conditions.

The rate of the fluorescence change represented by the slope of the fluorescence observations has also been indicated as another culture parameter reflecting metabolic changes (Ristroph et al. 1979) occurring at different stages of the culture and characterising the culture behaviour in different media (Boufflette et al. 1987). The instantaneous slopes of fluorescence profiles for the *C. acetobutylicum* fermentation conducted at $S_0=50$ g/l and pH 4.6 with associated metabolic culture events are plotted in Fig. 6. Exclusive solvent production was observed for $dF/dt > 25$. The rate $dF/dt$ represents a suitable parameter for on-line characterisation of the intracellular culture metabolic activities as well as for judging the normal culture performance in repetitive batch cultivations.

The requirement for higher levels of cell NADH for good butanol production has been reported in the literature (Datta and Zeikus 1985; Meyer and Papoutsakis 1989). However, the recent report by Rao and Mutharasan (1989) concluded that there was an inverse relationship between the raw NADH fluorescence and butanol accumulation. The above authors also indicated that the turnover rate between the reduced and oxidised states of NAD, rather than the NADH pool size, is a more important factor for good solvent production. The results of the present investigation (eight pH-stat batch cultivations, Fig. 7) support a direct relationship between the net $F$ and butanol accumulation [for butanol concentration $(B) > 1$ g/l] and suggest that high cellular levels of NADH favour pronounced solvent accumulation. Solvent production related to the levels of NADH in the culture, although contradicting the conclusions of Rao and Mutharasan (1989), has been further established by an independent study under steady-state continuous conditions (Srivastava 1990).

In conclusion, the methodology of using on-line fluorescence measurements and a theoretical stoichiometric framework to elucidate the direction of electron flow in *C. acetobutylicum* has been demonstrated. However, application of the above procedure to unconventional high-pH culture conditions failed to highlight the need for another suitable process parameter to assist in defining the culture physiology. The $H_2/CO_2$ ratio proved to be very useful in this sense, assisting in distinguishing between the two phases of culture metabolism. So did the $dF/dt$ parameter, which can also be

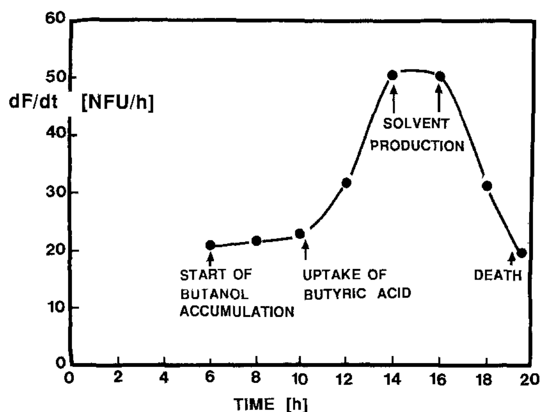


**Fig. 6.** Time derivative of fluorescence with respect to different metabolic events for a batch fermentation at $S_0=50$ g/l and pH 4.6

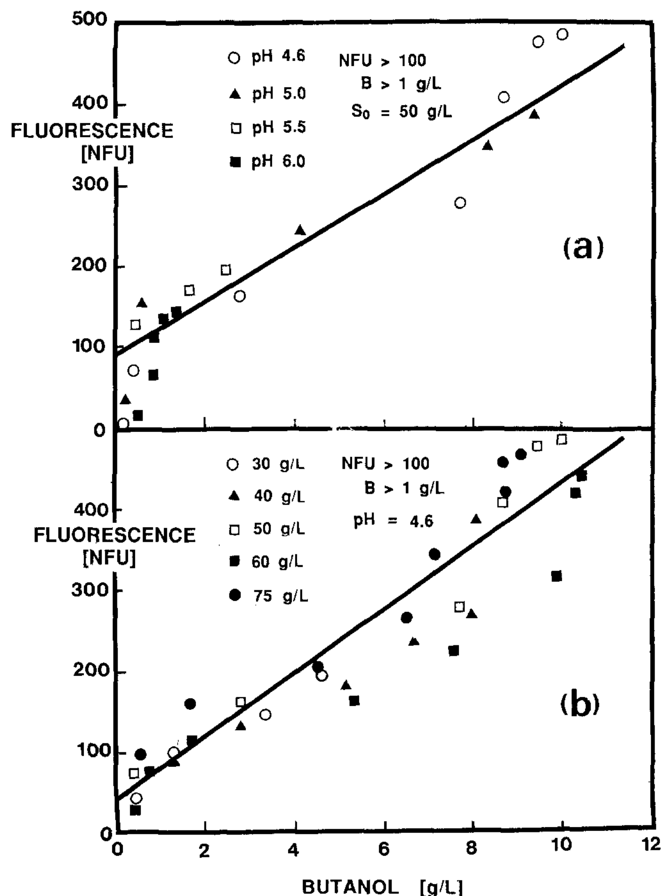


**Fig. 7a, b.** The correlation between net fluorescence values ($F$) and butanol concentration ($B$) under different culture growth conditions. **a** $F=109.8+24.6B$ (for pH 4.6–6.0). **b** $F=45.8+33.9B$ (for $S_0=30$–$75$ g/l)

employed in on-line characterisation of the culture metabolism.

The value of $F/X$ constitutes a parameter for characterisation of the culture physiological state. Acidogenic culture metabolism was invariably observed for $F/X =$ or $< 20$, whereas solventogenic culture metabolism featured $F/X > 65$.

The direct correlation between $F$ and $B$ under constant pH culture conditions observed in this investigation supports the hypothesis of a direct relationship between the culture NADH concentration and butanol production. This correlation suggests the possibility of using the fluorescence probe also as a butanol probe in this fermentation.

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