New trends in measurement and control of biotechnological processes

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Abstract - To improve the biotechnological production processes, improved on-line ana-
lysis techniques and better process control is necessary. The present review reports
mainly on recent developments in on-line techniques, such as on-line aseptic sampling,
on-line continuous flow analysis, on-line flow injection analysis, on-line HPLC, and on-
line mass spectrometry. Methods for the determination of the concentration and biologi-
cal state of the cells are also taken into account as well as the necessity for sophisticated
control.

INTRODUCTION

For the biosynthesis of cell mass, primary, and secondary metabolites as well as of enzymes for
industrial and diagnostical applications and of proteins for therapeutical use, coordinated interplay
of several enzymes is necessary. In order to achieve the full biological potential of the cells, the
optimal environmental conditions of cell growth and product formation must be maintained in the
bioreactor, at least with regard to the most important key parameters.

The activity of a particular enzyme is controlled by the concentration of several cultivation medium
components. Well-known are enzyme regulations by carbon compounds (e.g., glucose), nitrogen
compounds (e.g., ammonia), phosphates as well as induction of enzymes by their substrates. Gene-
rally speaking, high concentrations cause repression or inhibition, low concentrations reduce the
growth and the product formation rate. At times, this optimal concentration range is very narrow.

Several possibilities are available for controlling the concentration of key components: frequently,
components of low solubility (e.g., peanut flour) are employed, or substrates are used (e.g., lactose)
which can only be consumed slowly by the microorganisms. However, with strain improvement, the
optimal concentrations of the key compoments may vary. Such flexible operation can only be real-
ized by fast *in situ* or *on-line* process analysis suitable for process control.

The control of the concentration of the limiting component is particularly difficult due to the low
concentration level. In that case, the concentration of the key component often is calculated from
measurable components by means of a mathematical model.

In the following, new *in situ* and *on-line* measuring techniques for fast analysis of medium compo-
nents and characterisation of cell properties and their use for process control are presented.

ON-LINE MONITORING OF CULTIVATION MEDIUM COMPONENTS WITH LOW-MOLECULAR WEIGHTS

In situ techniques are restricted to the measurement of dissolved oxygen (pO_2), dissolved CO_2
(pCO_2), hydrogen ion (pH), and redox potential (ref. 1). The concentration of the other medium
components are measured *ex situ*, outside the reactor. Since most bioreactors are operated mono-
septic, aseptic sampling from the reactor is necessary for on-line analysis.

The most common on-line sampling systems use cross-flow filtration modules which are integrated
into a medium recirculation loop (e.g., BIOPEM of B. Braun Diessel Biotech, Melsungen) (ref. 2)
tubular *in situ* filters (e.g., *In situ* Filter of ABC Bioverfahrenstechnik/ Biotechnologie, Puchheim)
(ref. 3). Contrary to the *in situ* tubular filters, the cross-flow modules cannot be used for cultivations
of shear-sensitive (e.g., animal) cells, microorganisms which quickly change their metabolism under
oxygen-limited conditions (e.g., *S. cerevisiae*) and of recombinant microorganisms.

Most classical analytical instruments are used for *ex situ* analysis. The main problems are the on-line sample conditioning, calibration, and blank measurements.

The continuous air-segmented flow analyser (CFA) is popular in chemical industry (Technicon System of Bran + Lübbe, Skalar Analytica). However, if used for cultivation broth sampling after three days of operation, it usually breaks down due to protein precipitation and cell growth in the analyser system. This technique is also impaired by necessitating high amounts of reagents and samples and long analysis times (ref. 4).

The flow injection analyser (FIA) uses a carrier flow with a reagent in which a small amount of sample pulse is injected (ref. 5).This causes a large dilution of the cultivation medium and reduces the probability for cell growth in the analyser considerably. The analyte forms a product with the reagent, the concentration peak height of which is measured in the detector. Requiring a low amount of reagent and sample, short reaction times, and high flexibility are properties which explain its increasing popularity.

High Performance Liquid Chromatography (HPLC) is a standard technique in chemical industry (ref. 6). However, it is rarely applied for on-line process monitoring in biotechnology. When using very pure chemicals and degassed solvents, stable, long-range operation is possible (ref. 7).

Inexpensive mass spectrometers (MS) have become popular for monitoring exhaust gas composition of cultivations. Recently, dissolved gas and volatile components have also been analysed on-line (ref. 8).

Fully automated analyser systems allow the real-time elemental balancing and evaluating of non-measurable process variables (e.g., cell concentration) (ref. 9). The on-line measurement of the concentrations of all precursers and key medium components in bioreactors allowed the investigation of the *in vivo* biosynthesis of secondary metabolites during their production as a function of the medium composition (ref. 10) as well as the evaluation of dynamic relationship between cell regulation and reactor control.

These are the prerequisites for the development of a realistic dynamic model for advanced process control of the cell/reactor system.

ON-LINE MONITORING OF CULTIVATION MEDIUM COMPONENTS WITH HIGH-MOLECULAR WEIGHTS

The on-line measurement of the concentration of biopolymers during their production is still rarely applied.

For on-line monitoring of extracellular enzyme activities, usually model substrates are used which are converted into a product, the concentration of which is measured in CFA or FIA (ref. 11).

Immunoassays and HPLC are used for measuring a particular protein concentration in the cultivation medium.

On-line protein monitoring has been developed with turbidimetric immunoassays (Figure 1) as well as with homogeneous (Figure 2) and heterogeneous competitive immunoassays (Figure 3) (ref. 12, ref. 13). Figures 4, 5, and 6 show three examples for the on-line monitoring of the enzyme pullulanase, monoclonal antibody IgG, and Antithrombin III.

The response time of turbidimetric immunoassays is low enough (some minutes) to control the production process. Faster analysers are required, however, for the control of protein recovery and purification processes. In recent years, superfast HPLC techniques (of some seconds analysis time) have been developed for protein analysis (ref. 14). These techniques, however, have not been used for process control.

Mass spectrometric analysis of biopolymers has not been possible until recently, because of the high polymer masses. Different ionization techniques have been developed of late, which extended the use of the mass spectrometer to biopolymers.

By fast atom bombardment, mass spectrometry (FABMS), biopolmer ions with neutral atoms are produced. A useful range is up to 10 kDa (ref. 15). Liquid secondary ion mass spectrometry (LSIMS)
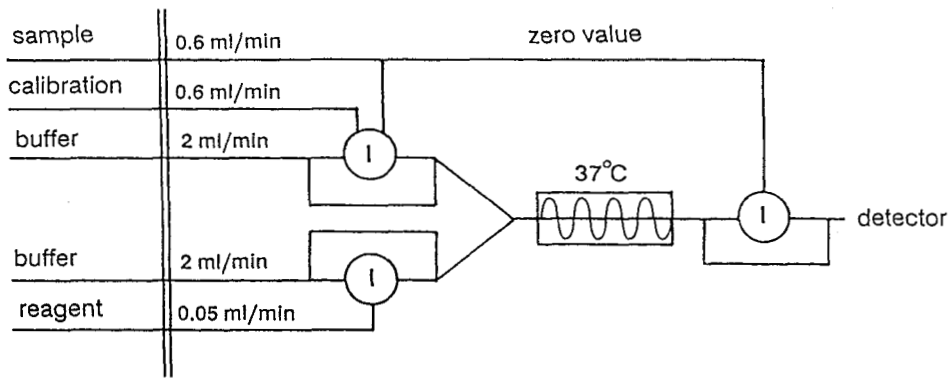
Fig. 1 Turbidometric immunoasay flow injection analyser. The antigen (Ag) in the sample and the antibody (Ab) in the reagent form an Ag-Ab complex which aggregates and scatters the light in the detector. The concentration of Ag is calculated from the extinction.
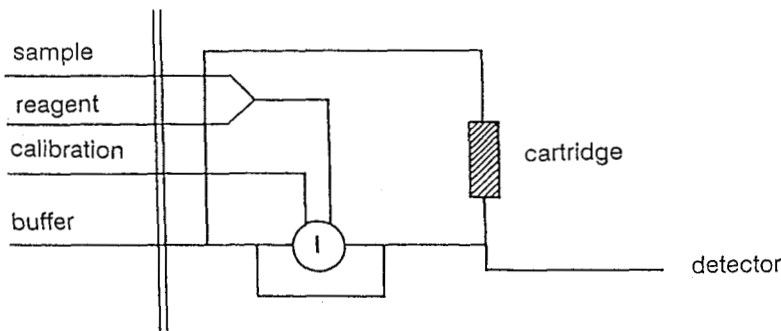

Fig. 2 Heterogeneous competitive immunoassay flow injection analyser. The Ab is immobilized on the surface of a carrier in a cartridge. The unlabeled Ag in the sample and the fluorescence dye-labeled Ag in the reagent compete for the binding sites of Ab. After forming the Ab-Ag complex, the cartridge is rinsed, the complex is displaced, and the fraction of the labeled Ag is measured in the detector.
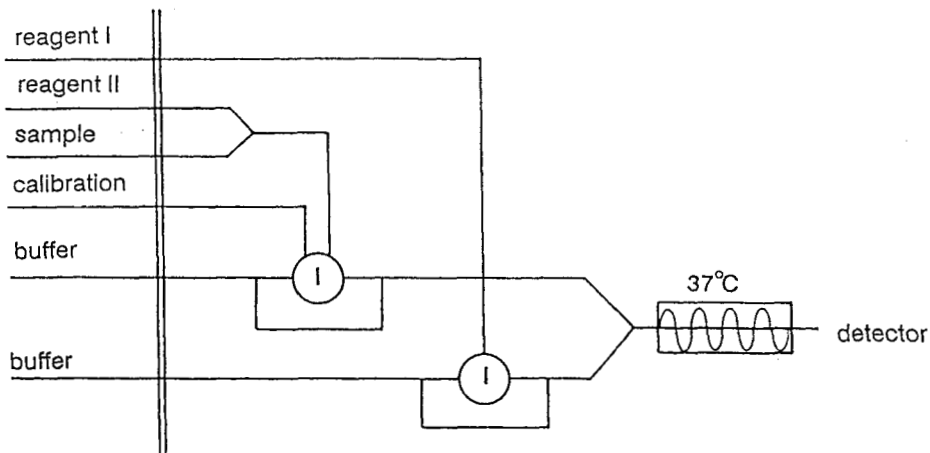

Fig. 3 Homogeneous competitive immonoassay flow injection analyser based on the energy transfer between labeled Ab and labeled Ag.
Fluorescence dye-labeled Ab in reagent I and fluorescence dye-labeled Ag in reagent II form an Ab-Ag complex which emits fluorescence light if excited in the detector with UV light. The unlabeled Ag in the sample competes for binding sites of Ab with labeled Ag, but it does not emit fluorescence. The higher the fluorescence intensity, the lower the concentration of Ag in the sample.
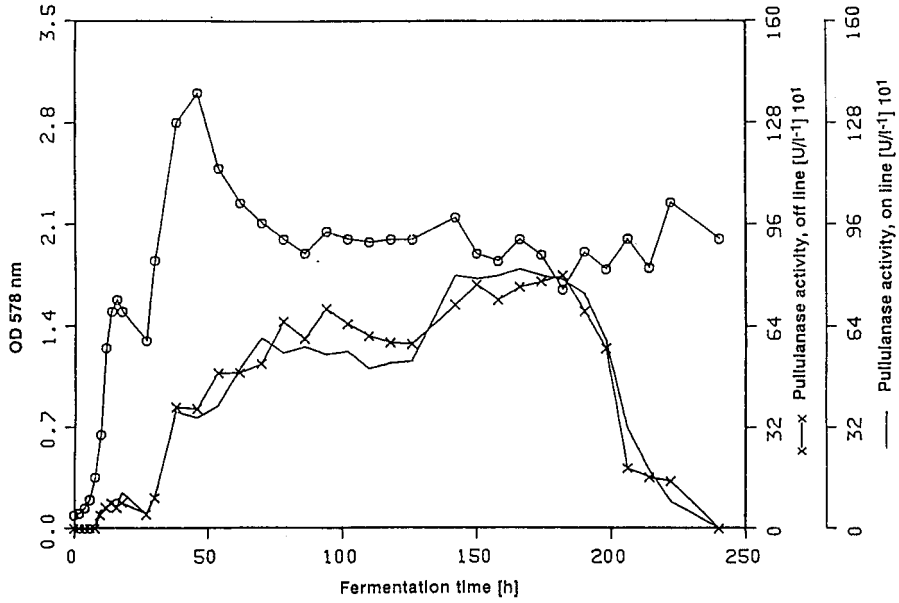
Fig. 4   Mean pullulanase concentration measured on-line (TIA) and off-line
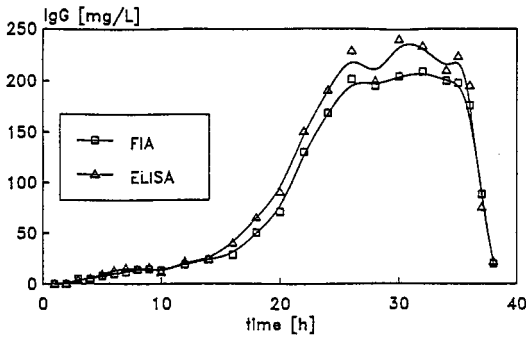   (reference assay) during a *C. thermosulfurogenes* cultivation.

Fig. 5
Correlation between turbidometric flow
injection analysis (FIA) and ELISA for
the determination of IgG in a simulated
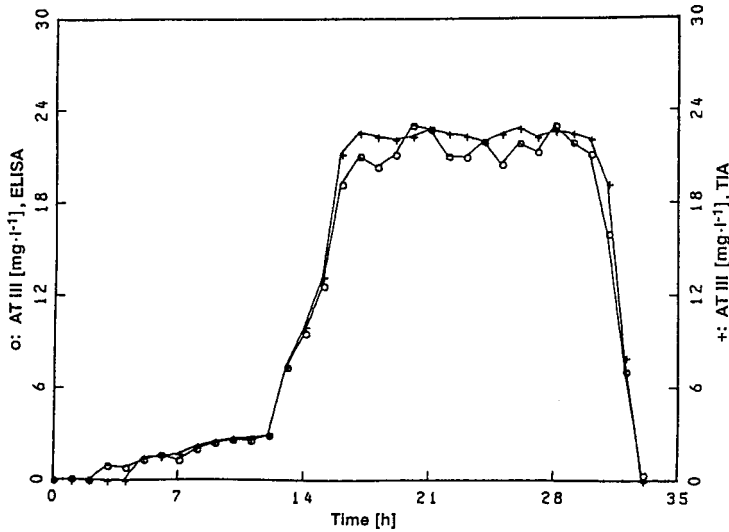fermentation.

Fig. 6   Simulation of a cultivation of AT III-producting cells. AT III is determined hourly by
         the turbidometric assay (TIA) and reference ELISA. Values given for the on-line
         assay are the mean value of three measurements, while the ELISA data are averages
         of two assay measurements.

uses energetic ions (e.g., Cs ion) for ionization (ref. 16). Electrospray mass spectrometry (ESMS) employs a high voltage field to produce a dispersion of charged fluid droplets. Multicharge protein ions desorb from the fluid surface and can be detected (ref. 17). "Ionspay" and "thermospray" (ref. 18) are variants, where gas and heat, respectively, assist the ion evaporation process (ref. 16).

Up to now, no on-line monitoring of biopolymers with mass spectrometry is known.
These new ionization techniques will enlarge the application of mass spectrometry for biopolymers in the future.

EVALUATION OF THE CONCENTRATION AND BIOLOGICAL STATE OF THE CELLS

The concentration of microorganisms and animal cells are important state variables. The most common methods to determine the cell concentration (biomass comcentration) in situ in cultures of microorganisms are the measurement of the transmitted light (turbidometry) (Bonnier Technology Group, EUR-Control, Models MEX 2/OD-10/5 and MEX 3/RD-10/5) and scattered light (nephelometry) (ref. 19). These methods, however, are not selective enough. They cannot distinguish between solid particles, dead, and viable cells.

The dielectric permittivity of suspensions is sensitive to the electrical properties of the particles. Hence, it is possible to make a distinction between cells and suspended solid particles in the culture that have different electrical properties. The BUGMETER of ABER INSTRUMENTS uses this principle. Nipkow et al. (ref. 20) tested this instrument and found, however, that its sensitivity was too low for low cell concentrations, and at high cell concentrations the conductivity of the cell culture was too high for measuring adequately.

Calorimetric or fluorometric methods can be used to measure the concentration of the viable cells.

Metabolically-active microorganisms produce heat having a direct relationship with the consumed oxygen (ref. 21). When using a calorimeter for cell cultivation, the cell mass concentration can be estimated from the heat produced by the cells under well-defined cultivation conditions (ref. 22). A heat flux calorimeter, called "Reaction calorimeter RC1", developed by Ciba Geigy AG, Basel, and commercialised by Mettler Instruments, is on the market.

NADH and NADPH in the vital cells can be excited by UV light, and the induced fluorescence can be measured by suitable detectors at 460 nm. Two instruments are on the market: FluoroMeasure of Biochem Technology and FLUOROSENSOR of Dr. W. Ingold.

This NAD(P)H fluorescence has a large potential as a tool to monitor, study and control cultivations (ref. 23, 24, 25). Zabriskie and Humphrey used culture fluorescence for monitoring viable cell mass concentration (ref.26). They found the following relationship between the fluorescence intensity I_f and the cell mass concentration X:

$$X = [e^{-b} \cdot I_f]^{1/a},$$

where a and b are constants, characteristic for the biological system and the reactor. In some special cases there is a linear relationship between X and I_f (e.g., for *Zymomonas mobilis* under nonlimited growth conditions (ref. 27), *Methylomonas mucosa* (ref. 28) and *Pseudomonas putida* (ref. 29).

Since the culture fluorescence is affected by several medium components which are fluorescence-active, and by the variation of the biological state of the cells, which has a severe effect on the NADH pool in the cells, the use of culture fluorescence for on-line monitoring of biomass concentration is a fairly difficult problem, whose solution needs a great deal of experience (ref. 30).

The determination of the biological state of the immobilized cells is especially difficult. It was possible to observe the increase of the viable immobilized cell concentration qualitatively by culture fluorescence (ref. 31).

In situ determination of the biological state of the cells is an extremely difficult problem. Different techniques are used to gain more information on the cell state, i.e.,

-    mass balancing,
-    fluorometry,
-    microcalorimetry, and
-    their combination.

The main problem with these techniques is the interpretation of the measured signals.

The use of the ratio of the CO_2 production rate in relation to the O_2 uptake rate, which is called respiratory quotient, RQ, is the most common technique of all mass balancing methods. RQ < 1 designates oxidative, and RQ > 1 denotes oxidative-fermentative metabolism. Several research groups used the RQ-control for baker's yeast production (e.g., ref. 32, 33).

By means of the intensity of the culture fluorescence due to intracellulary NADH, the aerobic-anaerobic transition, the influence of the dissolved oxygen concentration, dilution rate, substrate concentration, diauxic growth, synchronous culture, glycolytic oscillation, metabolic shifts, etc. can be investigated. In Table 5 of ref. 19 all of these investigations are compiled.

On account of the fast response, the culture fluorescence and its non-invasive character can be used for process control in combination with the CO_2 production rate and the respiratory quotient (ref. 34).

The on-line determination of the intracellulary enzyme activities is a difficult job. However, examples have been published on the determination of penicillin G amidase activity in recombinant E. coli with CFA (ref. 35) and the ß-galactosidase activity in E. coli with FIA (ref. 36).

Off-line methods are used for the determination of the intracellular key components (DNA, RNA, proteins, lipids) after their staining employing a laser flow cytometer (e.g., ref. 37, 38).
One can also distinguish between plasmid-free and plasmid-carrying cells, provided, the plasmid contains an antibiotics resistence gene, and the cell is cultivated in the presence of antibiotics (ref. 39, 40, 41, 42).

Under favourable conditions the gene expression can be observed by measuring the cell size and protein content quasi on-line with a flow cytometer (ref. 43).

Several different nuclei can be used to measure the intracellular pH by Nuclear Magnetic Resonance Spectroscopy (NMR), including ^1H and ^{13}C (ref. 44), ^{14}N and ^{15}N (ref. 45), ^{19}F (ref. 46) and ^{31}P (ref. 47). Nearly all investigators, however, used ^{31}P for measuring.
Intracellular pH measurements by in vivo ^{31}P NMR have become fairly common and have been used to study metabolic processes in different organisms (e.g., ref. 48).

NMR measurements take from hours to days. It is necessary to provide the cells with nutrients, including oxygen, during these investigations. Special mini-reactors were used for this purpose (ref. 49). However, they remain a laboratory technique that are used for basic research purposes.

PROCESS CONTROL

For the optimal operation of bioprocesses they have to be observable and controllable. By means of in situ and on-line monitoring of the control and state variables of the process, the system is being observed. These observed variables are used to maintain the optimal level of the control variables. Control of bioreactor systems can be divided into (ref. 50):

– Low-level control:
    sequence control (e.g., control of a batch process),
    single-loop control of environmental variables (e.g., temperature-, pH- or stirrer rpm-control),
    advanced process control,
    feedforward control (before the disturbance occurs, a corrective control action is performed in anticipation of the expected effect)
    adaptive control (when the characteristics of the process change with the time, the operationg conditions may need to be changed. This includes controller parameters as well as setpoints).
– Optimization and high-level control:
    constant setpoint is optimized for low-level control and for continuous bioreactors, profile optimization of fedbatch operation (e.g., baker's yeast production).
– Adaptive optimization and control:
    Determination of the optimal operation conditions for bioreactors that may be unknown or may change with the time.

On account of the high complexity of biological systems, it is not possible to model them without simplification. Furthermore, cell cultivation and product formation are not deterministic processes. Also, frequently, important control variables cannot be measured on-line. Therefore, at times, a

state estimator and adaptive control are necessary for optimal process operation.

The advanced control techniques are abeing developed and have partly been applied in laboratories. However, they are not yet reliable enough for application in biotechnological production processes. It is ex-pected that their reliability will be improved within the next years, and that they will be introduced gradually into industrial practice.

CONCLUSION

It is expected that on-line process analysis will be extended to additional medium components by using biosensors integrated into FIA systems. Intracellular components will be analysed by non-invasive methods, such as NADH fluorescence, and by on-line cell disruption and CFA or FIA analysis of the intracellular enzyme activities. Analysis instruments will be made more intelligent by suitable software, and on-line process analysis will be more sophisticated by means of knowledge-based evaluation of the measurements.

Off-line techniques such as flow cytometry measurements will be speeded up and will be operated in an on-line mode. A first successful endeavor has already been reported on (ref. 51).

NMR methods will only be used for basic research investigations because of high costs and time-consuming measurements.

Advanced control techniques will be used not only in laboratories, but also in production, if their reliability is improved further.

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