Review

Sampling and analytical strategies in on-line bioprocess monitoring and control

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Abstract

The use of on-line systems for the determination of small organic molecules during biotechnological processes is reviewed. Since sampling of part of the culture medium is an inherent and crucial part of the use of on-line systems, several sampling systems are described and compared and a categorization of sampling techniques — non-membrane, dialysis and ultrafiltration — is provided. Further, several analytical approaches — based on flow-injection or chromatographic analysis — and their relative merits for on-line measurements are discussed. Selected examples from the recent scientific literature serve to illustrate the applicability for on-line monitoring and control of bioprocesses.

Keywords: Reviews; Sampling methods; Process monitoring; Biotechnological processes; Saccharides

Contents

1. Introduction

Over the past years, the use of biotechnological processes (fermentations) for the production of a wide range of low- and high-molecular-mass compounds has increased sharply. In order to ensure economical viability, one of the primary objectives in their application is the improvement of the product yield. Traditionally, this is achieved by selecting micro-organism strains with elevated performance or optimizing the bioreactor configuration. In addition, the past few years have seen an increasing interest to

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utilize analytical techniques for the monitoring and control of the physicochemical and biochemical growth conditions in the culture medium. This bioprocess monitoring encompasses the measurement of widely divergent parameters and, consequently, involves the application of a variety of analytical methods. The present review is restricted to an area which has shown many interesting developments in recent years, viz. the determination of small organic compounds, such as many nutrients, metabolites and fermentation products. For reviews on the monitoring and control of other important parameters such as cellular biomass, microbial activity, pH and off-gases the reader is referred to the literature [1–4].

The determination of dissolved organic molecules can be achieved in a number of ways. Traditionally, analyses are performed *off-line*, which involves manual sampling of part of the medium, followed by transport of the sample to the laboratory for analysis. Chromatographic techniques, in particular column liquid chromatography, are frequently used, which implies that some sort of pretreatment of the highly complex sample has to be performed to ensure a proper protection of the analytical system. If the equipment is located near the bioreactor, sample pretreatment and analysis can take place *at-line*. Obviously, these laborious and rather time-consuming procedures easily give rise to infrequent and delayed data and are, as such, not very attractive for monitoring purposes and certainly not for an efficient control of the bioprocess. Therefore, present interest tends to shift to more sophisticated methods. So-called *non-invasive* techniques, which use spectroscopic probes in the bioreactor wall and measure the reflection of emitted radiation, are very attractive since there is no direct contact between the analyser and the fermentation broth. Very few organic molecules have, however, been monitored in this way; one example is the monitoring of the production of ethanol by near-infrared spectroscopy [5]. By installing an analytical device (sensor) directly in the culture medium, *in-line* measurements can be performed. These processes are usually based on the electrochemical detection of the analyte itself or, as in the case of biosensors, on the electrochemical detection of substrates or products involved in the enzymatic conversion of the analyte. A major advan-

tage of in-line analysis is that there is no need for sample withdrawal and pretreatment, which means that measurements can be performed without time delay and, in principle, continuously. Although it is expected to receive greater attention in future, the use of sensors for monitoring of organic compounds during bioprocesses has been rather limited so far, mainly because of problems associated with fouling of the sensors by broth components and their inability to withstand sterilization. For further information, the interested reader is referred to Ref. [6].

It is generally assumed that in the near future the application of *on-line* methods for the determination of organics in biotechnological samples will be most attractive and the present paper focuses mainly on this approach. In this case, a representative part of the medium is withdrawn from the bioreactor and after pretreatment, if any, introduced directly into an analytical system without any human intervention. The spectroscopic, chromatographic and enzymatic methods used for off-line and at-line analyses can also serve for on-line monitoring, provided that the analytical equipment is properly interfaced to the bioreactor. It is the aim of this paper to give an overview of the variety of approaches that can be followed for the on-line monitoring of bioprocesses. Several types of sampling devices will be compared and the applicability of different analytical methods will be discussed.

2. Requirements

Before providing a categorization of systems for on-line bioprocess monitoring, it is important to outline the major requirements which such a system should fulfil [7]. From the biotechnologist's point of view, maintaining *asepticity* is probably the most important demand; that is, the use of the analytical device should not increase the risk of contamination of the culture by external organisms. In addition, the course of the bioprocess should not be disturbed in any other way. In order to ensure long-term *stability*, the sampling system should remove any sample component that can negatively affect the performance of the analytical system. Furthermore, for a

proper process control, i.e. to allow instantaneous manipulation of the bioprocess on the basis of the results obtained, a *minimum delay time* between sampling and availability of the data is necessary. In addition, it is essential that the (pretreated) sample which is introduced into the analytical device is a *representative* fraction of the fermentation broth. This means that the analyte recovery preferably should be quantitative or, if not, at least be accurately known. Finally, a monitoring system should be *flexible* and it should be possible to utilize it with a wide range of analytes and fermentation conditions.

Unfortunately, none of the presently available on-line monitoring systems (nor any of the others) fulfils all these requirements simultaneously. The major drawback of on-line analysis compared with non-invasive and in-line techniques is its relatively long response time (typically several minutes), particularly when chromatographic methods are involved. It should be realized, however, that in many cases the response time as such is not the most important parameter, but rather the response time relative to the time constant of the bioprocess [8]. Indeed, a response time of several minutes turns out to be satisfactory in many instances. Advantages of on-line monitoring are the possibility to apply a wide variety of reliable and established analytical techniques for the determination of essentially all analytes of interest and the favourable long-term stability of the total system.

3. Sampling

3.1. Full culture medium

Although most sampling systems described in the scientific literature contain a membrane-based device for the removal of cellular and particulate matter from the sample, it is also possible to withdraw an aliquot of a full fermentation broth for analysis. In this case, due precautions must be taken to stop the cellular activity in the sample immediately after withdrawal and thus prevent changes in the concentrations of the analytes during the delay time between sampling and analysis. To this end, the fermentation sample is generally mixed with a

compound inhibiting the metabolic activity of the microorganisms. In one example, part of the broth was pumped into a small (1.4 ml) glass vial, where it was mixed with a 0.2 M chloramine solution [9]. As the vial was autoclavable and because of the presence of the inhibitor, the sampling system functioned as a sterile barrier between the fermentor and the analytical system and no contamination of the fermentation broth was experienced even after one year of continuous use. By using a coaxial catheter (Fig. 1), which was installed in the fermentor wall and featured an inner lumen into which the sample was sucked and an outer lumen delivering a flow of an inhibiting solution, metabolic activity could be stopped exactly at the time of sample withdrawal [10]. As the flow into the inner lumen was greater than the flow out of the outer lumen (typically two-fold), thorough mixing of the sample and inhibitor occurred and leakage of the toxic inhibitor into the fermentor was negligible. The probe was used for
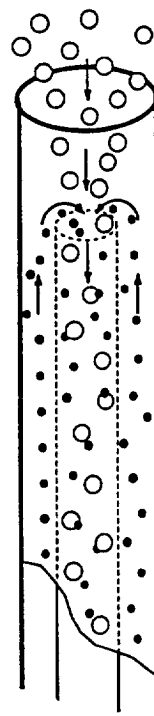

Fig. 1. Schematic diagram of a coaxial catheter used for sampling of a full culture medium: O, represents the biological sample; •, represents the inhibitor. From Ref. [10].

the monitoring of glucose and lactate in a 12-h *Lactobacillus delbreucki* batch culture, using 10 mM potassium cyanide as the inhibiting solution. It should be emphasized that the deactivated cells, which still are in the sample after addition of the inhibitor, generally have to be removed prior to the analysis. In the two cited applications, this was achieved by coupling a dialysis cell to the sampling unit. One may therefore conclude that the described approach does not have any major advantage over the direct use of dialysis and filtration for sampling from fermentors, provided that these membrane devices can be readily sterilized.

3.2. Membrane-based sampling systems

Since the mid-1980s several on-line monitoring systems involving a membrane-based sampling device have been described. The (porous) membrane effects the removal of cells from the fermentation broth, thus stopping the cellular metabolism in the filtrate, and it also retains other compounds which interfere with the subsequent analysis, such as proteins, as much as possible. Membrane-based sampling systems can be categorized according to two points of view. A first distinction can be made based on the membrane technique used, viz., dialysis and ultra- or microfiltration [11]. In dialysis, a concentration gradient is the only driving force for transport through the membrane and molecules of appropriate size diffuse from the sample to a so-called acceptor phase on the other side of the membrane, which typically consists of water or a buffer. In filtration, a pressure is applied to force the analyte molecules as well as the solvent through the membrane pores and, consequently, no acceptor phase is necessary. Microfiltration membranes (pore size typically 0.1–1 μm) are used to retain cellular material, whereas ultrafiltration membranes (1–100

nm) also remove macromolecular compounds. Advantages and disadvantages of both techniques for use in on-line bioprocess monitoring are summarized in Table 1.

Because of the non-quantitative character of dialysis, the system needs proper calibration in order to accurately determine the recovery and calculate the concentration of the analytes. In addition, it is essential that the analyte recovery does not change during a fermentation run. Since dialysis membranes are not very susceptible to fouling, this normally is no major problem. Speed and recovery are coupled in dialysis, which means that a higher recovery normally can be obtained at the expense of a longer dialysis time. In principle, filtration yields a representative sample (i.e., with 100% recovery of small analytes) as soon as a pressure is applied across the membrane. A drawback of filtration is that losses of the fermentation broth occur, since part of the filtrate is used for analysis. Because of the use of an acceptor phase, this does not happen with dialysis. Membrane fouling is a bigger problem in filtration than in dialysis, because compounds larger than the pores are, reversibly or irreversibly, forced against the membrane. To be precise, the reversible accumulation of retained compounds on the membrane is called concentration polarization and only the irreversible blockage of membrane pores fouling. Both processes contribute to an increased resistance to mass transport and give rise to a decreased filtrate flow through the membrane.

Secondly, the location of the membrane is of importance [12]. Internal or in situ devices are placed inside the fermentor below the broth surface level and a cell-free acceptor phase or filtrate is continuously aspired and introduced into the injection loop of an analytical system. If an external sampling system is used, part of the broth is pumped out of the fermentor, through a membrane device and

Table 1
Comparison of dialysis and filtration for on-line bioprocess monitoring

Technique	Advantages	Disadvantages
Dialysis	No sample losses	No quantitative recovery
	Minimal membrane fouling	Calibration required; relatively slow
Filtration	Quantitative recovery	Sample losses
	Relatively fast	Prone to membrane fouling

(generally) back to the fermentor. Dialysis or filtration therefore takes place outside the fermentor. Advantages and disadvantages of these approaches are summarized in Table 2.

When using an external device, part of the broth must be taken out of the controlled environment of the fermentor, which may lead to disturbance of the bioprocess. The microorganisms may suffer from shear forces caused by the high linear flow velocities in the membrane device and connective tubings, and problems may arise with respect to maintaining aerobic or anaerobic conditions and temperature. However, if the total volume of the sampling system is kept as low as possible, the volume fraction of the broth outside the fermentor can typically be less than 1% and by using high pumping velocities the time spent in the membrane device generally will be in the order of only 10–20 s. If necessary, external units can be replaced during a run, an advantage which is not shared by the internal devices. However, replacement of an external membrane-based sampling system increases the risk of contamination of the fermentation broth considerably and is not advisable. Since most external systems use a sample flow tangential to the membrane, its surface is kept relatively clean because fouling substances are swept away to a major extent. Internal devices generally do not have this possibility and are more sensitive to fouling.

3.2.1. Dialysis

Apart from the examples mentioned above, where full culture medium is sampled, dialysis membranes are only seldom used as part of an external sampling system. An external dialysis cell was used for the monitoring of glucose during the production of white wine, but only to dilute the sample to a glucose concentration compatible with the analytical system (dialysis recovery, 5%) and not primarily for the removal of cellular material [13]. On the contrary, in order to avoid yeast cells and particles from clogging
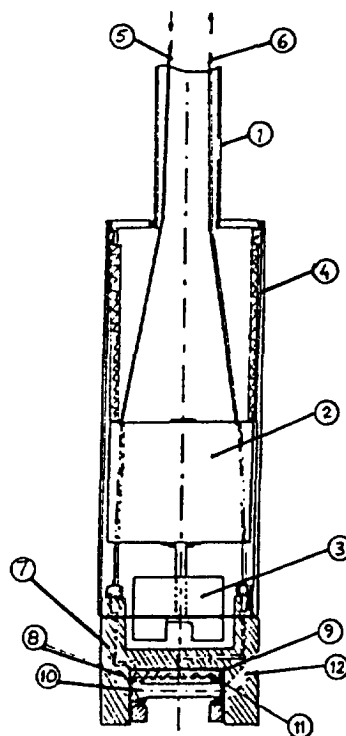

Fig. 2. Cross-section of an internal dialysis probe: 1, stainless-steel shaft; 2, autoclavable motor; 3, permanent magnet; 4, stainless-steel housing; 5 and 6, in- and outgoing acceptor phase; 7, polycarbonate membrane holder; 8, membrane; 9, membrane support with spiral groove; 10, magnetic stirring bar; 11, PTFE washer; 12, channels for air escape and improved circulation. From Ref. [14].

the channels and adsorbing to the membrane of the dialysis cell, a microfilter was incorporated into the sampling system between the fermentor and the dialysis unit.

More applications of internal dialysis systems have been reported. As an example, an autoclavable dialysis probe containing a membrane with a molecular weight cut-off (MWCO) value of typically 30 kDa and a magnetic stirring bar close to the membrane surface to effect a tangential sample flow and minimize fouling, is depicted in Fig. 2 [14]. The

Table 2
Comparison of internal and external membrane devices for on-line bioprocess monitoring

Technique	Advantages	Disadvantages
Internal	Minimal disturbance of bioprocess	No replacement possible; prone to fouling; relatively long response time
External	Replacement possible; relatively fast	Risk of disturbing bioprocess

probe can be used for sampling of low-molecular-mass analytes, such as glucose, in complex solutions for at least two weeks without exchanging the membrane.

The applicability of a miniaturized version of such an internal dialysis unit has been reported in a number of papers. The technique, called microdialysis, was originally developed for sampling of drugs from blood and tissues of animals and humans and typically uses a probe as shown in Fig. 3. The probe contains an inner and an outer cannula and a tubular membrane at the tip. Acceptor phase is pumped down through the inner cannula, passes through two holes into the outer cannula and is pumped upwards again. In the probe tip, the acceptor phase is led along the membrane and here the dialysis process takes place; the acceptor phase which contains the analyte(s) — the dialysate — is finally pumped out of the probe into an injection loop, from whence it is introduced into an analytical system [15]. The dialysis performance was tested with a standard aqueous mixture of saccharides both before and after monitoring a complex fermentation

sample for 33 h and no significant changes were found. The analyte recovery, in microdialysis generally referred to as the relative recovery (RR), is defined as the ratio between the analyte concentration in the dialysate and the analyte concentration in the fermentation broth. In practice, RR always is lower than 100% and strongly depends on the acceptor-phase flow-rate [16]. Fig. 4 shows that near-quantitative recoveries can only be obtained at very low flow-rates (<1 μl/min), which inherently leads to long response times. In the quoted study, a flow-rate of 4 μl/min was chosen as a compromise, which corresponds to a RR of 45% and a sampling frequency of at least 12 per hour.

3.2.2. Filtration

An internal filtration unit contains a tubular membrane of which one end is closed and the other is connected to a peristaltic pump. The latter continuously aspires a cell-free sample and introduces it into the analytical system. A home-made sampling module was used for the monitoring of a *Penicillium chrysogenum* fermentation [17]. Because of the highly complex composition of the broth, which contains high concentrations of proteins and oils, the module had to be placed in a part of the fermentor where a relatively high tangential flow of the medium existed to prevent blocking of the membrane. A filtrate flow of 0.3 ml/min, leading to a response time of 35 min, could be maintained for 300 h. However, blocking of the membrane could
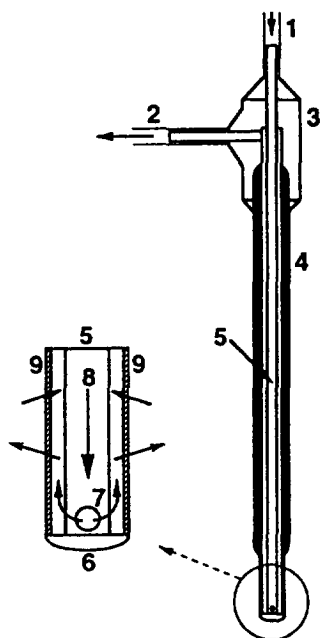
Fig. 3. Drawing of a microdialysis probe, with its tip magnified: 1, inlet tubing; 2, outlet tubing; 3, plastic head; 4, outer cannula; 5, inner cannula; 6, glue; 7, laser-drilled hole; 8, acceptor phase; 9, membrane. From Ref. [16].
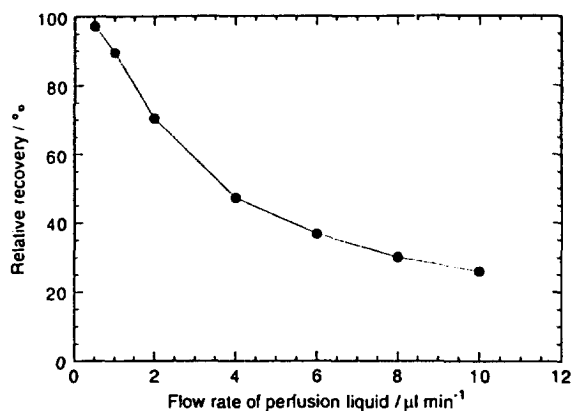
Fig. 4. Relative recovery (RR) of ethanol vs. acceptor phase flow-rate. From Ref. [16].

not be totally avoided and sometimes air was introduced into the sampling system, giving rise to erroneous results. Similar filtration units were applied for the monitoring of other complex fermentations, such as the production of cephalosporin C by *Cephalosporium acremonium* [18,19]. The major conclusion of these studies was that blocking of the membrane occurs to a lesser degree if the pore size is increased, i.e., if microfiltration instead of ultrafiltration membranes are utilized. However, microfiltration membranes with pore sizes of 0.2 μm generally were not sufficient to provide a proper protection of the analytical system.

In order to decrease this obviously problematic membrane fouling of internal filtration modules, a membrane device was constructed that can rotate at a speed of 0–6000 rpm (Fig. 5) [20]. The centrifugal force thus created removes fouling substances from the membrane surface and yields a constant filtrate flow. In the present application a filtrate flow of 1 ml/min was obtained at a rotation speed of 5000 rpm. A static variant, which is also depicted in Fig. 5, was sufficient for the monitoring of an alcoholic fermentation, whereas the (apparently more complex) lactic acid and polysaccharide fermentations studied required the rotating module for reliable sampling.

At least one internal filtration unit is commercially available, it is the ABC module marketed by Advanced Biotechnology (München, Germany). It consists of a unit supporting the tubular membrane, which can be made of a variety of polymeric and
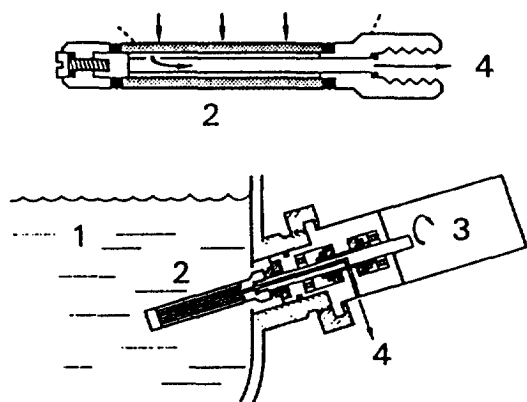

Fig. 5. Drawing of (top) static and (bottom) rotating internal filtration modules: 1, fermentation medium; 2, membrane; 3, motor; 4, filtrate recovery. From Ref. [20].

ceramic materials and can have a wide range of pore sizes. A 0.2-μm polypropylene membrane was used in this module for monitoring glucose and ammonium [21] and penicillin [22] during penicillin fermentations. Although the filtrate flow decreased from 2.0 to 1.0 ml/min during the fermentation, reliable sampling was possible and the same membrane could be used during several fermentation experiments for more than 1200 h.

Two types of external filtration modules have been reported, they contain either planar or hollow-fibre membranes. Planar membranes are generally clamped between two half-blocks, at least one of which contains a flow channel in a meander or spiral form. The fermentation sample is pumped through this flow channel at a relatively high flow-rate (up to 500 ml/min) and by restricting the diameter of the outlet tubing, a pressure is built up which drives a filtrate through the membrane pores. One of the oldest and most widely used systems, the Biopem (Braun, Melsungen, Germany), is equipped with a magnetic stirrer above the membrane surface which creates the sample flow across the membrane. It has been described to have a relatively long response time, because of the large dead volume (175 ml) of the stirred chamber [21]. Other commercially available modules include the Minitan system (Millipore, Bedford, MA, USA) which has been successfully used for sampling from a *Candida rugosa* culture growing in a defined medium, employing two 0.45-μm membranes [23]. The total volume in the filtration loop was 60 ml (1.2% of the fermentor volume) and a sampling frequency of 40 per hour was obtained. The A-SEP filtration device (Applikon, Schiedam, Netherlands) was utilized for determining acetate and phosphate during an *Escherichia coli* culture [24] and the filter/acquisition module (FAM), marketed by Waters (Millipore), for monitoring of the production of ethanol by *Saccharomyces cerevisiae* in a complex and viscous growth medium, spent sulphite liquor (SSL) [25,26]. Both with a 0.22-μm and a 0.45 μm-membrane, recoveries of essentially 100% were found for several small carbohydrates in SSL, whereas recoveries of about 50% were found for amino acids from SSL. The reason for this surprising behaviour remains unclear. The FAM module is depicted in Fig. 6 as a typical example of an external filtration device.
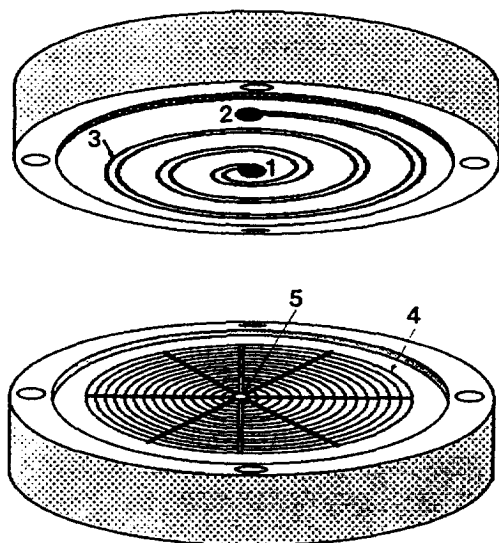
Fig. 6. Drawing of a typical planar external filtration module: 1, sample inlet: 2, sample outlet; 3, spiral groove; 4, filtrate collection; 5, filtrate outlet. The membrane is clamped between the two blocks. From Ref. [25].
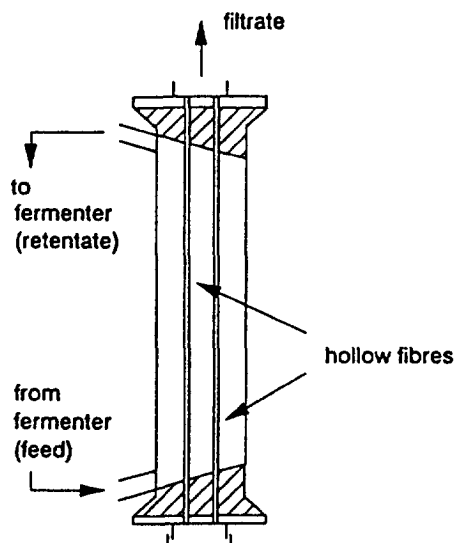

Fig. 7. Drawing of an external hollow-fibre filtration module, with two of the ca. fifty hollow fibres shown. The hatched areas indicate the potting material used to seal the fibre bundle in the housing. From Ref. [7].

In all these applications, concentration polarization and membrane fouling were kept at an acceptable level and no negative effect on the sampling performance has been reported when bacterial or yeast cultures were filtered. On the other hand, handling of samples containing filamentous fungi generally is impossible with these modules because the inhomogeneous broth components easily clog the flow channels [15,21,27]. Internal filtration and dialysis units do not have these problems to such an extent and, therefore, seem to be preferable in this context, although one external ultrafiltration module has been described to work satisfactorily with filamentous fungi cultures [27]. Here, a wide-bore (3 mm) spiral flow channel in a perspex ultrafiltration block featuring a 30-kDa polysulphone membrane, was used for the monitoring of several *Aspergillus niger* cultures. No clogging was observed during various 72-h fermentations and a constant filtrate flow of 1 ml/min was obtained, yielding a sampling frequency of about five per hour.

An important requirement for a high filtrate flow and, thus, a high sampling frequency is a large membrane area or, rather, a large membrane-area-to-volume ratio, which favours the application of

hollow-fibre membranes. An example of a hollow-fibre ultrafiltration module, available from Bio-Flo (Glasgow, UK), is depicted in Fig. 7. Several rather different fermentations have been monitored using this module, including a gluconic acid and a beer fermentation [7] and the production of a recombinant protein by a manipulated *Escherichia coli* [28]. The importance of concentration polarization and fouling was indicated in a study with a wild-type *Escherichia coli* [29]. Increasing the fibre diameter from 200 to 500 μm, and the sample flow-rate to >40 ml/min, helped to maintain a turbulent flow profile through the fibres and minimized concentration polarization. By closing the filtrate outlet of the module as often as possible and, thus, removing the pressure difference over the membrane, the degree of membrane fouling could be reduced. Both approaches resulted in a filtrate flow that decreased to a lesser extent as compared to the original situation. A further important parameter influencing the filtrate flow is the sample viscosity. Fig. 8 illustrates that the filtrate flow is a linear function of the applied pressure for pure water; if the sample contains components larger than the membrane pores, a concentration polarization layer is formed
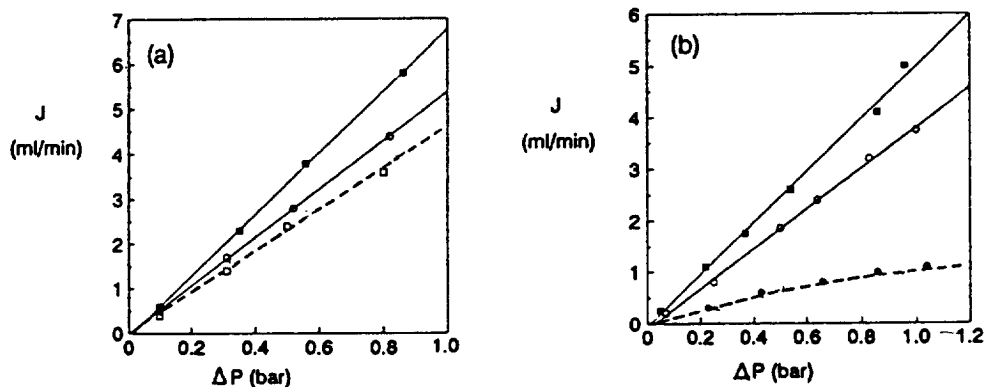
Fig. 8. Filtrate flow (J) vs. applied pressure (ΔP) for water (solid lines) and fermentation media (dashed lines). (a) Gluconic acid fermentation. Sample: water, temperature 30°C (■), 22°C (○); sample, fermentation medium, temperature 30°C (viscosity relative to water: 0.95) (□). (b) Beer fermentation. Sample: water, temperature 22°C (■), 10°C (○); sample, fermentation medium, temperature 10°C (viscosity relative to water: 2.33) (●). Adapted from Ref. [7].

and the flow–pressure relation deviates from linearity. In addition, the highly viscous beer fermentation medium yielded a much lower filtrate flow than water, whereas that of the synthetic gluconic acid medium — which has a viscosity comparable to water — deviates much less. A consequence of this behaviour is a difference in maximum sampling frequency: five per hour for the beer fermentation against twelve per hour for the gluconic acid fermentation. Finally, by using materials impermeable to oxygen, the sampling system could be used to monitor a beer fermentation without disturbing the anaerobic conditions in the broth.

4. Analysis

Two techniques are widely employed for the on-line analysis of fermentation samples, viz. flow-injection analysis (FIA) and chromatography. The choice between these techniques depends on several factors, i.e., the number of analytes, the sample complexity, the required analysis frequency, whereby

personal preference also is of importance. In FIA, an aliquot of a pretreated sample is injected into a carrier stream, typically a buffer, which is directly introduced into a detection device. When using chromatography, the aliquot is injected onto a separation column; because of the aqueous nature of most samples, column liquid chromatography (LC) is used much more frequently than gas chromatography (GC). Advantages and disadvantages of both techniques are summarized in Table 3.

At present, FIA clearly dominates bioprocess monitoring; this can be attributed to its speed, simplicity and relatively low price. As no separation of sample components is effected, a sufficiently selective detection mode should be used in order to ensure that only one compound is indeed determined. Most frequently, FIA is therefore combined with an enzyme-based detection system. The major advantage of chromatographic techniques is, of course, that many analytes can be determined in one run. On the other hand, the application of so-called multi-channel FIA systems, in which several flow channels and detection systems operate in parallel can increase the

Table 3
Comparison of FIA and chromatography for on-line bioprocess monitoring

Technique	Advantages	Disadvantages
FIA	Fast; simple; inexpensive	Typically one analyte per analysis; not applicable for all analytes; selective detection required
Chromatography	Multi-compound analysis; applicable for any analyte	Slower; relatively expensive

number of analytes to about five. As mentioned before, a drawback of chromatography is the relatively long time of analysis, which typically is in the order of 10 min, but can be as long as an hour for complex separations. FIA with its typical analysis frequencies of about once per min compares relatively favourable; however, the analysis frequencies used in practice often are much lower, not seldom around once per 10 min! In addition, the recent development of LC columns with smaller dimensions and particle sizes has caused a considerable decrease of run times and multi-component mixtures can now be separated in 1–2 min. Chromatographic columns require a more thorough sample pretreatment than FIA systems, primarily to remove macromolecular sample constituents which easily clog the columns and ruin the separation efficiency. Therefore, chromatography is nearly always combined with ultrafiltration or dialysis membranes featuring MWCO values below 50 kDa, whereas microfiltration membranes normally are sufficient for FIA.

4.1. Flow-injection analysis

A typical set-up for the determination of glucose (one step) and maltose (two steps) by FIA is depicted

in Fig. 9 [30]. Glucose is converted into gluconic acid by glucose oxidase (GOD) which is immobilized on a support material; the reaction requires one molecule of oxygen and produces one molecule of hydrogen peroxide. In this case, the oxygen consumption rate is measured with an amperometric oxygen electrode, but alternatively the hydrogen peroxide which is formed can be determined by a chemiluminescence reaction with, e.g. luminol [31], which has the advantage of being more sensitive. The disaccharide maltose is enzymatically converted into the monosaccharides α-D-glucose and β-D-glucose by immobilized α-glucosidase in a first cartridge, and the β-D-glucose formed is thereupon determined by means of GOD in a second cartridge in the same way as described above. These FIA systems were used for the on-line determination of glucose and maltose during the production of alkaline protease by Bacillus licheniformis and good agreement was found between the on-line FIA determination of glucose and an off-line spectrometrical method. By co-immobilizing α-glucosidase and GOD on the same support material, the total process can be carried out in a single cartridge, as was shown during the monitoring of recombinant protein production by Escherichia coli [32]. Depend-
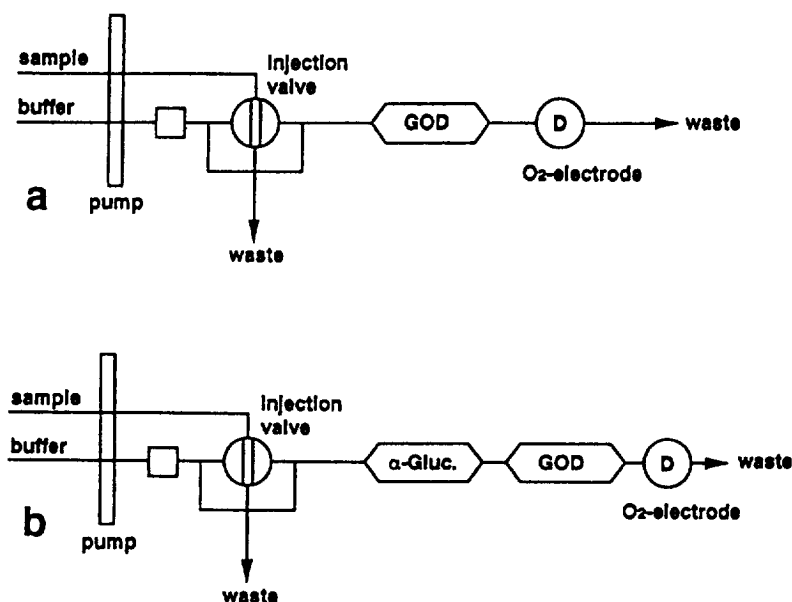

Fig. 9. Schematic drawing of FIA systems for (a) glucose; (b) maltose. From Ref. [30].

ing on the analyte, the detection system can be quite complex. Sucrose, for example, requires three enzyme systems: (i) invertase to split sucrose into α-D-glucose and β-D-fructose; (ii) mutarotase to convert α-D-glucose into β-D-glucose; and (iii) GOD to measure the β-D-glucose formed [30]. Another difficulty arises if disaccharides have to be determined in the presence of glucose, since the GOD detects both the glucose originally present in the sample and that originating from the converted disaccharide and special software may be necessary to separate the two signals [32].

One further aspect worth mentioning is the possible lack of selectivity of the detection mode used. In complex samples several compounds may well be present which can interact with the enzyme and, thus, generate a signal. To quote an example, a biosensor based on co-immobilized alcohol oxidase and peroxidase (conversion of ethanol by the former enzyme and subsequent amperometric determination by the latter enzyme of the hydrogen peroxide formed) was found not to be sufficiently selective for the determination of ethanol in a complex broth [16]. A short chromatographic column was therefore inserted in the monitoring system to effect a separation between ethanol and interfering short-chain aliphatic alcohols. Galactose oxidase, used for the determination of galactose during lactic acid fermentations, suffers from interference by lactose, which has to be circumvented by determining lactose in a separate analyser and correcting the result afterwards [33]. Interference of other sample components can also occur after the enzymatic hydrolysis step. For example, the determination of glucose during a penicillin fermentation was found to be hindered by the presence of penicillin V [21]. The hydrogen peroxide formed upon the oxidation of glucose by GOD also oxidizes the sulphur present in the penicillin molecule and as the detection was based on a chemiluminescence reaction of hydrogen peroxide with luminol, a strong reduction of the glucose signal was found if penicillin V was present in the sample. The problem could be solved by favouring the chemiluminescence reaction relative to the oxidation process: a high luminol concentration was used (12 mM, which is 12-fold higher than initially used) and a co-oxidant was added to the carrier buffer. This enabled glucose determination in the presence of up

to ca. 12 g/l of penicillin V. The various examples clearly illustrate that possible selectivity problems may well occur in FIA.

In an exhaustive review article on FIA for bioprocess monitoring published in 1991 [6], Bradley et al. reported that in 47% of all applications glucose was the analyte of interest. Other analytes frequently determined were lactate (16%), other saccharides (13%) and ethanol (11%). This pattern has not changed a lot in more recent papers. The strong preference for these compounds is without doubt to some extent caused by their importance in many bioprocesses. It should, however, also be realized that a proper FIA system needs a good stability of the enzymes, substrates and reagents involved. The relatively high stability of the enzyme systems (glucose oxidase, lactate oxidase and alcohol oxidase) has certainly also contributed to the widespread attention for just these analytes.

Further interesting applications include the determination of penicillin V, formed during a *Penicillium chrysogenum* fermentation, by β-lactamase immobilized on a standard pH electrode, which measures the change in the proton concentration resulting from the enzymatic hydrolysis [22]. The linear range of the electrode is 0–35 g/l penicillin V, which is sufficient for monitoring a complete 200-h fermentation. Compared to an alternative method, which is based on the enzymatic conversion of penicillin to penicillinoic acid, followed by a classic iodometric determination of the penicillinoic acid, it was found to be more stable and easier to handle. Amino acids, which also are of great importance in many bioprocesses, can be determined by amperometrically measuring the oxygen consumption as a result of the conversion of an amino acid into an α-keto acid by amino acid oxidase [30]. As the enzyme is group- rather than compound-specific and thus responds to any amino acid, the method is primarily applicable for fermentation samples containing only one amino acid, a situation which is certainly not always encountered. Alternatively, more selective enzymes can be used, such as glutaminase for the important amino acid glutamine. Also in this instance, a two-step detection is necessary as glutamine is first converted into glutamate, followed by the conversion of glutamate into α-ketoglutarate by the enzyme glutamate dehydrogen-

ase. The latter enzyme requires the cofactor NAD^+, and produces NADH, which is spectrophotometrically determined. A problem encountered here, which is a more general one when using several enzyme systems, is the large difference in pH optimum for the two enzymes (pH 5 and 8, respectively). Therefore, a combination of two FIA systems was necessary utilizing carrier buffers of different pH values.

4.2. Chromatography

As with FIA, the use of chromatography in on-line bioprocess monitoring has so far mainly focused on the separation of mixtures of saccharides and/or other simple organic molecules such as organic acids and alcohols. For the determination of several carbohydrates an LC cation-exchange column is typically used with water as the eluent; its selectivity can be influenced by properly selecting the cation with which it is loaded. Lactose, glucose and fructose can conveniently be separated by a cation-exchange column in the Ca(II) form [29], whereas a column in the Pb(II) form showed the best performance for the separation of the monosaccharides glucose, xylose, galactose, arabinose and mannose [26]. The simultaneous determination of saccharides, organic acids and alcohols can be achieved using a special mixed-bed LC column which contains a cation-exchange resin in the H^+ form and is eluted with dilute acid, typically 5 mM sulphuric acid. Information about the consumption of sugars and the production of metabolites such as organic acids and alcohols can help to get a better understanding of the complex biochemical mechanisms that play a role during fermentations. The possibility to abstract information about many important compounds in one straightforward analytical run, makes the use of this kind of columns a strong tool for bioprocess monitoring, in particular for research and development work. Examples of this approach include the determination of the substrate lactose and the product lactic acid during a sour whey fermentation [34], the monitoring of glucose, the product gluconic acid and minor metabolites 2- and 5-ketogluconic acid during a *Gluconobacter suboxidans* culture [7] and the monitoring of a beer fermentation with the saccharides glucose, maltose and maltotriose and ethanol as major analytes [7]. Fig. 10 shows a chromatogram obtained during an
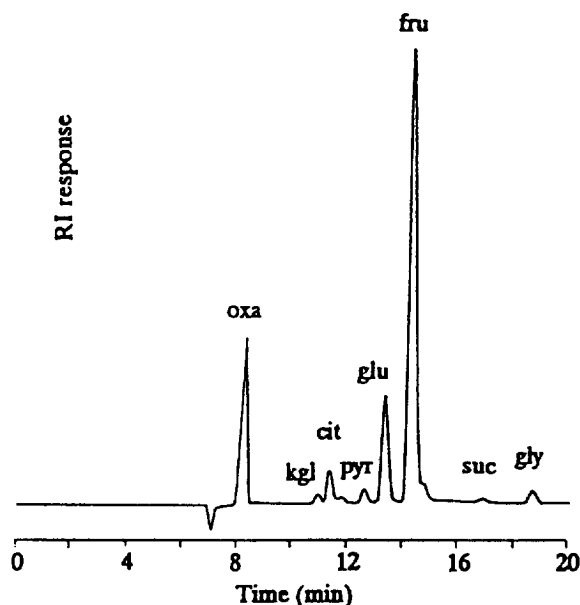

Fig. 10. LC–refractive index (RI) chromatogram of an *Aspergillus niger* NW101 fermentation medium sample. Oxa=oxalate; kgl= 2-ketoglutarate; cit=citrate; pyr=pyruvate; glu=glucose, fru= fructose; suc=succinate; gly=glycerol. From Ref. [27].

Aspergillus niger culture growing on a double carbon source (glucose and fructose), and illustrates that information about a wide variety of compounds can be obtained in one chromatogram. Run times typically were 10–20 min.

Other types of analytes studied are penicillin V, its precursor phenoxyacetic acid and important byproducts and degradation products [17] and cephalosporin C and several other compounds involved in its biosynthesis [19]. Both separations were performed under standard reversed-phase LC conditions; run times were typically 10–20 min. The data obtained in the first example were used to control the feed of the rather expensive precursor and to simultaneously establish the optimal harvest time, which is essential since once the maximum penicillin V concentration has been reached, it rapidly decays by different decomposition pathways. The chromatographic method used for monitoring of cephalosporin C was compared with a FIA method [18], which can not discriminate between cephalosporin C itself and other cephalosporin derivatives present in the sample, and was found to be superior. Information about the consumption of amino acids during fermentations

was obtained by analysis on an ion-exchange column with gradient elution followed by post-column labelling with o-phthalaldehyde/2-mercaptoethanol [28]. Here the number of analytes was fifteen and the analytical run time 40 min. The information obtained was helpful to optimize the composition of the growth medium of a recombinant *Escherichia coli*: by adding an extra amount of those amino acids that were found to be preferred by the organisms, a significantly higher product yield could be obtained.

Since the analytes which are most frequently studied during bioprocesses (sugars, acids, alcohols) lack chromophores, detection is often accomplished using a refractometric index (RI) or low-wavelength UV detector, or a combination of both. Although these are simple to operate, a distinct disadvantage is their relatively low selectivity and sensitivity. This means that in practice only high concentrations of analytes can be reliably determined in complex samples. Fortunately, the concentrations of these important compounds in fermentation samples often are in the g/l range. With lower concentrations, quantitative and qualitative errors can arise, for example if co-elution of the analytes and other matrix components occurs as a result of insufficient

chromatographic resolution. This was shown in an interesting study using LC in combination with diode-array UV and mass-spectrometric detection for the characterization of sugars [35].

For highly complex fermentation media, such as the SSL mentioned earlier, additional selectivity has to be introduced into the system. On-line clean-up of SSL filtrates on a solid-phase extraction column for the removal of phenolic compounds was found to be favourable prior to the LC–RI determination of five carbohydrates [25,26]. Alternatively, the extra selectivity may be introduced at the detection side. The determination of low mg/l concentrations of reducing sugars [29] or ketogluconic acids [7] could be achieved by a simple post-column derivatization with p-aminobenzoic acid hydrazide (ABH). Fig. 11 clearly shows the strong increase in sensitivity and selectivity obtained for the determination of several sugars; detection limits were improved up to 100-fold compared with RI detection and up to 1000-fold compared with UV detection at 190 nm.

For the determination of volatile fermentation products, GC can be used as the analytical technique, as was done for the monitoring of acetoin, acetic acid and D/L- and meso-butane-2,3-diol, the principal
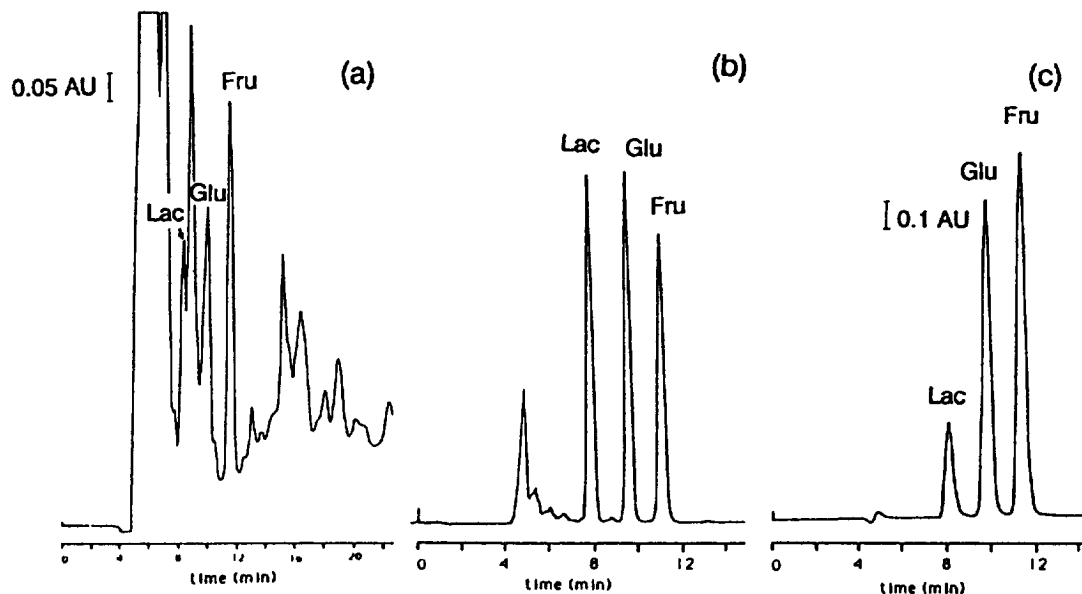

Fig. 11. LC chromatograms of a brain–heart infusion sample containing 15 g/l of lactose (Lac), glucose (Glu) and fructose (Fru): (a) UV detection at 190 nm; (b) RI detection; (c) UV detection at 410 nm after post-column derivatization with ABH; sample diluted 20 times with water. From Ref. [7].

metabolites present in a *Bacillus subtilis* culture [36]. Here, the filtrate was pumped into a degassing unit from which 1-μl samples were transported to an injection valve. The analysis time was less than 8 min and the capillary column performed well for over 13 000 injections. On-line GC analysis was also performed for the vicinal diketones diacetyl and 2,3-pentanedione during a beer fermentation [37]. Because of the high volatility of the analytes, a 0.2-μm pore size gas membrane was used for sampling, the acceptor side of which was flushed with nitrogen. For monitoring of the precursors of the vicinal diketones, α-acetolacetate and acetohydroxybutyrate, a liquid-phase heat conversion into the diketones and subsequent GC analysis proved to be the most satisfactory approach.

5. Conclusions

In recent years, the manual sampling and analysis of biotechnological samples for monitoring and control of fermentations is increasingly being replaced by on-line, automated methods which yield faster, more frequent and often also more reliable data. By far most of the on-line sampling systems use a membrane for the straightforward and efficient removal of interfering cellular (and macromolecular) material from the sample, dialysis and filtration being the most common techniques. Both dialysis and filtration have been used successfully for bioprocess monitoring; they both have their advantages and disadvantages and can be considered more or less complementary. The geometrical construction of a membrane-based sampling device and parameters such as pore size and membrane material often are more important for successful sampling than the question whether dialysis or filtration is the applied technique. With the sampling equipment available today many types of highly complex bioprocesses can be reliably monitored and controlled for periods of up to several weeks and sampling frequencies of typically once per five minutes are feasible.

The choice of the analytical technique is determined by parameters such as the number, concentration and type of analytes and the complexity of the fermentation sample. Flow-injection analysis combined with a sufficiently selective detection system is typically used for a single analyte. Chromatographic techniques, mostly LC, can best be used for multi-analyte studies; non-selective detectors such as RI or low-wavelength UV may suffice for high analyte concentrations and 'clean' samples; otherwise, additional sample clean-up and/or a more selective detection system have to be used. GC is only seldomly used, viz. for volatile analytes.

Because detailed and valuable information can be extracted about the often complex biochemical mechanisms involved in a fermentation and because of the possibility to control the fermentation conditions, it is to be expected that the use of on-line monitoring systems will continue to grow. For research and development, where a better understanding of the bioprocess is crucial, chromatographic techniques will probably be preferred because they provide details about the consumption and production of many important compounds in a single run. For controlling large-scale fermentations, where speed is the most important factor and often only one or a few compounds need to be measured, flow-injection analysis probably is the best alternative. In this context, more work will have to be carried out to adapt monitoring systems to being used under the extreme conditions of an industrial environment; so far, most applications have been with small-scale laboratory fermentations.

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