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Literature review of on-line bioprocess monitoring
Report

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1 Summary

On-line bioprocess monitoring is becoming increasingly important. To begin with, bioprocesses themselves are becoming more important as more pharmaceuticals are produced that way and sustainable production of oil-replacing fuels and chemicals is in many cases based on bioprocesses. Thus, on-line monitoring is needed more often to verify how the process develops and control it to run more efficiently. In addition, measurement and fabrication technologies have also developed providing more possibilities for on-line monitoring. In general, monitoring approaches can be divided to in situ probes and automated at-line systems. In situ probes are placed inside the bioreactor and require no sampling whereas in automated at-line systems sampling and subsequent sample analysis outside the bioreactor have been automated. Both approaches have their pros and cons related to sterilization, specificity and operation. In situ probes are mainly based either on optical or electrical measurement principles as they withstand steam sterilization often applied in bioprocesses. The key in reliable automated at-line systems is often sampling, but after that several different measurement techniques are available; including devices based on chromatography, electrophoresis, mass spectrometry and microscopy to mention a few. Approaches where biological components, mainly proteins, are used to recognize the analytes are also available and under further development. In general, considering the development of the devices for on-line bioprocess monitoring, the range of different bioprocess matrices and analytes is large from slurries to watery media and from bioethanol to monoclonal antibodies.

2 Purpose and scope

The purpose of this deliverable is to collect a summary of the state of the art in on-line bioprocess monitoring. The scope of the summary is quite large covering and exceeding the area of on-line measurement techniques than are actually being developed in the Nanobe EU-project. Different approaches can be applied to measure same parameters in processes which justifies the broad coverage of analytical approaches also in this summary. The summary is based on literature in peer-reviewed journals and on companies own publications especially on the Internet. Thus, the summary contains both scientific prototype devices and commercial devices in various applications. All applications are not necessarily directly in bioprocess monitoring, but could be applied in the field.

3 Introduction

The measurement of products, by-products and other physicochemical parameters in real-time during cultivation of cells in bioreactors is becoming increasingly important. For example, the process analytical technology (PAT) initiative by United States Food and Drug Administration (FDA) is about to revolutionize biotechnology-based processes. The PAT initiative enables using of real-time process monitoring and controlling tools for deviating from strict manufacturing recipes in production of pharmaceuticals within certain boundaries (design space) (1). In addition to production of pharmaceuticals in bioprocesses, also biorefinery applications for sustainably replacing oil-based fuels and petro chemistry using microbial bioprocesses are becoming more and more important. Better monitoring of the production process allows verifying and tracing back the integrity and quality of the products in production of pharmaceuticals. On the other hand in the case of producing bulk chemicals in biorefineries the efficiency of the process becomes more important and on-line monitoring tools can help to run the processes more efficiently.

An obvious advantage of the automated measurements is the possibility to refrain from using manual steps in the analysis procedure. Normally, the manual operation requires obtaining the sample, separating the cultivation medium from the cells, in some cases further extraction of the sample, storing the sample for analysis and performing the analysis. All steps, but especially manual steps, are candidates for more errors in the final results. The more steps one can avoid the more time it is possible to save in addition to cutting down error propagation. Overall, automated product measurement tools have potential to shorten the time required for strain selection, process development and process control. Such a development path from a range of potential organisms to production scale is demonstrated in Figure 1.

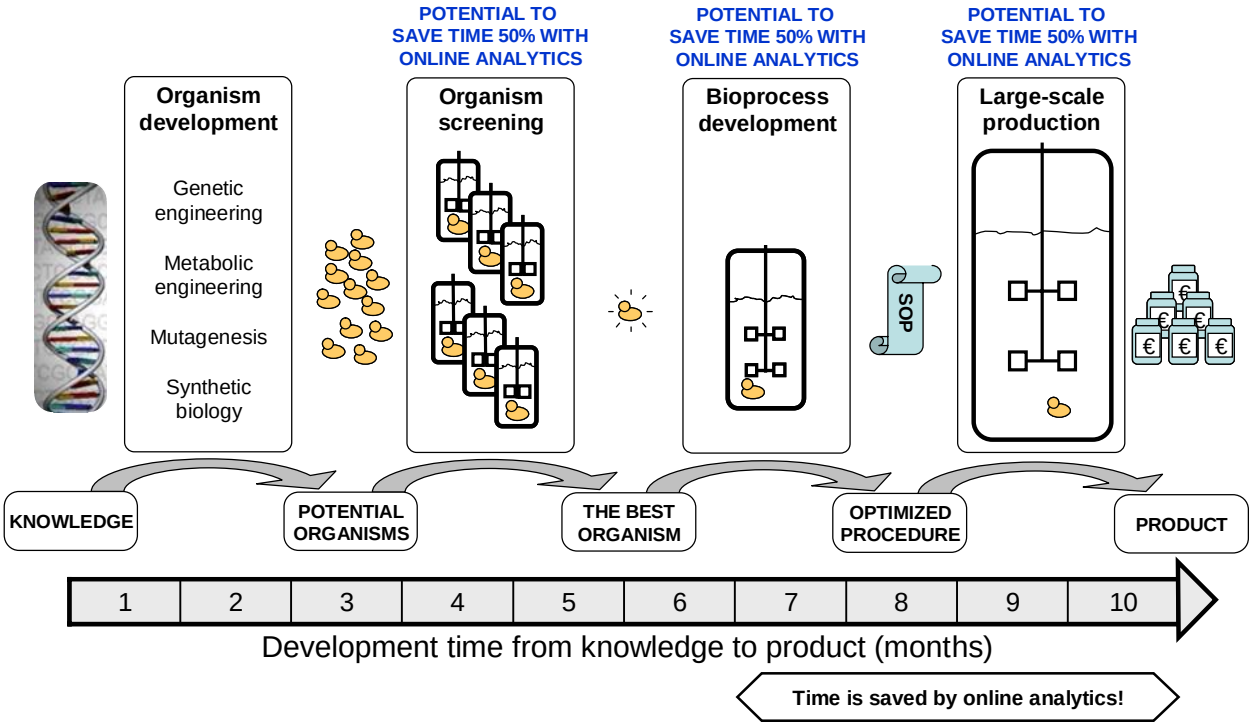

Figure 1. Application of on-line analysis tools during the development of a bioprocess right through from initial concept (e.g. product gene or a target metabolic pathway) to production. 1.) Organism development creates a great number of strain variants. On-line analysis of the performance of the strains will aid in screening of strains, and selection of the best production organism in miniaturized or small scale bioreactors. 2.) Production conditions are optimized using on-line analysis tools in bench-top bioreactors to accelerate scale-up to full production. 3.) On-line analysis can be applied in process control, monitoring, and quality control of the production phase.

There are basically two main philosophies how automated analysis can be performed – either without taking a sample, but applying a measurement probe inside the reactor or by automated sampling and subsequent sample analysis. There are many terms that have been used to describe the different approaches; such as on-line, in-line, at-line, in situ, real-time, etc., but their use is not always consistent. Nevertheless, the decision is anyway whether a sample is taken or not. Here we will call the prior approach without sampling “in situ” and the latter approach with sampling and subsequent sample analysis “automated at-line”. The pros and cons of the two approaches are summarized in Table 1. Basically, automated at-line systems have the possibility to be more versatile in terms of range of analytes as once you have the sample you have the possibility to apply many analytical techniques that are developed and applied in off-line analyses. Hence, they can also be tailored to measure specifically the analyte of interest. The main benefit of in situ probes, however, is that they provide really real-time measurement information. Automated at-line approach requires always additional time to perform the sequence of steps. In addition, in situ probes can be considered safe for the process

as long as they can be sterilized prior to the cultivation. When you take a sample from the process you have to always keep in mind that you cannot risk the process integrity and you have to maintain a sterile barrier between the process and the contaminants of the outside world. However, the sterilization is often the biggest hurdle for in situ probes; especially for the ones that contain biological components. Biological components such as enzymes or antibodies could be applied in in situ probes in order to improve the specificity of the analysis, but proteins do not withstand the heat of steam sterilization that is applied in most of the bioreactors. Thus, in practice in situ probes rely mainly on direct optical or electrical measurement principles and in most cases this limits their specificity for a certain analyte.

Table 1. General comparison of the properties of the on-line monitoring approaches based on in situ probes and automated at line analyzer systems.

	In situ probes	**Automated at-line systems**
Sampling	In situ probes do not require sampling, hence they are quite safe for the process once they have been sterilized and placed safely inside the reactor	Requires sampling, but how representative is sampling, how much sample is withdrawn, and how to clean and close after sampling
Sterilization	Sterilization is often the biggest hurdle for in situ probes especially if probe contains biological components. Either gamma irradiation and single use or autoclavation and repeated use.	Most sampling probes withstand sterilization well and are easy to sterilize. Actual analytical device does not require sterilization being outside the reactor.
Calibration	Calibration is quite problematic as the probe sits inside the reactor. For many optical probes the calibration is very process specific and they are difficult to calibrate specifically for certain analyte.	The analytical device can be calibrated even several times during a cultivation and the calibration can be more analyte specific not depending that much on the actual process.
Multiplexing	Requires at least one probe per reactor, but with fiber optics in some cases possible.	Can be multiplexed for measuring from several reactors with one analytical device

What to measure then? We asked a few companies applying bioprocesses what they would like to measure on-line. The priority can be summarized simply as 1) cell density 2) cell viability, 3) substrates, 4) products and 5) biomarkers. Cells are the catalysts of the bioprocess and keeping them alive and productive is good for the business, but you do not want to have too much of them unless that is your final product. Cells can be measured at three levels; density, viability and vitality. Cell density gives the number or mass of the total population and it is probably the first priority for on-line measurements. Cell density is in many cases measurable using optical in situ probes. Cell viability gives the ratio of the living cells over the whole population and has traditionally been measured using microscopy of the stained cells. Alternatively, electrical, capacitance-based in situ probes have been used to measure the total number of intact cells. Kiviharju and co-workers have reviewed the in situ probes applicable for on-line biomass monitoring (2). The final finesse of measuring cells is the measurement of cell vitality. Cells may be alive, but they are not necessarily metabolically active; vital. There are no direct measurements for cell vitality, but it is possible to get some indications based on indirect

measurements of various biomarkers such as intracellular levels of energy precursors ATP, ADP and AMP. For a review about various approaches for measuring viability and vitality of yeast cells in brewery applications see the article by Heggart and co-workers (3). Measurement of substrates such as glucose or glutamine in the extracellular medium can be considered in general to be the third priority. Based on the measurement of substrates it is possible to control the feed of substrate keeping the concentration at certain level. In most organisms substrate concentration triggers various control mechanisms such as substrate inhibition. Thus, in general, controlling the substrate concentration it is possible to control what the cells do; for instance select between the products of primary growth-related and secondary non-growth-related metabolism. Products can be considered the fourth priority for on-line measurements. Obviously, range of products is endless; cells, proteins, enzymes, antibodies, peptides, various nucleic acid molecules, large and small metabolites. The main reason for measuring products is to assure that the production process is working properly. Especially, the measurement and assurance of product quality in pharmaceutical industry is very critical and could be monitored already during the process. It should be noticed that products can also be intracellular. The range of biomarkers can be considered even larger than the range of products. Production of by-products can also be considered as a form of biomarkers as formation of by-products can be considered as an indication of less optimal conditions. The main purpose of measuring biomarkers can be considered to be the estimation of cell vitality; how active the cells are to perform the conversion from substrate to products. Furthermore, with the biomarker measurements it may be possible to predict how active the cells will be if the conditions are kept the same. Especially measurements of gene expression can in some cases give indications about cells preparing for changing their operation. An example of this is a diauxic shift from one substrate to another.

Figure 2 presents a hierarchy of different approaches applicable for on-line bioprocess monitoring. To begin with, the approaches are divided to measurement technologies and sampling technologies. Sampling technologies contain also different sample treatment approaches such as filtration for separating cells from the liquid and lysis of the cells in order to enable the analysis of intracellular components. Measurement technologies is a much broader field and is here divided in to five sub categories – optical measurements, electrical measurements, chromatography-like measurement where analytes are first separated and then detected, direct mass spectrometric measurements, and measurements where biological component is used in recognition of the analyte such as various antibody-based assays. The differentiation between in situ probes and automated at-line systems is not maintained in the hierarchy, but majority of the technologies is applicable only for automated at-line approach. In situ probes are discussed mainly in optical and electrical measurements. In the following chapters we proceed to present various approaches for bioprocess monitoring along the hierarchy presented in Figure 2.

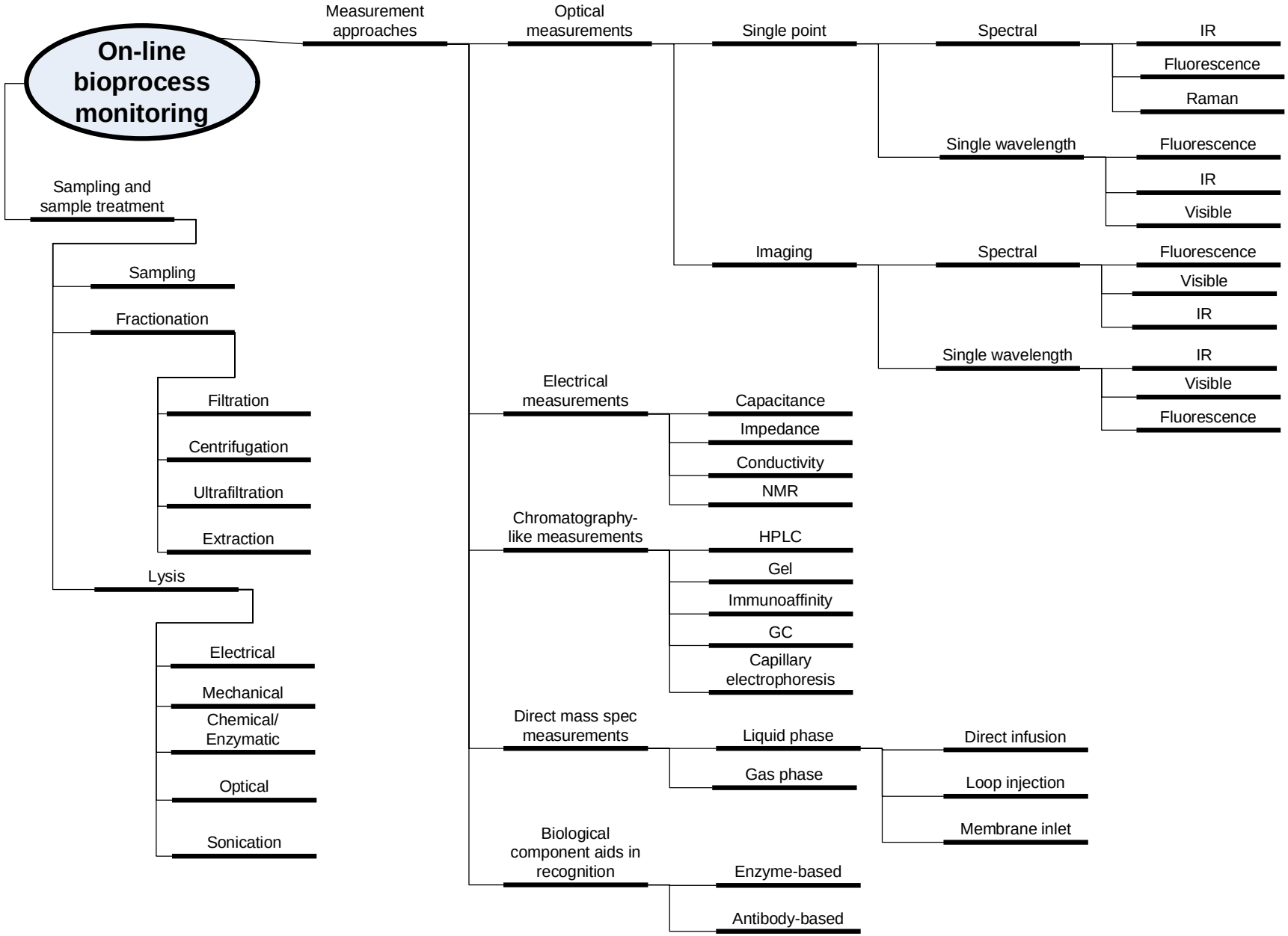

Figure 2. Hierarchy used in this review for on-line measurement and sampling approaches that are applicable for bioprocess monitoring.

4 Measurement approaches

Majority of this summary is about various measurement approaches that are applicable for automated measurements. We have divided the measurement approaches in five categories; optical measurements, electrical measurements, chromatography-like measurement where analytes are first separated and then detected, direct mass spectrometric measurements, and measurements where biological component is used in recognition of the analyte such as various antibody-based assays.

4.1 Optical measurements

Optical measurement approaches are further divided in two categories; single point measurements and imaging measurements. Optical single point measurements are those that are best suitable for direct in situ measurements in reactors. Examples of optical in situ probes are for instance Fourier transform infrared (FT-IR) or fluorescence probes. Imaging measurements are of course various microscope techniques which basically take the measurements to individual cell level. In general, both single point and imaging approaches can be applied at different wavelengths of light; visible, ultraviolet and infrared. Certainly, also fluorescent techniques can be applied with both single point and imaging approaches.

4.1.1 Optical single point measurements

Infrared and fluorescence spectroscopy provide a versatile means for in situ measurements. The most simple and cheapest optical probes are those with one wavelength applicable for the analysis of cell density measuring transmitted light. Such probes are available at least from Finesse Solutions and Mettler-Toledo. Trucell2 probe by Finesse Solutions is illustrated in Figure 3. Trucell2 operates at 800 nm wavelength at near-infrared region.

Figure 3. Trucell2 optical probe and transmitter for in situ cell density measurements by Finesse Solutions.

By measuring full spectrum rather than single wavelength it should be possible to identify correlation for more analytes. Fourier-transform infrared spectroscopy (FT-IR) with attenuated total reflection (ATR) crystal has been applied for on-line monitoring of glucose and ethanol concentrations during yeast fermentation (4). Infrared spectroscopy at the near-infrared region of the spectrum (NIR) has been applied for monitoring of reducing sugars and volumetric mass in white wine fermentation (5). There are several companies, who have commercial IR systems available such as Matrix F FT-NIR by Bruker (6), and XDS Process Analytics NIR by FOSS

(7) illustrated in Figure 4. Other companies with IR spectroscope products are at least Thermo Scientific, Perkin Elmer and Varian.

Figure 4. In situ near infrared spectrometer devices by Bruker (Matrix F FT-NIR) and by FOSS (XDS Process Analytics NIR).

Two dimensional multi-wavelength fluorescence spectroscopy is another spectroscopic technique enabling in situ measurement of products having biogenic fluorophores such as tryptophan, tyrosine, phenylalanine, vitamins (pyridoxine, riboflavin) and coenzyme (8). It seems to be nicely suitable for analysis of protein production (9). However, recently it was also applied for the measurement of biomass, glucose and ethanol (10).Two dimensional multi-wavelength fluorescence spectroscope is commercialized by Delta under trademark Bioview. The device is illustrated in Figure 5.

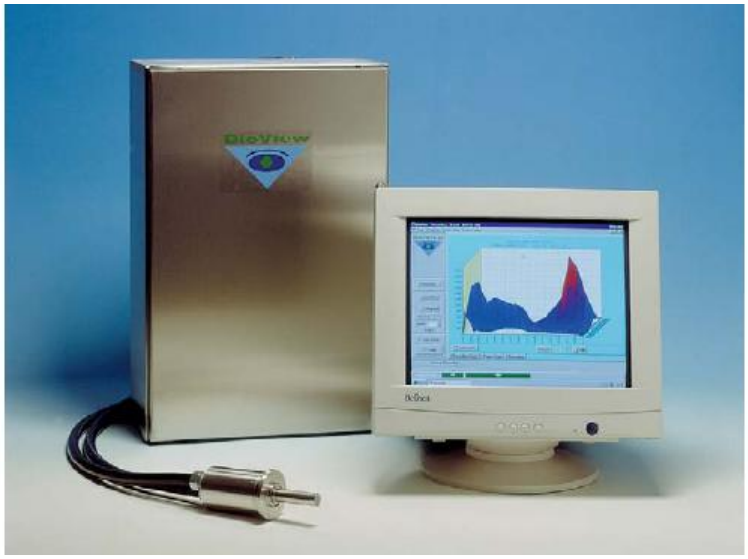

Figure 5. Bioview in situ two-dimensional multi-wavelength fluorescence spectroscope by Delta.

In general, issues with spectroscopic techniques are the complexity of the spectrum and the fact that the cultivation conditions in general have such a large effect on the spectrum. As a result, calibration of spectroscopic sensors for the analysis of specific compounds requires often an extensive experimental approach (11).

4.1.2 Imaging measurements

Even though in situ microscopes exist for imaging cells directly in bioreactors (12), most of the commercial microscopes for bioprocess monitoring rely on taking a sample from the reactor,

staining the sample and then counting the cells as it makes a more versatile product allowing also off-line analyses. Staining the cells is used mainly to provide information about the viability of the cell population. Obviously, staining would not be possible inside the reactor. Automated microscopes are made commercially available at least in Cedex™ Standard and HiRes models by Innovatis; now part of Roche (13), in Vi-Cell™ by Beckman Coulter (14), Countess™ by Invitrogen (15) and in TC10™ by Bio-Rad (16). They all apply trypan blue staining, hemacytometer principle, and brightfield light microscope and in addition to cell number and cell viability provide information also about cell morphology and cell size. The devices are mainly intended for mammalian cell work, but some could be applied also for yeast studies with methylene blue staining. In addition, only Cedex models and Vi-Cell seem to be automated with at-line process analysis in mind. The two are illustrated in Figure 6.

Figure 6. Automated at-line microscopes for analysis of cell number and viability with trypan blue staining; Cedex HiRes by Roche Innovatis and Vi-Cell by Beckman Coulter.

Another staining approach is fluorescent staining, which is applied in Nucleocounter devices by Chemometec. Nucleocounter is based on fluorescent microscope and fluorescent dye propidium iodide that binds to DNA accessible in dead cells (17). There are different models for yeast (YC-100) and mammalian cells (NC-100). However, the device is not yet available as an automated at-line device.

One way to automate cell imaging is of course imaging of cultivations on microtiter plates. This is practically in situ monitoring even though it cannot be applied as such in production scale, but is applicable for screening and molecular biology. Examples of on-line microscopes for microtiter cultivations are Cellavista™ by Roche Innovatis (18) and Cell-IQ 2 by Chip-Man Technologies (19) which are large systems with both brightfield and fluorescent imaging options and several automated image analysis properties. Major task in making the automated imaging systems work reliably is indeed the image analysis and how to convert the information in the image to numerical parameters about the process and there are several approaches trying to achieve such automated, reliable analyses (20).

4.2 Electrical measurements

Electrical measurements can be used to detect the state of a cell culture or of individual cells separately. The electrical impedance is a parameter combining the conductive behavior of the measured system with its insulating behavior, and can be performed at various frequencies to characterize the system's capacitance and conductivity. Nowadays, various systems use

electrical measurements for bioprocess monitoring, such as the Aber capacitance probe (21) and an impedance probe developed by Fogale (22). Aber probe is illustrated in Figure 7.

Figure 7. Aber cell density probe based on capacitance measurement.

A very early example of the impedance measurement systems is the Coulter counter, developped in the 50's and first miniaturized by Larsen and co-workers (23). This system uses a small aperture through which cells can pass, thereby influencing the aperture resistance. Coulter counters are commercially available from Beckman Coulter. Idea of Coulter counter has been further developed by Gawad and co-workers. Using multiple frequency measurements to discriminate between different cell types (24) or between living and dead cells (25). This technique has also been integrated on chip with optical measurements as complementary characterization (26). Latest commercial development in Coulter counters is the handheld Scepter by Millipore illustrated in Figure 8 (27). Scepter is of course not automated for process monitoring, but the technology inside the device could be applicable for automation in bioprocesses.

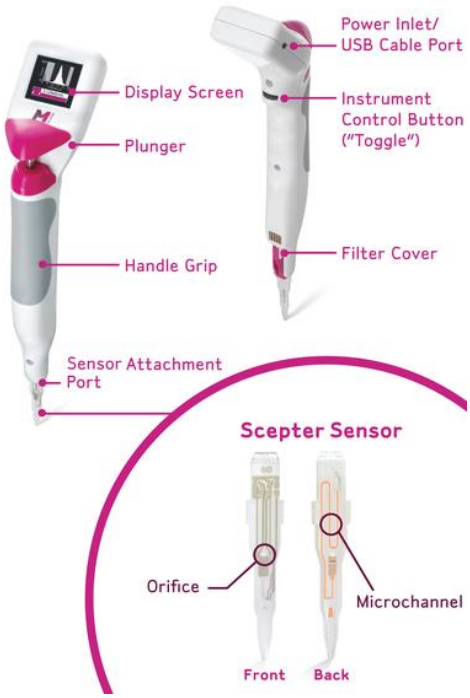

Figure 8. Handheld Scepter Coulter counter by Millipore.

Impedance measurements are not limited to single cells, and have also been used to sense a population of cells for various purposes, such as culture growth (28) and tissue reaction after implantation (29). In the context of online bioreactor monitoring, tools have been developed to

monitor the viable cell density and culture growth (30) (31) as well as more subtle factors such as lipid storage in yeast cells (32).

In vivo nuclear magnetic resonance spectroscopy (NMR) is able to measure intracellular variables although mainly as an overall signature rather than measuring certain specific compounds directly (33). In addition, the reactor contents need to be circulated through the NMR device providing an additional perturbation to the experimental setup (34). Furthermore, the sheer size and price of NMR devices means that NMR is not applicable for monitoring in production phase. However, NMR devices with automated sample handling could be suitable for reaction monitoring in screening stages.

4.3 Measurements based on chromatography-like separation

Liquid chromatography, gas chromatography and capillary electrophoresis, which are the most popular off-line analytical techniques applied in biotechnology, are applicable also in on-line monitoring. Compared for instance to optical in situ probes, these separation-based analytical techniques require some time for the analysis. Time is required for the analytes to migrate out from the capillary or the column. The benefit is that there is a large array of methods available that can be further developed to measure exactly the desired analytes. This is often not possible with the optical probes.

4.3.1 On-line capillary electrophoresis

Capillary electrophoresis (CE) involves a family of related separation techniques that use narrow-bore (usually 50 μm) fused-silica capillaries to separate a complex array of large and small molecules. The separation is carried out in high electric field strengths and molecules are separated due to their differences in charge, size and hydrophobicity. The advantages of capillary electrophoretic techniques include high separation efficiency, low analyte and solvent consumption, and short analysis time. At the moment commercial equipment are not available, so all the published work has been done with CE devices applying the off-line philosophy where sample is brought to a sample vial at the autosampler of the CE device. Such a system is for instance available from Groton Biosystems (35).

CE has been coupled with bioreactors for the monitoring the production of organic acids where a pneumatic autosampler with membrane interface was used for the sampling (36). This set-up has also been used for the on-line monitoring of enzymatic conversion of adenosine triphosphate (ATP) to adenosine diphosphate (37) and for on-line monitoring of bioaccumulation of heavy metals by bacteria (38).

On-line CE has been used for process monitoring in pulp and paper industry. A device that used a laboratory-made sample flow cell was developed for the analysis of dissolvable inorganic anions and cations as well as some organic acids from circulation waters of pulp and paper machines (39) (40).

4.3.2 On-line liquid chromatography

On-line liquid chromatography is rather commonly used analytical tool for on-line monitoring of different processes. Assembling the device is much easier than with capillary electrophoresis because of the loop-injection. There are at least three companies worldwide that manufacture

on-line LC devices. Waters® (USA) has developed PATROL™ UPLC™ Process Analyzer that is based on ultra-performance liquid chromatography (UPLC™), Dionex (USA) has DX-800 Process Analyzer that exploits both ion chromatography (IC) and high-performance liquid chromatography (HPLC), and Bayer (Germany) has BaychroMAT® process analysis system that enables HPLC, gel permeation chromatography and gas chromatography. On-line HPLC devices of Dionex and Waters are illustrated in Figure 9. Groton Biosystems (USA) has developed a sampling system interface for HPLC called Automated Reactor Sampling System (ARS™) that provides automated sampling and sample preparation. It can interface to a variety of bioreactors, fermentors, and other process liquids and offers analytical interface options for fraction collection, nutrient monitoring, capillary electrophoresis, cell counting, flow through spectrophotometry, and the HPLC interface. Groton Biosystems works in collaboration with Agilent Technologies applying Agilent's off-line HPLC and CE systems for enabling on-line analyses when connected to Groton's sampling system (41).

Figure 9. Two on-line HPLC systems; DX-800 by Dionex and PATROL by Waters.

On-line HPLC has been used for the monitoring of sugar consumption and organic acid production of *Escherichia coli* fermentation (42) and for on-line monitoring and controlling of ethanol fermentation of *Zymomonas mobilis* (43). Extracellular metabolite concentrations have been monitored during a batch cultivation of *Saccharomyces cerevisiae* by real-time HPLC (44). One of the future aspects of on-line liquid chromatography is to produce enough data for automated process analysis in combination with other on-line measurement tools. Analytical information is then utilized as inputs to mathematical models which should be able to generate the values of the critical variables from the modeled biological mechanisms (45).

Wastewater treatment is also of great importance. On-line HPLC is applicable for process monitoring of the anaerobic degradation of wastewater from baker's yeast production where several different components, e.g. anions and organic acids, has to be analyzed simultaneously (46). Textile industry produces complex wastewater from dyeing processes. Since some of degradation products of the dyes are toxic, and legislation determines the decoloration rate of

wastewaters, on-line HPLC is a powerful tool for process monitoring (47). Using mass spectrometer as a detector for HPLC makes the tool even more versatile (48).

4.3.3 On-line gas chromatography

On-line analysis of gaseous samples can be obtained with gas chromatography (GC). One of the drawbacks of using GC is derivatization that makes on-line analytics difficult. Dihydroxyacetone and glycerol was determined in fermentation broth by using pyrolytic methylation-GC with vertical microfurnace pyrolyzer. With this on-line pyrolytic methylation in presence of an organic alkali, compounds were converted into their corresponding methyl ethers and were analyzed by GC with flame ionization detector (FID). (49)

Combining to GC systems to perform on-line two-dimensional gas chromatography (GCxGC) gives the analysis of extremely complex samples selectivity and sensitivity as well. GCxGC connected to FID and mass spectrometer gives a qualitative and quantitative analysis method for the analysis of highly complex hydrocarbon mixture in petrochemical pilot plant. (50)

GC can also be used for the analysis of aqueous samples. On-line GC-MS system for automated monitoring of crude waste water at a production site enabled analysis of 140 volatile and semi-volatile compounds. A laboratory-made two-stage injector consisted of upper part that vaporized the sample and deposited the matrix, and of lower part where adsorption on a packing material and subsequent thermal desorption occurred. (51)

4.4 Direct mass spectrometric measurements

Most of the on-line mass spectrometer (MS) applications are performed in connection with some of the above separation techniques; especially with gas chromatography. However, MS devices can be applied also without prior separation techniques. MS is inherently well suitable for analysis of gas phase samples from bioreactor exhaust gas. Hence, very many MS devices are applied in this purpose for measuring oxygen consumption and carbon dioxide production during cultivations. In addition, some volatile products are also directly measurable in the exhaust gas such as ethanol. Membrane inlet mass spectrometry (MIMS) is a way to do on-line mass spectrometry from the liquid fraction. MIMS is a technique that allows analytes to be transferred from a complex aqueous sample to the ion source of mass spectrometer via a semi-permeable membrane. This technique can be used for the analysis of volatile and semi-volatile organic compounds (52). For instance ethanol can be measured from the cultivation broth through a membrane with MIMS. In addition, MIMS allows the measurement of volatile flavour compounds for instance in the brewing process (53).

MS devices applied as LC detectors could be applied also in loop injection or direct infusion mode for the on-line analysis. However, mass spectrometers in general are very expensive, large and power-consuming devices. Microfabricated mass spectrometers would impact MS-based analytical systems in various ways. It would give compact size and low power consumption which would allow portability and less demanding working space, and low cost and relative simplicity. There are still challenges to overcome, for example increasing the mass range, mass resolution and ion throughput. There are two main groups and companies developing the miniaturized mass spectrometer; Professor R. Graham Cooks (54) at Purdue University, West Lafayette, Indiana involved in ICx technologies (55) and Professor Richard Syms (56) at Imperial College, London, England involved in Microsaic Systems (57). However, neither of the groups claim to have the miniaturized LC-MS yet available.

The absence of a viable, commercially available miniature LC-MS, in particular for process monitoring applications, was the motivation for Microsaic Systems' involvement in Nanobe.

Microsaic in developing a miniature MS with an electrospray interface in collaboration with its partners in Nanobe. The technology developed by Microsaic in Nanobe will be marketed as a process monitoring solution. Currently, Microsaic has made significant progress and has demonstrated mass spectrometry of enzymes and other process analytes in a miniature format. This miniature detector will be coupled with VTT's capillary electrophoresis device and applied as part of the Nanobe platform.

4.5 Measurements where biological component aids in recognition

There are varying approaches where biological component is used as an aid in recognition of the analyte of interest. In most of the cases proteins and especially antibodies are used in recognition. In addition to recognition, also a way to create an electronic read-out signal must be realized.

In situ autoclavable sensors with immobilized glucose oxidase enzyme have been developed for specific glucose measurements (58). However, in general probes containing enzymes are not autoclavable as the enzymes do not withstand steam sterilization. Similar specific sensors have been developed also for fructose, glycerol and ethanol sensing (59). Yellow Springs Instrument's YSI 2700 system can incorporate up to three simultaneous automated *ex situ* assays chosen from roughly 20 compounds which have the test available. YSI devices are based on immobilized oxidase enzymes and subsequent amperometric detection of generated hydrogen peroxide (60). Principle of the YSI sensor is illustrated in Trace Analytics' TRACE-products use the same enzymatic principle with amperometric detection as YSI for the measurement of glucose, ethanol, lactate and methanol (61) Even though majority of the enzyme probes do not withstand autoclavation they benefit from their specificity enabling measurements from cell-containing samples.

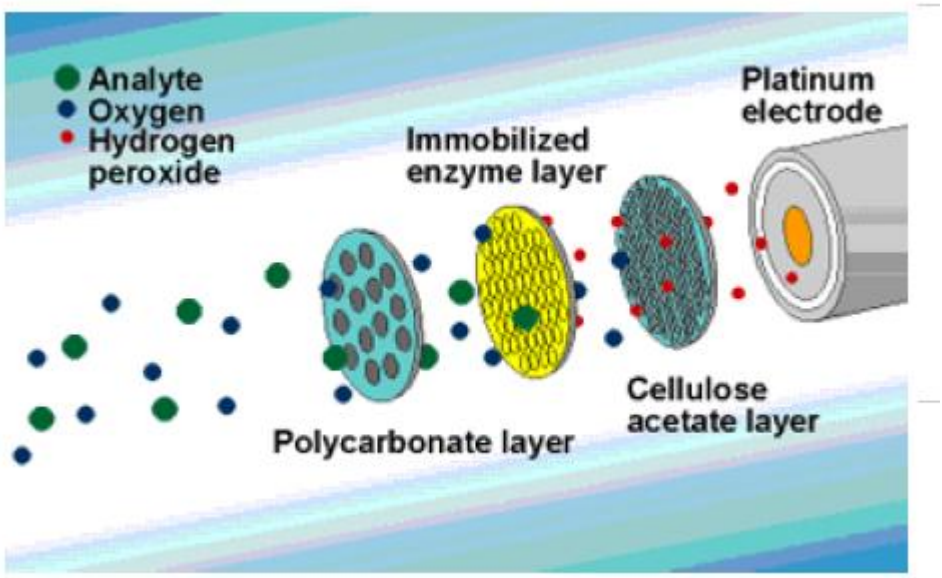

Figure 10. Operation priciple of Yellow Springs Instruments enzyme sensors based on oxidizing enzymes and amperomteric electrode.

5 Sampling and sample treatment approaches

As stated in Table 1 in the Introduction, sampling and sample treatment are among the key technologies for automated at-line measurement approaches. Clearly, they play no role in the analyses based on in situ probes. There are several aspects good sampling system should take into account; 1) is sampling safe for the process not possessing any risks for contamination

(aseptic sampling), 2) how representative is the sample compared to rest of the cultivation, 3) how much sample is taken and how much sample is wasted during sampling (dead volume), 4) how well the sampling system can be cleaned and sterilized before the next sample, 5) is it possible to quench the sample right after sampling and how fast, 6) from what kind of conditions it is possible to take a sample in terms of pressure and viscosity, 7) what is the size of the sampling system and in how large vessels it can be used. Obviously, for automated measurements sampling must be automated. After sampling the sample can be used as such for instance for microscope analysis or it can be diluted or incubated with a reagent depending what should be analyzed. However, quite often liquid phase and solid phase of the sample need to be separated for further analysis as substrates and often products as well are in the liquid phase. Furthermore, if intracellular analytes need to be analyzed, cells need to be lysed and the interesting analytes possibly separated from the rest of the cell lysate.

5.1 Automated sampling

Various automated sampling devices have been patented even by the authors of this review (62) (63), which actually exemplifies how difficult it is to develop a proper automated sampling system that is able to handle full range of various samples and would be capable also for separation of cells and liquid. There are also several companies who offer automated sampling systems and automation of subsequent sample treatment and analysis. Such systems for samples close to viscosity of water are at least bioPROBE by BBI-Biotech (64) illustrated in Figure 11, GPA 1000 by Groton Biosystems (65) illustrated in Figure 12, Seg-Flow by Flownamics (66) illustrated in Figure 13, MultiTRACE by Trace Analytics (67), and different modules by Biospectra (68). Groton and Flownamics can provide multiplexing from eight bioreactors, Trace from six and Biospectra from four bioreactors.

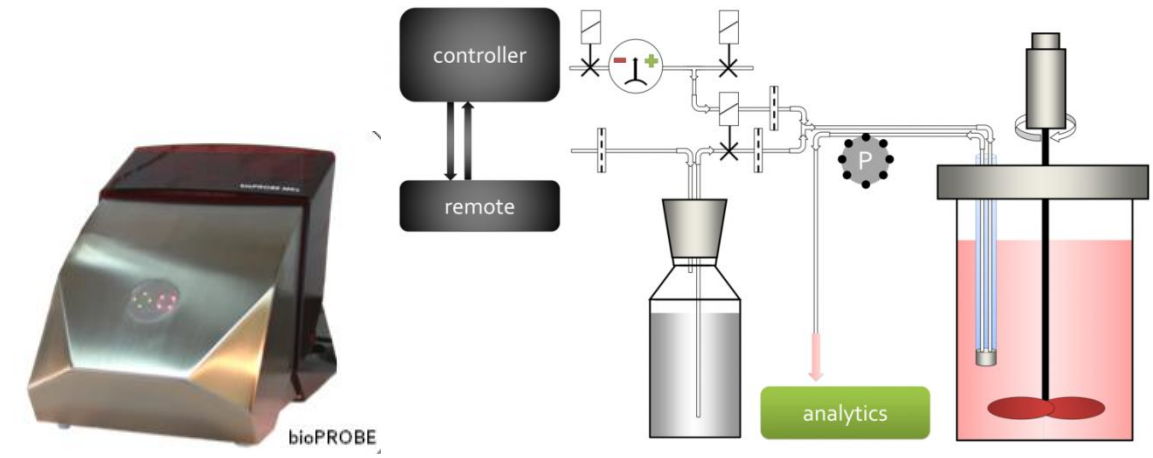

Figure 11. Automated sampling system bioPROBE by BBI-Biotech and flow scheme for sampling.

Figure 12. Automated sampling system Seg-Flow 804m by Flownamics for sampling from eight parallel bioreactors.

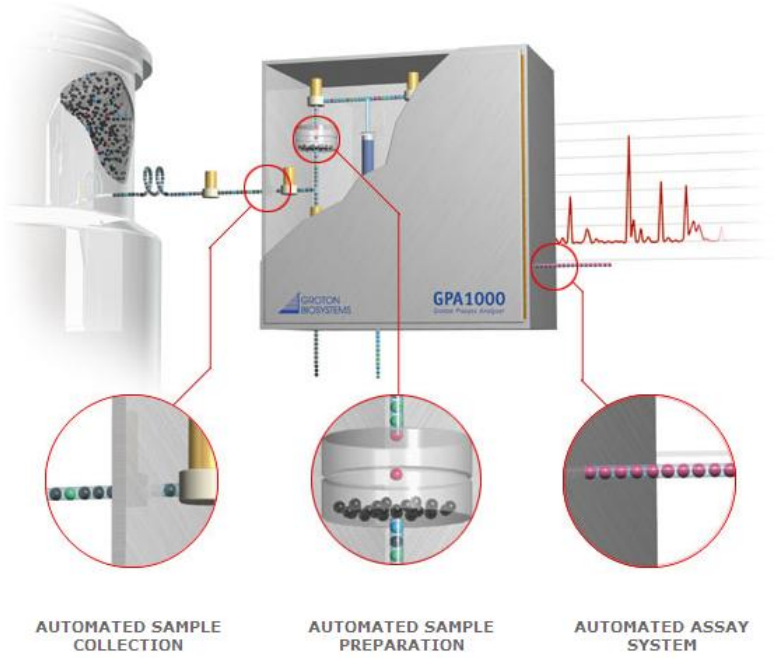

Figure 13. Scheme of automated sampling system GPA 1000 by Groton Biosystems.

Sentry Equipment provides sampling systems also for sampling highly viscous cultivations (69). Operation principle of Sentry's two sampling pistons is demonstrated in Figure 14. Pitkänen and co-workers at VTT have developed this approach further enabling dead-end sample filtration in the inner piston (70).

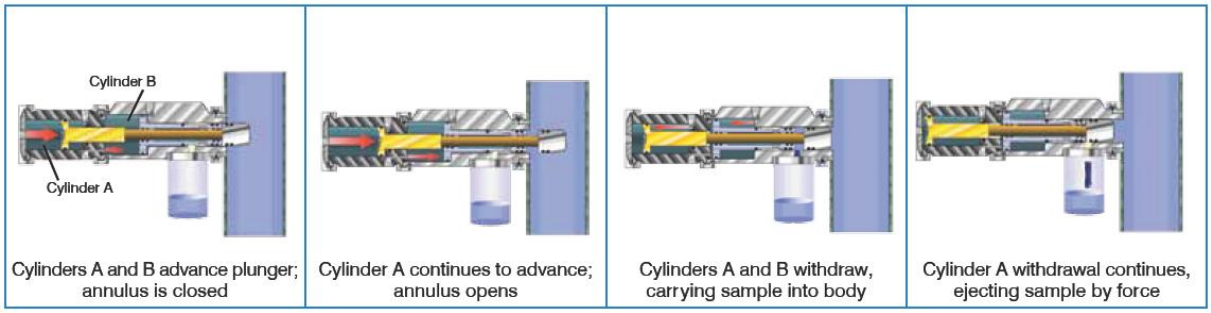

Figure 14. Sampling process of highly viscous samples using ISOLOK MSE sampling system by Sentry Equipment.

5.2 Sample fractionation

The same companies that provide systems for automated sampling have also solutions for taking cell-free samples. At least Groton Biosystems, Trace Analytics and Flownamics (71) have a filtration probe where a filter probe is placed inside the bioreactor as illustrated in Figure 15. In addition to filtration probe, Trace Analytics has also dialysis probe, which basically does not require any volume for sampling, but in principle changes the concentrations of the analytes in the liquid (72). Another disadvantage of the in situ filtration and dialysis probes is potential biofilm formation on the filtration surface eventually blocking the filtration. One proposition to prevent such biofilm formation has been to vibrate the filtration probe (73). Biospectra's solution to cell separation is tape filtration outside the bioreactor after sample has been withdrawn. In tape filtration the filtration tape is rolled forward so that fresh piece of filter paper is available for each new sample.

Figure 15. In situ sample filtration probe C-series by Flownamics.

Separating different cell types can be performed by many techniques. Density gradients have been used for decades to separate cells depending on their cytosol density, either using the Earth's gravitational field for sorting by sedimentation velocity (74) or in combination with centrifugation (75).

In competition with this label-free method, more specific methods have been developped to separate various cell populations depending on their surface antigens (76). The most famous of these techniques is the fluorescence-activated cell sorting (FACS) (77), which is still nowadays the benchmark technique in cell sorting research. Antibody-coated magnetic particles have also been used to sort cells by magnetic-activated cell sorting (MACS) (78).

More recently, intensive research has been focusing on label-free cell sorting, which does not require the use of expensive antibodies, at the cost of the specificity. In 1999, Yang et al. developped a cell sorting technique based on the balance between the dielectrophoretic force and sedimentation (79). This technique has been further developed using multiple-frequency dielectrophoresis to sort living and dead yeast cells in continuous flow, based on their dielectric properties (80).

Other intrinsic properties can be used to sort cells and/or particles. Acoustophoresis uses acoustic waves to sort cells and/or particles depending on their mechanical rigidity (81). Pinched-flow-fractionation makes use of the flow profile in microchannels to sort cells/particles as a function of their size (82).

The best sorting technique depends very much on the cell types studied and on the cell properties that should be highlighted.

5.3 Cell lysis

In most cases, in order to analyse what is inside the cells the cells must be lysed. There are various techniques how to break cells and different techniques work for different organisms. As an example, mammalian cells are easy to break compared to yeast cells. The main techniques

for cell lysis are chemical (83), enzymatic (84), electric (85), mechanical, optical (laser) (86), and sonication. A nice approach for achieving sampling, quenching, lysis and extraction at the same unit operation for analysis of intracellular metabolites has been proposed by Dauner and co-workers applying hot plug flow reactor for precipitating the cells (87).

6 Conclusions

Number of different analytes related to bioprocesses is huge and in this summary we have only scraped the surface of it. There are various ways to measure the analytes that are considered most important by the companies working with bioprocesses. For cell density and cell viability even in situ probes exist although more reliable viability values can be obtained by microscopy of a stained cells at-line. Alternative approach without staining is the Coulter counter approach where even handheld devices have emerged (27). For the analysis of substrates the range of technologies is very wide from in situ infrared and fluorescent spectroscopes to enzyme sensors and various on-line HPLC systems. The same devices are potentially applicable also for the analysis of small metabolite products – certainly depending on what is the product. On-line HPLC systems seem to be applicable also for the analysis of proteins. But in most cases proteins and especially antibodies have been analysed using various flow injection analysis – type assays or sandwich assays using recognition antibodies for the analyte antibodies. Mass spectrometry has been applied only in few cases as a detector in on-line HPLC (48). An obvious explanation would be the sheer size and price of the current mass spectrometers applied in LC-MS off-line analyses. There should be no shortage of possible sampling systems – especially for the cultivations where viscosity stays relatively low. However, there are not so many systems where cell lysis or analyses of intracellular components would have been yet automated for bioprocess monitoring. In general, there is activity in the field of on-line bioprocess monitoring, but there is no single system that would solve all the analytical needs.

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