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In situ fluorescence measurements—clarifying or blurring the picture?

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Abstract - Several studies on fermentation systems have been made with *in situ* fluorescence probes during the past fifteen years. These probes normally contain a stable UV light source and filters for selection of the appropriate excitation and emission wavelengths. The objective of different studies has varied. Culture fluorescence has been used for on-line biomass estimation, for monitoring of metabolic changes and for process control purposes. In this short review, different applications of *in situ* fluorescence measurements are discussed.

INTRODUCTION

Fermentation monitoring and control is a subject of increasing interest as the field of biotechnology matures and processes begin to reach production scale. Process control requires fast and on-line measurement techniques. Several on-line monitoring techniques have been developed such as flow injection analysis (FIA), mass spectrometry (MS) and on-line high performance liquid chromatography (HPLC) (refs. 1, 2). However, most of the available techniques are useful only for the measurement of the abiotic phase, e.g. measurement of the substrate concentration or off-gas analysis. Few on-line methods are available for the measurement of intracellular metabolite or cofactor concentrations, i.e. direct measurement of the "biological state" of the microorganisms. Measurement of culture fluorescence is one of these possible methods.

The basic principle behind culture fluorescence measurements is that a suitable fluorophore, preferably naturally present in the microorganism, is excited by light of a certain wavelength. The fluorophore emits light of a slightly longer wavelength, which is detected by either a photodiode or a photomultiplier. The geometric arrangement for an *in situ* probe is normally an open-ended one, which means that the back-scattered fluorescence is measured. Most probes have been tuned for the detection of NAD(P)H, which emits light with an intensity maximum at 460 nm when excited by light of the wavelength 340 nm.

In 1957 Duysens and Amesz (ref. 3) were the first investigators to use fluorimetric methods for the study of intracellular NAD(P)H concentrations in commercial Bakers' yeast. They concluded that the fluorimetric methods were probably more selective than the previously used absorption spectrophotometry for *in vivo* studies of NAD(P)H. The first on-line measurements were made by Harrison and Chance (ref. 4) in 1970 on chemostat cultures of *Klebsiella aerogenes*. With the commercialization of fluorosensor probes (FluoroMeasure System, BioChem Technology; Fluorosensor, Ingold AG), fluorescence measurements grew increasingly popular in the 1980's. Below, papers have been classified according to the different objectives of the studies.

ESTIMATION OF BIOMASS CONCENTRATION

On-line estimation of biomass concentration is far from trivial in fermentation processes. The traditional off-line method of cell concentration determination by measurement of turbidity (in the visible region

550-650 nm) is not that easy to apply on-line. The cell concentrations are normally too high, which gives a poor sensitivity. However, several turbidimetric sensors using IR-LEDs have recently reached the market (MEX3, BTG Källe Inventing; Cell Growth Probe, Wedgewood Technology). A different approach is to measure the dielectric permittivity, and a commercial instrument based on this priciple is available (BUGMETER, Aber Instruments).

Culture fluorescence can be used to measure biomass concentration, if one can assume a constant intracellular concentration of NAD(P)H. This will obviously not be true when the metabolism of the microorganism is changing, but may well be a reasonable assumption during balanced growth. Several investigators have correlated measured fluorescence to biomass concentration (Table 1).

TABLE 1. Investigations aimed at biomass estimation using culture fluorescence.

Authors	Ref.	Microorganism	Type of correlation
Zabriskie and Humphrey	5	*Saccharomyces cerevisiae*	log-log
		Streptomyces sp.	log-log
		Thermoactinomycetes	not found
Meyer *et al.*	6	*Bacillus subtilis*	linear
		E. coli (transformed)	not found
		Sporotrichum sp.	not found
Scheper *et al.*	7	*Zymomonas mobilis*	linear
		Penicillium sp.	not found
Luong and Carrier	8	*Methylomonas sp.*	linear
Samson *et al.*	9	*Saccharomyces cerevisiae*	linear
		Pseudomonas putida	linear
MacMichael *et al.*	10	Hybridoma cells	polynomial
Boyer and Humphrey	11	*Pseudomonas putida*	linear (partly)
Greer *et al.*	12	*Xanthobacter sp.*	linear
Li and Humphrey	13	*Candida utilis*	log-log
		Pseudomonas putida	log-log
		Saccharomyces cerevisiae	log-log
Li *et al.*	14	*Saccharomyces cerevisiae*	log-log
		Candida utilis	log-log

Not all attempts to correlate the biomass concentration with the fluorescence signal have been successful. Problems with a too low fluorescence signal (ref. 6), changing metabolism during fermentation (ref. 5), or excretion of fluorophores (ref. 6) or of compounds absorbing excitation light (ref. 11) sometimes makes it impossible to obtain a correlation useful for biomass estimation.

When a correlation is found, it is normally either a linear correlation between the biomass concentration and the fluorescence signal, or a correlation between the logarithm of the biomass concentration and the

logarithm of the fluorescence signal. Linear correlations are reported for low biomass concentrations (up to approximately 2 g dwt./l), whereas the logarithmic correlations are found for higher biomass concentrations (up to 50 g dwt./l). These differences are probably due to the intrinsically nonlinear nature of open-ended fluorescence probes. Several factors are important for this nonlinearity, one of which is the so called inner filter effect, which was taken into account in the model of Srinivas and Mutharasan (ref. 15). Besides the inner filter effect, geometric factors are also important (refs. 16, 17). When these factors are taken into account, a rather complex model results, containing several parameters. However, it is possible to lump some of the parameters and obtain a simplified two-parameter model valid for a wide concentration range (ref. 16). A linear model may be sufficient for low concentrations, but a separate calibration should be made in each fermentor.

Another question of interest is whether perhaps there is a better intracellular fluorophore for biomass monitoring than NAD(P)H present in microorganisms. This was examined by Li and coworkers (ref. 13, 14) by multiple excitation fluorometry. The fluorophores examined, apart from NAD(P)H, were tryptophan, pyridoxine and riboflavin and the authors conclude that tryptophan and pyridoxine may indeed be a better choice than NAD(P)H for biomass monitoring, at least for some microorganisms.

MONITORING OF METABOLIC CHANGES

As only the reduced forms (NADH and NADPH) but not the oxidized forms (NAD^+ and $NADP^+$) fluoresce, the fluorescence signal is sensitive to any metabolic change, which influences the ratio between oxidized and reduced forms. Therefore, culture fluorescence has been used to study changes in the metabolic state (Table 2). Apart from NAD(P)H fluorescence, fluorescence of intracellular F_{420}, a coenzyme present in methanogenic bacteria (ref. 33), and intracellular FAD (ref. 26) have also been investigated. Interestingly, not only suspended cells but also immobilized cells can be studied (refs. 23, 25, 28).

The studies can be divided into studies of rapid metabolic changes (aerobic/anerobic transitions, substrate puls responses, toxic pulse responses) and slow metabolic changes (diauxic growth, acetone-butanol fermentation). The quantity of interest is often the specific fluorescence value (i.e. the fluorescence signal divided by the biomass concentration). For rapid metabolic changes the biomass concentration can be assumed to be approximately constant and the measured fluorescence changes can be interpreted directly as changes of the specific fluorescence. For slow changes, however, an independent measurement of the biomass concentration is necessary to obtain the changes of the specific fluorescence. Also other aspects, such as background fluorescence changes and rheological changes, are more important and must be considered. Care must also be exercised with e.g. the use of antifoam in the fermentor broth (ref. 35).

In Fig. 1 fluorescence measurements on an anaerobic fermentation of xylose by *Pichia stipitis* is shown. The biomass concentration during the fermentation decreases slowly, but the fluorescence value increases. The background fluorescence, measured after separation of the cells, does not show a corresponding increase. The increase in fluorescence is thus not caused by the excretion of an extracellular compound. When compared to on-line HPLC measurements (Fig. 1), it is seen that maximum fermentation rate is obtained at a relatively constant fluorescence level. This information may possibly be used for fed-batch control of a xylose fermentation.

The prospects of bioprocess control using culture fluorescence have been tested by some investigators. Control of fed-batch of *C. utilis* was tested by Watteeuw *et al.* (ref. 18). In this paper, the authors managed to decrease the acetate formation and thereby increase the biomass productivity. Fed-batch control of *C. acetobutylicum* was demonstrated by Srivastava and Volesky (ref. 29) by a manual addition scheme, by which the authors managed to maintain a high butanol productivity. Meyer and Beyeler (ref. 20) used culture fluorescence in combination with carbon dioxide evolution rate for maximizing the biomass productivity in a continuous culture of *S. cerevisiae.*

TABLE 2. Studies on metabolic changes using culture fluorescence

Authors	Ref.	Microorganism	Comments
Watteeuw *et al.*	18	*Candida utilis*	Fed-batch culture
Beyeler *et al.*	19	*Candida tropicalis*	Aerobic/anaerobic transitions, pulse experiments
Meyer and Beyeler	20	*S. cerevisiae* *S. uvarum*	Synchronous growth, control Dynamic change of dilution rate
Armiger *et al.*	21	*S. cerevisiae*	Aerobic/anaerobic transitions, pulse experiments
Scheper and Schügerl	22	*S. cerevisiae*	Continuous culture, synchronous growth
Müller *et al.*	23	*S. cerevisiae*	Immobilized cells
Lidén *et al.*	24	*S. cerevisiae*	Batch culture
Anders *et al.*	25	*S. cerevisiae*	Immobilized cells
Siano and Mutharasan	26	*S. cerevisiae*	Aerobic/anaerobic transitions pulse experiments, FAD and NAD(P)H
Siano and Mutharasan	27	Hybridoma cells	Aerobic/anaerobic transitions Substrate pulse experiments
Reardon and Bailey	28	*Clostridium acetobutylicum*	Immobilized cells
Srivastava and Volesky	29, 30, 31	*C. acetobutylicum*	Continuous culture, Batch culture Substrate concentration changes, Dilution rate changes
Rao and Mutharasan	32	*C. acetobutylicum*	Continuous culture Induction of solventogenesis
Peck and Chynoweth	33	methanogenic bacteria	Measurement on coenzyme F_{420} and NAD(P)H
Lidén and Niklasson	34	*Pichia stipitis* *Pa. tannophilus* *Candida utilis*	Aerobic/anaerobic transitions

Other applications

Fluorescence measurements have been used also for other purposes than biomass concentration monitoring or monitoring of metabolic changes. As examples could be mentioned that the use of a NAD(P)H fluorosensor probe in an optical biosensor system for measurement of lactate and pyruvate (ref. 36) has been reported, as well as pH monitoring using the fluorophore 1,4 dihydroxyphthalonitrile (ref. 37).

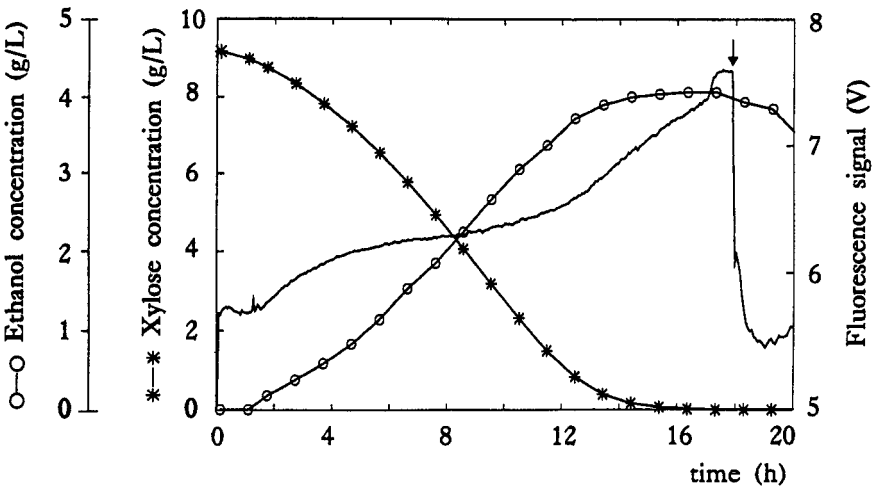

Fig. 1 Fluorescence measurements on an anaerobic batch fermentation of xylose by *Pichia stipitis* (CBS 5773) in a defined medium. The figure also shows on-line HPLC determinations of xylose and ethanol concentrations. The steady flow of nitrogen through the fermentor (0.2 vvm) was exchanged for air at the time indicated by the arrow in the figure. The cell concentration at the beginning of the fermentation was 1.9 g d.w./L and the cellconcentration at t=17 h was 1.2 g d.w./L. The temperature during the fermentation was 30 °C and the pH was controlled at 4.5 (G. Lidén and C. Niklasson, unpublished results).

CONCLUSIONS

The interpretation of an *in situ* fluorescence measurement is not trivial as the signal is influenced by many factors such as bubbling, interfering fluorophores, temperature, absorbing compounds, dissolved oxygen tension and pH. However, the technique offers several advantages. It is fast, continuous, and gives information about intracellular concentrations, an information very difficult to obtain on-line with other methods. So far, most studies have concerned NAD(P)H fluorescence, both for biomass concentration monitoring and for the monitoring of metabolic changes, but other fluorophores will probably be considered in the future, especially for monitoring of the biomass concentration.

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