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Online Analysis for Industrial Bioprocesses: Broth Analysis

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23.1 Introduction

A bioprocess can be described as a process in which a pure or mixed population of microorganisms consumes various substrates and uses them to grow and produce metabolites. In parallel with the improvement in the microorganisms' strains and the capability of assessing their viability and vitality during a bioprocess, it is also desirable to optimize the bioprocess conditions, in particular the growth temperature and/or production, the pH, and the composition of medium, aiming to increase the process yields [1–3].

The capability of controlling bioprocesses automatically and accurately in their optimal state is of paramount importance to most industries, enabling them to reduce or contain production costs and increase yields while maintaining the products' quality. Because of increased competitiveness, strategies based only on empirical knowledge and incorrect attempts are no longer sufficient or effective. The availability of advanced sampling techniques coupled with automated measurement tools (e.g., traditional analytical techniques; new sensor technologies, probes, and analyzers) may greatly reduce the time required for strain selection, process development, and process control by diminishing the number of steps in the production/cultivation process, especially manual steps, and cutting down error propagation. This is highlighted by the Food and Drug Administration, which advises the use of new monitoring and control strategies to improve and ensure the quality of products, especially for the pharmaceutical industry [4]. For instance, the implementation of process analytical technology (PAT) is viewed as a practical tool for the ongoing monitoring and adjustment of production processes to guarantee the consistent quality and specifications of biologically active pharmaceutical ingredients [5]. Nevertheless, for bioprocesses the implementation of PAT is a challenging task owing to the great complexity of cell cultures and fermentation processes and the variability of the raw materials. On the other hand, the successful of PAT

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implementation as well as any other monitoring and control strategy depends on: (1) the availability of robust, reliable, low-cost, easy-to-use online/at-line sensors; (2) a complete understanding of the variability inherent in the bioprocess and introduced by the raw materials; and (3) the availability of sufficient time to develop a process that is amenable to the application of any online monitoring or control procedure during manufacturing [5]. However, it may be difficult to implement monitoring and control strategies because they require the use of experimental design tools, which for some industries such as biopharmaceuticals implies a high-cost, time-consuming procedure.

On the other hand, several studies showed the advantages of developing mathematical models for the design, optimization, and bioprocess control. However, the vast majority of industrial processes are still controlled and optimized without the explicit use of these models. Nevertheless, the development of mathematical models that can describe bioprocesses has become essential because it is usually cheaper to model a system and simulate its operating conditions than to perform laboratory experiments. In some cases, apart from the economic point of view, there are other practical reasons, such as safety and ethical questions that make experimentation impracticable in real systems [6,7].

Mathematical modeling, monitoring, and the real-time control of bioprocesses is a major challenge for biotechnologists and control engineers, leaving them the task of creating communication platforms among themselves and the industry so that the novel techniques that are developed can be used on an industrial level.

The development of modeling strategies, real-time monitoring, control, and optimization is necessary to guarantee operational reproducibility, quality control, and run-to-run consistency for both developmental and scale-up purposes [8].

However, the significant uncertainty of the models' structure and parameters, and the nonlinear and dynamic nature of these systems make bioprocesses modeling, monitoring, and control a difficult and challenging task. Also, implementation of the most suitable type of automated analysis is a main difficulty.

Automated analysis can be performed in two ways: by using a measurement probe inside the reactor, avoiding the need for sampling; or by automated sampling and subsequent sample analysis. The main drawback to the first approach is the scarcity or even lack of inexpensive and robust probes able to allow direct and in-line measurements of state variables. For the latter, expensive, time-consuming, and mostly offline methodologies (e.g., chromatography, electrophoresis, and mass spectrometry) are usually applied that required highly skilled technicians. Nevertheless, although it is rarely available, timely process information about fermentation biomass, substrates, intermediates, products, and nutrients is required to make effective control decisions [9,10]. For instance, in a bioprocess it is crucial to keep cells alive and productive, but unless they are the final product, cell density should be kept at a desirable level. In many cases cell density is measurable using optical in situ probes or intact cell mass spectrometry (ICMS) implemented on matrix-assisted laser desorption−ionization time-of-flight mass spectrometry (MALDI−TOF−MS) [11], near-infrared spectroscopy (NIR), and Fourier-transform−infrared spectroscopy (FTIR) [12,13] coupled with chemometric tools. Also, several methods based in particle and image analysis

and in micromechanical devices may provide quantitative morphological data. Krull et al. [14] compiled a set of sophisticated techniques to characterize fungal growth on the micro- and macromorphological and process-scale levels; knowledge about these may allow better bioprocess performance to be obtained with filamentous fungi. Cell viability (i.e., the ratio of the living cells over the whole population) may be measured offline using microscopy of the stained cells [15] or using electrical or capacitance-based in situ probes. However, the direct measurement of cell vitality [i.e., cells may be alive but not necessarily metabolically active to perform the conversion from substrate(s) to product(s)] is not yet possible, although some indications may be drawn indirectly from measuring biomarkers (e.g., intracellular levels of energy precursors adenosine triphosphate, adenosine diphosphate, and adenosine monophosphate). The measurement of substrates in the extracellular medium is also of utmost importance, because by controlling the substrate feed, the substrate concentration is kept at a certain level, which triggers several control and inhibition mechanisms, allowing the cell state to be controlled and thus selection between the products of primary growth-related and secondary nongrowth-related metabolism. Measuring products (e.g., cells, proteins, enzymes, antibodies, peptides, various nucleic acid molecules, large and small metabolites) is essential to ensure that the production process is working properly. Indeed, the possibility of rapidly assessing information about a fermentation progress may allow secondary metabolite production to be optimized by influencing specific fermentation parameters in a timely manner [11]. Besides being used as an indirect way to assess cell vitality, in the case of by-products (that can also be considered a form of biomarkers), the measurement of biomarkers may also give important information about a departure from optimal conditions. Substrate, product, and by-product levels during a bioprocess may be monitored using a diverse number of analytical techniques coupled or not to chemometric tools, including in situ biosensors [16−18], optical sensors or photometry [19,20], electronic noses [21], UV spectrophotometric methods [22,23], offline classical analytical techniques such as liquid chromatography [24,25], dot blot methodology coupled with image-processing algorithms [24], capillary electrophoresis [26], cyclic and square-wave voltammetry [27], or spectroscopic methods such as Raman spectroscopy [28,29], electrochemical impedance spectroscopy [27], FTIR [13,30], NIR [8,19,31−36], two-dimensional (2D) fluorescence spectroscopy [37], or dielectric spectroscopy [38].

In the literature, different terms have been used to refer to timely measurements of the fermentation broth media that could be used directly for bioprocesses monitoring and control strategies, such as online, in-line, at-line, in situ, and real time, among others, but their use is not always consistent. One possibility would be to name an approach "in situ" without sampling and another approach "automated at-line" with sampling and subsequent sample analysis. Both approaches have known pros and cons, such as at sampling, sterilization, calibration, and multiplexing levels [39].

The monitoring step must include the acquisition and analysis of data collected by either in situ physical sensors or automated at-line procedures. Soft sensors, which are mathematical models coupled with a limited set of state variables that can easily be measured, have been the focus of several studies, and represent an interesting

alternative to the traditional automated analysis (in situ sensors or automated at-line approaches), allowing the online monitoring of state variables that affect the bioprocess but cannot be measured in real time [40−49]. In real time, soft sensors can estimate process variables that are difficult to measure online or those whose measurement by analytical procedures is tedious and time-consuming [46]. Despite the complexity of the underlying theory, some soft sensors have been proposed, although there are not many documented examples of applications to complex bioprocesses described by dynamic models consisting of several balance equations and complex kinetics. Thus, the development and experimental validation of these algorithms need to be carried out because they may be helpful tools in controlling and optimizing industrial processes, overcoming the lack of physical sensors, and minimizing the reluctance of the industry to use new physical sensors, such as for problems of sterilization.

On the other hand, during bioprocess optimization, advanced sampling techniques, new sensor technologies, and analyzers have been applied, resulting in large and complex data sets with underlying multivariate interactions. Thus, multivariate data analysis (MVDA) has emerged as a practical tool for dealing with these complexities and extracting relevant information from these highly correlated multivariate data sets [50]. In the literature, several works highlight the use of MVDA tools in bioprocessing applications such as in cell culture operations [51−55].

23.2 Bioprocess Broth Characterization

In fermentation processes, a selected strain of a microorganism, plant, or animal cell line is cultivated in an appropriate nutrient medium using a bioreactor whose operating conditions are carefully controlled, allowing the desired product to be produced. The product can be the cells themselves (e.g., baker's yeast), some cellular component (e.g., DNA or protein), or a metabolite synthesized by the cells (e.g., organic acids, vitamins, or recombinant human proteins) [9]. Most bioprocesses are three-phase systems. The cells are dispersed as a solid phase in a liquid medium phase which is aerated by a gas phase, which results in a set of complex solid−liquid−vapor interactions. Assessment of these interactions is crucial because biological components (i.e., microorganisms) are sensitive to any change in the reaction's environment (e.g., temperature, substrate or nutrient concentrations, pH, pO_2), which may affect the cells' activity or the reproducibility of the process [46,56].

In most processes, multiple substrates are present to fulfill the microorganisms' requirements regarding the carbon source, the energy source, the nitrogen source for protein synthesis, the phosphate source, growth factors, and mineral salts. In certain fermentation processes, the media should also supply the required precursor to increase the yields of the desired product. Indeed, bioprocesses are characterized by their complex dynamics such as the inverse response, dead time, and strong nonlinearities, which are crucial biochemical variables and/or parameters hard to measure online; if induced

cultures are used, high variations may happen in the morphology, energy metabolism, and macroscopic composition of the cells, and quantification is a hard task that is not straightforward [46]. In large industrial-scale fermentations (e.g., 150 m^3), nutrient sources may even be undefined because different types of raw material are used, such as crude nutritive sources, mainly owing to economic requirements. Saccharine-based materials (such as sugar cane, sugar beets, molasses, and fruit juices), cheese whey, starchy materials (such as cereals, roots, and tubers), cellulosic materials (sulfite waste liquor, wood molasses, and rice straw), hydrocarbons (gas oil and n-paraffins), vegetable oils, and nitrogenous materials (for example, corn steep liquor, soybean meal, or oils) are used as carbon and nitrogen sources in fermentation industries. The chemical composition of those media can be highly variable from batch to batch and depends on the quality and the intrinsic diversity of the raw material and on the processes involved in manufacture, because they are usually by-products of other industries.

In fermentations for which the media are often chemically defined, such as animal cell culture media, many individual nutrients are present and frequently complex supplementation is needed (e.g., amino acids), which results in complex mixtures [9].

Indeed, regardless of the type of fermentation, the initial medium often contains high levels of sugars such as glucose, fructose, or lactose (up to 60% in some cases) and other nutrients including vitamins (such as biotin, pyridoxine, thiamine, pantothenic acid, and inositol) and inorganic salts (such as calcium, iron, chloride, phosphorus, and sulfate). Some precursors and inducers compounds can also exist which generally improve the yield or quality of the product (for example, phenyl acetic acid and cobalt) [9]. It is also usual to apply compounds which act as antifoams and are not metabolized by the microorganisms (e.g., stearyl alcohol and octyl decanol, esters, fatty acids, cotton seed oil, linseed oil, castor oil, cod liver oil, etc., and silicones and sulfonates).

The chemical composition of the culture medium, the growth of microorganisms and inherent morphology changes, and the related consumption and production processes may result in changes in the culture broth's rheological properties during a bioprocess [33]. The viscosity of the culture medium may vary not only along the cultivation time but also from batch to batch and with the reactor scale, which increases the need to monitor the evolution of the fermentation, and which may turn out to be a difficult and complex task. For example, if the fermentation medium contains a polymeric substrate such as starch, the apparent viscosity will decrease during batch fermentation because of its enzymatic degradation and consumption, and a change from non-Newtonian to Newtonian behavior can occur. On the other hand, when a polymer is produced during batch fermentation, the viscosity of the broth will increase with an increase in broth concentration and in some cases non-Newtonian behaviors may appear, being the apparent viscosity time-dependent. As described, the chemical complexity of the fermentation broth varies considerably, which makes optimization of robust online monitoring and analysis challenging [9].

Mixed-feed bioprocesses have been used to enhance bioprocesses productivity. Typically, multiple carbon sources are co-fed: the primary substrate supplies energy for

growth (e.g., glucose or glycerol) and an assimilable secondary substrate is administrated to induce recombinant protein production (e.g., arabinose, lactose, or methanol). This strategy raises novel process-technological challenges for bioprocessing monitoring because the energy supply through the growth substrate and the energy drain through recombinant protein production must be independently controlled via mixed-feed approaches [44]. Sensor probes may provide important real-time information for all unit operations in a biological production facility. Although many types of online sensors and analytical techniques are available, only a few are commonly used in the biotechnology industry because the great majority have several practical shortcomings, such as that they are complex, expensive, labor-intensive, time-consuming, and not compatible with standard sterilization procedures. Therefore, noninvasive and versatile probes are still needed [56]. Usually three kinds of parameters should be evaluated when online bioprocess monitoring is envisaged: physical variables (e.g., pressure, temperature, viscosity, stirrer speed), chemical variables (e.g., pH, pO_2, nutrients, metabolites), and biological variables (e.g., biomass concentration, cell metabolism). For each variable, different analytical sensing techniques may be found (Fig. 23.1) [56].

Sensor probes developed for the in-line monitoring of bioprocesses used in industrial facilities are usually designed to be directly immersed in bioreactor medium broth, and so they must be sterilizable (i.e., they should resist to steam or $\gamma-$radiation) and temperature and pressure stable. Also, in-line sensor probes must be able to overcome problems caused by fouling or medium interference. Considering these basic requirements for industrial use of an in-line probe, it can be easily recognized that not every laboratory analysis method can be used for in-line monitoring of bioprocesses in an industrial setting [56]. On the other hand, any in-line or at-line analytical probe used for online monitoring of a bioprocess must fulfill other technical attributes such as accuracy (i.e., the degree of agreement between the measured and true values of the analyte), selectivity (i.e., the capability of measuring a specific parameter in the medium broth, overcoming possible interference from other parameters or background noise effects), and sensitivity (i.e., the capability of detecting and quantifying small changes in the desired parameter property if possible within a large dynamic range) [56]. Other attributes must be also taken into account for any sensor probe to be used for online monitoring, such as repeatability (i.e., agreement between different measurements of the same parameters made by the same operator under the same conditions at different times), robustness (i.e., the capability of a sensor to deliver a consistent signal, independent of process conditions and the duration of the analysis), stability (i.e., the capability of a sensor to provide reproducible results for a certain period of time, retaining sensitivity and selectivity), and linearity (i.e., deviation from a linear response, including detection and quantification limits). Finally, there is the response time of a sensor probe (i.e., the monitoring time delay), which should be small enough to ensure that it is possible to evaluate important process dynamics in real time; otherwise, the efficient control of the process will not be possible. Thus, ideally a sensor probe must provide high selectivity, sensitivity, robustness, repeatability, and stability and have a
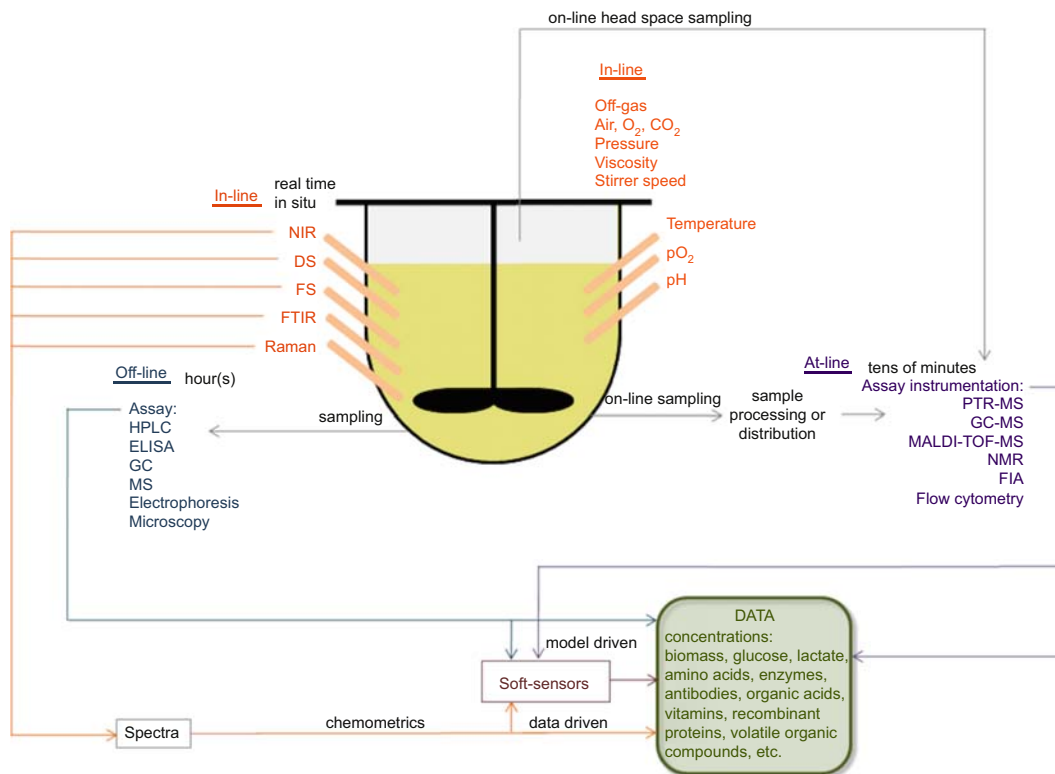
FIGURE 23.1 Schematic of bioprocess monitoring: variables and different analytical techniques. *NIR*, near-infrared spectroscopy; *DS*, dielectric spectroscopy; *FTIR*, Fourier-transform infrared spectroscopy; *FS*, fluorescence spectroscopy; *HPLC*, high-performance liquid chromatography; *ELISA*, enzyme-linked immunosorbent assay; *GC*, gas chromatography; *MS*, mass spectrometry; *PTR-MS*, proton transfer reaction—mass spectrometry; *MALDI-TOF-MS*, matrix-assisted laser desorption—ionization time-of-flight mass spectrometry; *NMR*, nuclear magnetic resonance; *FIA*, flow-injection analysis.

low detection limit and linearity with short response times and a long lifetime. Therefore, the development of online monitoring analytical techniques that could be implemented at industry facilities is still a challenging task. In the next section, some of the most common methods described in the literature for the simultaneous online monitoring of several parameters of a fermentation medium broth are reviewed together with their main advantages and shortcomings.

23.3 Online Techniques for Industrial Bioprocess Broth Analysis

Online monitoring techniques are needed to assess cell growth, substrate consumption, and product formation on a real-time basis, enabling effective control of the bioprocess, which results in increased yield, productivity, and reproducibility [36]. Thus, the

development of reliable analytical techniques capable of monitoring critical process parameters in-line is essential [34]. These real-time analytical tools must fulfill requirements such as being process-friendly, with minimal or no sample preparation and/or pretreatments, having high-speed data acquisition and simultaneous detection and/or quantification of multiple parameters, and having the possibility of being used for in situ measurements. However, the intrinsic variability of each bioprocess, the complexity of the biological systems, and the need, in many cases, to operate in a sterile environment, together with the gas phase or vibrational effects caused by agitation pose a number of difficulties and may partially explain the scarcity of real-time noninvasive measuring analytical techniques or probes. On the other hand, upstream bioprocesses usually used by industry are sensitive to several parameters such as the size and configuration of the reactor, the composition of the growth media, pH, temperature, dissolved oxygen level, agitation rates, and the chemicals used in growth and expression processes. Thus, to increase production yields and ensure product consistency and reproducibility, these parameters must be controlled, and therefore real-time monitoring is required [8]. Also, to automate large-scale microbial bioprocesses, a thorough real-time estimation of the state of the process must be achieved and critical quality attributes of the bioprocess must be monitored, preferentially in-line and in situ, allowing an assessment of the process during running, enabling its feedback correction toward the optimal quality profile, and minimizing perturbation of the process [32].

Indeed, in a great number of industrial fermentations the levels of some key parameters (e.g., carbon sources and/or precursors, metabolites) must be strictly controlled, which in some cases involves the integration of chemometric techniques and the dynamics of the fermentation process [33]. Also, sensor-based techniques usually generate a great amount of signals data that must be correlated to key variables of the bioprocess under study (e.g., substrate concentrations) through an appropriate mathematical model which then can be used to evaluate variable profiles or the process status using recorded online signals data. These correlations are usually established using multivariate data analysis to identify relevant information hidden over the entire data set [56].

The establishment of reliable chemometric models requires the ability to select an appropriate calibration procedure based on real-time sampling data, which must be able to take into account the intrinsic variability of a batch-to-batch process and its impact on complex system interactions [33].

However, real-time sensor technologies are mostly established for some critical process parameters (e.g., temperature, pH, dissolved oxygen, stirring speed) and used in process control, but for others parameters such as biomass and medium composition, research is ongoing [32,34]. Owing to the complexity of the general cell metabolism during growth and product formation, in situ data on the critical variables are not easily estimated based on knowledge of the critical process parameters [32]. Because of the scarcity or even lack of in-line sensor probes for online monitoring of critical variables, several industrial microbial cultivation processes are still monitored mainly using offline analysis of biomass, product, and carbon sources and require sampling procedures that

increase the risk of contamination and delay the determination of target analyte concentrations [32]. On the other hand, most commercial techniques available for the real-time analysis of biomass, product, and carbon sources are limited to cultures in which media matrix and measurement conditions are relatively simple. Indeed, the high complexity of microbial fermentations caused by the heterogeneity of the raw materials and the different stirring and aeration needs, resulting in constant changes in the growing microorganism morphology as well as the broth rheological properties, and which may differ within cultivations, from batch to batch, and with each reactor size, are difficult for developing real-time analysis strategies [33]. Nevertheless, several works have been published reporting the successful application of real-time analytical techniques. Among them, special attention has been paid to spectroscopy-based methods as well as other techniques for the online monitoring of bioprocesses.

23.3.1 Spectroscopic-Based Methods

The application of non-invasive spectroscopic methods to bioprocess monitoring turn out in a multitude of on-line signals indirectly related to either the cell population or compounds in the medium. Signals and spectra data contain valuable but masked cell physiology-relevant information which requires the use of chemometrics tools to establish correlations with offline bioanalytical data. This procedure allowed successful data-driven soft sensor systems to be developed for the online monitoring of complex variables such as concentrations of some organic compounds in the medium, cell density, cell growth, recombinant protein concentration, plasmid copy number, and metabolites [57].

Spectroscopic analysis is based on the interaction of electromagnetic waves and molecule bonds, and thus the infrared (IR) spectrum of a compound reflects energy absorption caused by the vibration of its chemical bonds over a wavelength range [58]. Bioprocess monitoring mainly uses spectroscopy methods in the IR region of the infrared spectrum subregions (near, 780−2526 nm; mid, 2500−40,000 nm; and far, >40,000 nm). With the use of spectroscopic sensors, no sampling is required (except for calibration), there is no interaction between the sensor and analytes, and the simultaneous and instantaneously assessment of several different process variables can be determined simultaneously [56].

Among spectroscopic-based techniques reported for the online monitoring of microbiological processes, most interest has been paid to dielectric spectroscopy (DS), NIR, FTIR, 2D-fluorescence spectroscopy, and Raman spectroscopy.

23.3.1.1 Dielectric Spectroscopy

DS has emerged as potential online, in situ, and in-line tool to monitor mammalian, yeast, and bacterial processes, even in the presence of turbidity or biomass aggregates [59,60]. This technique allows robust real-time, in-line measurements, simple linear calibrations for biomass estimation, and measurement selectivity to viable biomass [60,61]. Indeed, the recorded capacitance data may be related to the amount of living

cells because they behave as small capacitors when exposed to an electric field [38]. DS probes have also been applied to monitor lipid storage in yeast cells [62] and to monitor in situ and in real time bacterial cell growth, product formation, and the optimum time for harvesting polyhydroxyalkanoate from the cells as well as the consumption of volatile fatty acids [38]. Ehgartner et al. [60] described a novel method that combines soft sensors and dielectric measurements in dual-frequency mode to monitor in real time physiological changes in recombinant *Escherichia coli* bioprocesses producing an inclusion body product accumulating in the cell. Therefore, globally, studies carried out have shown that this tool may improve process understanding and optimization by implementing models and applying control strategies.

23.3.1.2   Near-Infrared Spectroscopy

NIR is a nondestructive analytical technique that requires minimal sample preparation and/or pretreatments; it is fast (i.e., high speed of spectrum acquisition) and allows the simultaneous detection and/or quantification of multicomponents to which it is possible to couple fiber-optic probes that can be immersed directly into the culture broth and steam sterilized with it, enabling the acquisition in situ of real-time information [34,36,63]. Also, the low-absorption coefficient enables high penetration depths, and therefore larger sample volumes can be analyzed [34]. To monitor complex industrial cultures using NIR spectroscopy, a wide range of wave numbers (740−1300 nm) in the NIR region of the electromagnetic spectrum is needed. In NIR analysis combinations and overtones of molecular vibrations are used rather than specific fundamental vibrations, although the measurements cover the specific wave numbers that correspond to the maxima absorbance of the typically bonds (i.e., the O−H bonds of alcohols, C−H bonds of aliphatic and aromatic carbon compounds, and N−H bonds of proteins) found in the multicomponent media broth [8,33,56,63]. In fact, the more different vibrational modes that can be excited, the more information can be gained and the more specific the determination of analytes can be [56]. NIR analysis may be carried out by transmittance (e.g., low−cell density fermentation broths, transparent liquids and suspensions), reflectance (e.g., high−cell density fermentation broths, opaque liquids and/or solids and powders) or transflectance (e.g., transparent and turbid liquids and semisolid samples) [58,64]. The spectrum acquired during a fermentation run is a fingerprint of all broth NIR active constituents, and because of its complexity and the overlapping bands, which are hard to assign directly to a specific analytes in the sample, qualitative/quantitative multivariate statistical tools must be used for spectral interpretation and modeling [31,34,36]. Despite the recognized potential of NIR for bioprocesses monitoring and control, some drawbacks are usually pointed out [8], such as the need to carry out frequent time-consuming model calibrations, and the failure to measure concentration data accurately for significant postcalibration periods or during high aeration, stirring, or temperature changes. Even so, several applications for NIR fiber-optic probes have been reported for in-line and at-line measuring of analytes in biological processes, such as monitoring of biomass growth of bacteria and mammalian cells, carbon source

consumption, by-product formation and consumption, and their corresponding products (e.g., glucose, lactic and acetic acids, glycerol, methanol) [19,65−67]. However, few of them report the implementation of NIR technique in situ, aiming for real-time monitoring application [31,36,63]. Also, because high dynamic ranges of agitations, air-flow rate, and biomass concentrations occur during the whole culture process of microorganisms, only few works report the application of in situ NIR probes [32]. Furthermore, several NIR-based models described in the literature for measuring carbon and nitrogen sources in microbial processes show high mean percentage errors, and so they are unsuitable for fermentation processes in which the toxic feed components must be kept at residual levels [33]. On the other hand, few works report the application of in situ NIR probes along the whole culture process of microorganisms, which enable coping with very high dynamic ranges of agitation, air-flow rate, and biomass concentrations. A novel soft NIR probe was proposed [33] to monitor critical nutrient ratios of linoleic acid, oleic acid, and ammonia during lipstatin fermentation by *Streptomyces toxitricini*. Goldfeld et al. [67] designed an innovative NIR spectroscopic method that could successfully monitor, in real time and online, concentrations of glycerol, methanol, and biomass during the production of a monoclonal antibody by a *Pichia pastoris* high−cell density process, with long-term stability. An in situ NIR fiber-optic probe combined with chemometrics tools [e.g., partial least squares (PLS) models] was used by Sampaio et al. [32] to monitor the cultivation of two recombinant *Saccharomyces cerevisiae* strains producing heterologous cyprosin B, enabling the real-time evaluation of the main critical variables involved in the cultivation process (e.g., biomass, glucose, galactose, acetate, and ethanol). Alves-Rausch et al. [34] described real-time multiparameter monitoring during *Bacillus* spore production based on multivariate statistical models established using NIR in-line spectroscopy data. The approach was successfully applied to a large-scale industrial fermentation process. The NIR spectroscopy resisted cleaning-in-place and sterilization-in-place of the harsh scale production environment with low maintenance. Moreover, the fast diode array technology used enabled the attenuation effect to be taken into account owing to the air bubbles, by applying a filter during spectra acquisition. Lopes et al. [63] demonstrated the ability of in situ to monitor *E. coli* cell cultures designed for plasmid bioproduction using an NIR fiber-optic transflectance probe combined with PLS models. The methodology allows the real-time acquisition of spectral information related to key process variables without the use of reagents and no risk of contamination. Hakemeyer et al. [31] described the application of at-line NIR transmittance spectroscopy on supernatant samples from Chinese hamster ovary (CHO) cell−based monoclonal antibody cultivation processes. An online monitoring process was developed by Cruz et al. [36] to produce polyhydroxyalkanoate (PHA) using cooking oil as the sole carbon source and *Cupriavidus necator*. NIR spectroscopy and PLS models were used successfully to monitor the production of PHA, biomass growth, and the consumption of used cooking oil, despite the intracellular nature of the product and the immiscibility of the substrate. Kim et al. [8] used real-time online NIR monitoring of glycerol concentration, methanol concentration, and relative cell density

to characterize and optimize monoclonal antibody production in glycol-engineered *P. pastoris* during high−cell density fed-batch fermentation. The online NIR monitoring potential was proved over 1 year, which enabled an accurate estimation of biomass, glycerol, and methanol concentrations without recalibration.

23.3.1.3 Fourier Transform Infrared Spectroscopy

Attenuated total reflection FTIR spectroscopy has become a powerful process analytical tool for in-line and at-line monitoring of bioprocesses [19]. It is a fast, easy, and nondestructive analytical technique showing the typical advantages of any spectroscopic technique over classical analytical methods employed for bioprocess analysis, such as the ability to quantify multiple target components simultaneously (e.g., substrates, desirable and undesirable metabolites) by applying common chemometric tools [e.g., principal component analysis (PCA), PLS regression, and multivariate curve resolution (MCR)] [30]. Experimental spectra data are acquired in situ without the need for sample preparation and can be provided in real time, which makes it well-suited for process monitoring. FTIR spectroscopy enables the identification of different functional groups (e.g., lipids, proteins, and carbohydrates) and organic compounds (e.g., exopolysaccharides and phycoerythrin) by evaluating transitions between vibrational states of the bonds contained within the molecule [13]. Although it is a promising technique, FTIR has several shortcomings, such as the strong overlap of the bands owing to the high similarity of the structure of the carbon sources, of the evolving species, or because of the presence of interferences that hamper perfect elucidation of the analytes being transformed [30]. Some works reported the successful application of FTIR-based methods for online monitoring of bioprocesses. Scholz et al. [68] presented a methodology based on FTIR spectroscopy coupled to PLS regression to determine the main variables of plasmid bioproduction with *E. coli* cultures: plasmid, carbon sources (glucose and glycerol concentrations), and a by-product (acetic acid concentration). The satisfactory results showed that FTIR spectroscopy was sufficiently sensitive to detect changes in the bacteria metabolism induced by changes in the chemical environment. Grassi et al. [30] showed that the combined use of FTIR spectroscopy and MCR alternating least squares technique facilitates the study of complex beverage bioprocesses, allowing beer fermentation to be monitored and full understanding of the evolution of sugars (maltose, maltotriose, fructose, sucrose, and dextrins) and ethanol during the biotransformation, independently of environmental conditions (i.e., different yeast inocula and temperature). The proposed methodology was suitable for monitoring and understanding the kinetics of the whole fermentation process, maximizing and standardizing the productivity of the batches. Fuentes-Grünewald et al. [13] used FTIR technology to characterize macromolecule biomass composition and the quality of the metabolites produced during microalgal production. The methodology enabled the evolution of macromolecules composition to be followed and characterization of the metabolites produced, such as phycoerythrin or exopolysaccharides in *Porphyridium purpureum* cultures. These examples demonstrate the effectiveness of FTIR

spectroscopy coupled with different chemometric tools for online monitoring of a wide range of bioprocesses, and their main advantages as well as some shortcomings.

23.3.1.4   *Raman Spectroscopy*

Raman spectroscopy is a simple, fast, nondestructive, and noninvasive technique that allows the analysis of molecular characteristics by evaluating vibrational modes in molecules, for which the effect of water interference is insignificant [28]. Raman-based techniques may be used to study transparent, translucent, opaque, and colored samples including solids, semisolids, suspensions, and solutions [69]. Raman techniques have progressed from traditional laboratory spectrometers to several miniaturized and tunable lasers, optical filters, spectrographs, interferometers, charge-coupled devices, microprocessor controls and probes. These have improved mobile applications to be noncontact and process-friendly and allow for remote analysis, enabling in-line analysis. Moreover, because Raman spectroscopy is an inelastic scattering technique of monochromatic light [29], the spectrum generated contains a large amount of chemical information that may be used to predict multiparameter concentrations [28,69]. Also, because Raman spectroscopy does not require a reference light path (which is needed for IR/NIR), it is amenable to fiber optics and allows for remote sampling. In many cases, it enables a better sample characterization than FTIR spectroscopy. However, Raman spectra can be affected by high fluorescence background signals, which distort the spectrum shape but can be removed using adequate software and algorithms, and by signal attenuation caused by the presence of light-scattering particulates, a drawback that has not yet been totally overcome [28,29,69]. Although process monitoring using Raman spectroscopy has not yet gained the same popularity as NIR, some studies have shown the versatility and promising performance of in situ Raman probes coupled with appropriate chemometric tools to monitor concentrations of substrates and products during fermentation processes (e.g., yeast fermentations and mammalian cell culture bioprocesses) [29]. Ashton et al. [70] showed that UV resonance Raman (UVRR) spectroscopy could monitor multicomponents in mammalian cell cultures, including recombinant protein titer. This specific Raman technique measures Raman spectra with UV lasers, showing no fluorescent background; it creates a resonance Raman effect that greatly increases the signal and is selective toward individual species in complex media when appropriate UV wavelengths are selected, although no UVRR probe is available to date. The potential of UVRR spectroscopy was further demonstrated by Ashton et al. [71]. These authors showed that UVRR could be used as a tool to monitor variations in residual DNA and RNA that may contaminate mammalian cell culture medium before and after purification of valuable recombinant proteins from the same medium, without interference from background fluorescence, which is often a major problem with conventional Raman spectroscopy. Ávila et al. [72] used coupled Raman spectroscopy and PLS regression models to establish an online monitoring system for glucose fermentation by *S. cerevisiae* yeast. The procedure allowed online monitoring of substrates (glucose), fermentation products (ethanol and glycerol), and biomass growth. These

authors also showed that multivariate control charts based on spectra data recorded enabled the detection of fermentation fault batches. Gray et al. [73] employed in situ Raman spectroscopy for the real-time monitoring of simultaneous saccharification and fermentation of corn mash by an industrial strain of *S. cerevisiae*. Based on spectra acquired during fermentation, these authors established PLS regression models to evaluate total starch, dextrins, maltotriose, maltose, glucose, and ethanol concentration changes during fermentation under typical industrial conditions. Iversen and Ahring [29] showed the potential of Raman spectroscopy for in situ and real-time monitoring, of a *S. cerevisiae* fermentation process. The instrument was equipped with a sapphire ball probe designed to minimize interference by particulates and to withstand the harsh environment during sterilization. The proposed Raman technique coupled with a simple univariate calibration model (typical multivariate analysis was omitted) allowed reasonable monitoring of concentration changes (e.g., glucose, ethanol, and acetic acid) during pretreatment, hydrolysis, and fermentation processes during the production of lignocellulosic bioethanol. Paudel et al. [69] presented an integrated framework that allowed the real-time estimation of glucose concentration during microalgae cultivation in a photobioreactor operated under mixotrophic conditions using Raman spectroscopy.

23.3.1.5   Fluorescence Spectroscopy

Attention has been focused on fluorescence spectroscopy, an optical and noninvasive method for process monitoring, because it allows instantaneous data acquisition and in-time evaluation of bioprocess variables. Independently of the known advantages of each spectroscopy-based method, fluorescence spectroscopy seems to be an interesting approach to bioprocess monitoring considering that NIR spectroscopy is not always suited to analyzing aqueous broth samples because of strong water signals and Raman spectroscopy. Although it is suitable for aqueous sample analysis, the Raman signals recorded for some analytes are weak and could be affected by matrix fluorescence interference [74]. The usefulness of the fluorescence technique relies on the fact that many cellular substances and media components are biogenic fluorophores. Indeed, fluorescence spectra contain valuable information concerning cells and process variables (e.g., biomass and cell density). Thus, fluorescence spectroscopy combined with chemometric tools offers deeper insight into biological processes resulting in easier bioprocess monitoring, optimization, and control [75]. In fact, multivariate statistical methods are needed because, similar to other spectroscopic methods, fluorescence spectra recorded during a fermentation process result in a high data volume, and so chemometric methods are usually needed [e.g., PCA, PLS regression, and artificial neural networks (ANN)] [37]. Rossi et al. [76] demonstrated that the metabolism of *Klebsiella pneumonia* under aerobiosis and anaerobiosis could be monitored online and predicted by the application of chemometric models and multiwavelength 2D-fluorescence spectroscopy. Biomass, glycerol, and 1,3-propanediol content were predicted using PLS and ANN models established using online fluorescence data, although the last two are not fluorescent compounds. 2D-Fluorescence spectroscopy combined with

multivariate linear (PLS) and nonlinear (ANN) models was also applied by Grote et al. [37] to predict online process variables (e.g., pH) and acidity during sourdough fermentation. Li et al. [74] showed that fluorescence excitation-emission matrix (EEM) spectroscopy combined with chemometric methods could quantitatively predict recombinant glycoprotein production cultured in a CHO cell fed-batch process. The feasibility of this technique relies on the fact that EEM spectra of complex solutions are sensitive to compositional changes, and thus during the cultivation progress, changes in the emission properties of several key fluorophores (tyrosine, tryptophan, and the glycoprotein product) could be recorded to enable culture progress via MCR-ALS to be followed. Overall, it was shown that the proposed approach could be successfully implemented for monitoring, at-line or offline, the productivity of industrial fed-batch mammalian cell culture processes from small to large scale. Ohadi et al. [77] also described the accurate potential of multiwavelength fluorescence spectroscopy to monitor viable and dead cells, recombinant protein, glucose, and ammonia concentrations for CHO cells during cultivation by means of PLS regression models based on recorded fluorescence excitation−emission data. The satisfactory performance of the proposed combined approach clearly shows that this methodology could be used for in situ monitoring of mammalian cell culturing.

23.3.2   Mass Spectrometry-Based Methods

One major shortcoming of noninvasive spectroscopic methods for online monitoring of bioprocesses is the time required to acquire accurate process data sets that will constitute the basis for developing reliable, data-driven predictive models, which limits the acceptance of this approach. Moreover, the practicability of validating and implementing such complex approaches in a standard industrial process environment leads to some skepticism in industrial fields. A possible alternative would be to apply online analyzers for the direct detection of physiologically relevant process variables. This approach should meet the high demands on sensor/analyzer systems in a bioprocess environment and the need for real-time evaluation of metabolites that may be enclosed in the cells without cell disruption and time-consuming sample preparation procedures [57]. One possibility could be to use online mass spectroscopic techniques.

23.3.2.1   *Proton Transfer Reaction−Mass Spectrometry*
Proton transfer reaction−mass spectrometry (PTR-MS) can be used to monitor metabolites released through the cell membrane during cell interaction with their environment. Among these, volatile organic compounds (VOCs) may be used for real-time, noninvasive bioprocess monitoring, because they are directly connected to physiologically relevant information and easily accessible via headspace sampling. PTR-MS is a promising tool for the quantitative analysis of VOCs and so could be used as a noninvasive technique with minimal requirements for sample preparation, which may guarantee sample authenticity. Moreover, the soft ionization of VOCs via this technique

allows for a low degree of fragmentation that is especially useful for the analysis of complex gas matrices of bioreactor headspace samples. Furthermore, VOC signals acquired by PTR-MS are quantitative and can be assigned to specific compounds. When they are coupled with multivariate statistical tools, they may be further used to predict particular variables [57]. The potential of PTR-MS as an advanced upstream process monitoring tool for noninvasive online headspace measurement of VOCs in recombinant *E. coli* fed-batch fermentations was demonstrated by Luchner et al. [57].

23.3.2.2   Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

MALDI−TOF−MS is a prominent technique in biological mass spectrometry, especially for analyzing intact cells (e.g., microorganisms). The technique has several advantages, such as simple sample preparation, the possibility of automated analysis over a wide mass range, and fast analysis. Helmel et al. [11] optimized and implemented an ICMS approach on two MALDI−TOF−MS instruments in the linear positive ion mode, allowing the growth stages of *Penicillium chrysogenum* to be monitored during batch or fed-batch fermentations by means of hierarchical cluster analysis. ICMS gives a unique mass spectral fingerprint of surface-associated proteins and/or peptides, enabling the identification of microorganisms from different species or strains. The technique proved to be a fast, nearly real-time screening method allowing biomass production to be followed during batch fermentations and penicillin-producing fed-batch processes. Steinhoff et al. [78] described a novel high-throughput method based on MALDI-TOF-MS and multivariate analysis (PCA) to monitor intracellular metabolite levels in fed-batch processes. The technique is based on a new microarray sample target and allows the detection of phosphorylated nucleosides and other metabolites (glutathione and nucleotide sugar) using stable isotope-labeled internal standards. The proposed method requires short sample preparation steps and is suitable for monitoring mammalian cell cultures such as antibody-producing hybridoma cell lines in industrial environments.

23.3.3   Other Methods

Apart from the most common spectroscopy and mass spectrometry−based analytical methods usually applied when online monitoring of fermentation broth is envisaged, a wide range of other methods have been reported in the literature. In this section, some of those methodologies are reviewed to cover novel and alternative methodologies.

23.3.3.1   Photometric Sensors

Photometric sensors have been reported as a promising low-cost alternative to spectrometers for the online monitoring of bioprocesses [19]. Photometric sensors have optical bandpass filters in spectral regions that are specific to the respective components to be analyzed. Also, these sensors have some advantages such as compactness, simple

calibration, and robust operation, as they are easily fitted to bioreactors. Photometric sensors are adequate for monitoring volatile compounds, such as ethanol; and because of vapor–liquid equilibrium their contents in the fermentation broth may be indirectly determined by photometric measurement of the concentrations in the exhaust stream. Beuermann et al. [19] developed an online carbon balance of aerobic yeast fermentation processes using a miniaturized optical photometric IR gas sensor to monitor ethanol and CO_2, and a fiber-optic backscatter setup for direct biomass evaluation. Dietzsch et al. [20] developed a novel online sampling analysis device with an automatic photometric robot for fast bioprocess monitoring of microbial cultures. The photometric device monitored glucose, glycerol, ethanol, acetate, phosphate, and ammonium concentrations during several batch cultivations of *P. pastoris* with no time-consuming recalibration or maintenance work.

23.3.3.2 Calorespirometric Methods

Calorimetry represents an additional method for the online monitoring and controlling of bioprocesses. Noninvasive calorimetric measurements, both in pilot-scale and large-scale bioreactors, may be made by determining the heat transfer coefficient and temperature difference between the inside of the reactor and the representative cooling liquid in the reactor jacket or by assessing the mass flow of the cooling liquid and the temperature difference between the inlet and the outlet of the reactor jacket. Calorimetry can be used to monitor both aerobic and anaerobic processes. A calorespirometric method was proposed [79] for the online detection of microbial lysine formation in a pilot-scale stirred tank reactor by comparing the heat generation of the bacterial strains with that calculated from the oxygen consumption of *Corynebacterium glutamicum* for batch and fed-batch cultures. The results proven that calorespirometry is a viable noninvasive technique to detect product formation during a fermentation process.

23.3.3.3 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a promising analytical tool. Its use for online monitoring of biological systems has been frequently constrained by the high complexity of custom-made NMR bioreactors, considerable costs for high-field NMR instruments, and the undesirable effect of aeration in NMR spectra acquisition [80]. With the aim of reducing instrumentation costs, low-field NMR instruments may provide a cost-efficient alternative. Thus, the use of low-field ^{1}H-NMR spectroscopy for noninvasive online monitoring of two different microbial systems was investigated by Kreyenschulte et al. [80]. For the yeast *Hansenula polymorpha* glycerol consumption could be accurately assessed despite the presence of high amounts of complex constituents in the medium. During cultivation of the fungal strain *Ustilago maydis*, which is accompanied by the formation of several by-products, concentrations of glucose, itaconic acid, and the relative amount of glycolipids could be quantified. The compact design combined with the high temporal resolution of spectra acquisition allowed online

monitoring of the respective processes, which showed that low-field NMR spectroscopy has a great potential for noninvasive online monitoring of biotechnological processes.

23.3.3.4   Biosensors

A biosensor is a self-contained integrated device that contains a biological recognition element (i.e., a biochemical receptor) in direct spatial contact with a transducer element (electrical or optical readout), and which provides through an amplification and signal conversion unit a quantitative or semiquantitative analytical response. Some biosensors can be sterilized by γ-irradiation, which may be an advantage for some applications. Some of these devices have been applied in monitoring strategies of fermentation processes. Biosensor devices have been designed and applied for direct sense key nutrients such as sugars and amino acids, to establish the limits of feasible operating parameters and for the high-throughput design of media [18]. However, as reviewed by Biechele et al. [56] few studies are described in the literature concerning the online monitoring of bioprocesses. Moreover, most enzyme-based biosensors commercially available are used as offline monitoring tools. Genetically encoded biosensors have been applied to sense extrinsic and intrinsic cellular stress, which shows the need to change operating parameters to improve cell health and productivity [18]. Bäcker et al. [16] developed a silicon-based biosensor chip with an integrated microfluidic channel for the simultaneous amperometric detection of glucose, glutamate, and glutamine in a batch hybridoma cell-culture medium. For that, glucose oxidase, glutamate oxidase, and a two-enzyme system made of glutaminase and glutamate oxidase were immobilized in platinum thin-film electrodes. Preliminary results showed the feasibility of the biosensor chip for monitoring the nutrient concentration in fermentation processes, whose performance was improved by coupling an optimized flow-injection analysis system. Schenkmayerová et al. [17] developed a microbial biosensor for 2-phenylethanol based on the bacteria *Gluconobacter oxydans* immobilized in a disposable polyelectrolyte complex gel membrane attached to a miniaturized oxygen electrode. The biosensor was successfully applied for bioprocess offline monitoring, but its application within an online configuration could be foreseen after minor adaptations.

23.3.3.5   Soft Sensors

Sensor or probe technologies have been used extensively for in situ real-time, near−real time, or at-line noninvasive bioreactor monitoring. These methodologies are simple, noninvasive, reusable, easy to maintain for sample source sterility, and stable in the long term [81]. Alternatively, soft sensors have been used to estimate process variables that are difficult to measure in real time or whose measurement by common analytical procedures is tedious and time-consuming. Soft sensors include a measuring device and a software-based estimation algorithm. Data recorded by the sensors from directly measurable variables or more complex multivariable measurements are used by the software algorithm to predict critical process variables that cannot be measured directly. Data-driven or model-driven soft sensors have been described. The first are based on

chemometric models (e.g., PCR, PLS, ANN) established using process data to predict other process variables online. The latter are based on mass and energy balances that describe physical and chemical process principles [44,46,56]. Software sensors based on standard online data and simple mathematical models can be used to monitor different state variables of fermentations processes (e.g., biomass concentration, substrates or products concentrations, specific growth rates, metabolic state of cells, oxygen transfer capacity, and the ratio between oxygen and energy substrate consumption). Model-driven soft sensors may be more adequate than data-driven soft sensors because process changes cannot always be balanced by multivariate data analysis. Moreover, because model-driven soft sensors do not require a training data set, they seem to be useful tools for the development of bioprocesses when no or few prior data are available [45]. In some cases, data-driven and knowledge-based soft sensors may be further fused using Kalman filter approaches [42,56]. Some works have demonstrated the applicability of soft sensors for online monitoring of complex bioprocesses (Fig. 23.2).

Sagmeister et al. [44] proposed a method for the efficient acquisition of reliable information on the physiological boundaries of mixed-feed *E. coli* fed-batch fermentation for the production of heterologous proteins. The methodology combined dynamic pulse and ramp experimentations with soft sensor−assisted control of specific substrate uptake rates based on real-time chemical data (residual substrate concentrations) obtained through an in-line FTIR technique. This same research group [45] used a soft sensor on the basis of a redundant equation system involving the degree of reduction and carbon balance for estimating the biomass concentration in real time, which was further used for closed-loop control of the specific uptake rates in a noninduced *E. coli* decellerostat culture using a mass balance−based control approach. Kana et al. [82] developed Web-enabled software for online and real-time monitoring of anaerobic biogas fermentation for biohydrogen, biomethane, pH, dissolved oxygen, temperature, and conductivity. The
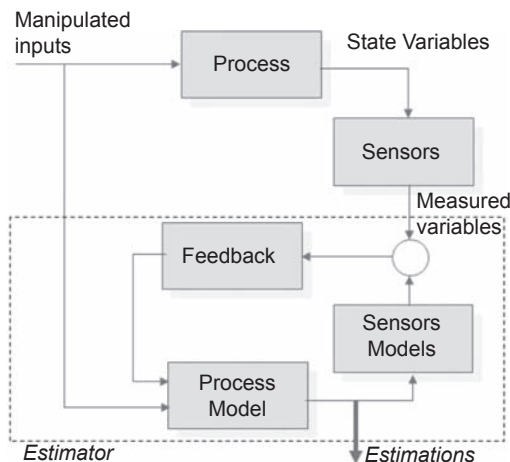

FIGURE 23.2 Schematic view of model-driven software sensor.

soft sensor was further evaluated via the continuous monitoring of dark fermentations for biohydrogen production using anaerobic sludge as inoculums and glucose as substrate. Sharma and Tambe [46] introduced a novel and exclusively data-driven genetic programming (GP) soft sensor approach to evaluate biochemical processes. The GP-based soft sensors were successfully applied to evaluate the extracellular production of lipase enzyme, allowing its prediction of time-dependent activity and the bacterial production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer and enabling determination of its accumulation. Krause et al. [48] presented a self-organizing sensor network capable of overcoming over sensor failures based on the combination of process knowledge and computational efforts. The strategy first uses multivariate linear and nonlinear models to create a search space based on the multisensor data set, aiming to establish simple correlations between raw data and several sensors, which are then applied to extract multivariate statistical process control trajectories. The optimal sensor–model combination is defined by swarm intelligence. The swarm-sensing method showed the ability to achieve robust online monitoring.

23.4 Conclusions and Future Prospects

Several methodologies have been described for monitoring different process variables in several biotechnological processes. Although they fulfill the basic requirements of any analytical tool (e.g., sensitivity, sensibility, repeatability, reproducibility, linearity), offline or even at-line monitoring traditional techniques (e.g., chromatography, electrophoresis, polymerase chain reaction, colorimetric methods) should be avoided because of the inherent risk of contamination caused by any sampling system. Online and in situ or in-line sensing probes should be then used as often as possible. Spectroscopic methods and optical-based sensors have great potential, as described in this chapter, for in situ and online monitoring of different yeast, bacteria, or fungi cultivations in either batch or fed-batch fermentation processes; in some cases, they have been successfully integrated using soft sensor approaches, in process control strategies, increasing bioprocess productivity. Indeed, for many variables and analytes, it is already possible to perform in situ measurements, but a wide range of technical equipment is needed, sometimes involving great complexity and requiring skilled technicians. Therefore, although some novel in-line approaches are reported almost on a daily basis by the scientific community, their applicability in real industrial facilities is still limited. For instance, for industrial in situ applications, the connectivity of the sensor to the bioprocess has shortcomings for many sensor-based technologies. The modular construction of sensor heads and detectors is envisaged by the industry, which require inexpensive sensor heads that could be incorporated several times in high-throughput screening systems or could even be disposable. In fact, innovation in this field is always driven by the requirements of state-of-the-art reactor technologies, which are expected in future to be more diverse than classical stainless-steel fermenters. Despite

the lack of real-time sensors to measure some critical process variables, difficulties in implementing sensor probes in situ/in-line in industrial facilities, or to avoid the time-intensive and expensive reapproval of the process owing to the inclusion of a new sensor technology, the use of soft sensors is seen as a promising and practical alternative strategy. Soft sensors may combine data from different sensor techniques with process knowledge, enabling an estimation of additional variables indirectly, which are hardly or indirectly measurable, including the metabolic activity of cells or growth rates. The main goal is to meet the need for a total process overview, aiming at steadily ongoing improvement of processes by online monitoring.

The utmost benefit of sensor patches is their potential use in disposable bioreactors, a new trend in modern bioprocessing that leads to the concept of single-use/disposable systems. This novel approach will promote the development of new sensor systems or adapter systems that enable the connection of "classical" sensors to single-use reactors. Thus, a new paradigm in the field of in situ and real-time sensor probes for the online monitoring of bioprocesses has emerged. Single-use bioreactors are presterilized, avoiding laborious and time-intensive sterilization and cleaning procedures but requiring a novel sensor philosophy. Conventional sensors are mainly reusable devices for long-term operation and cannot be employed in single-use bioreactors because they are closed and sterile systems. Also, the new single-use sensors for disposable reactor systems must be γ-irradiation sterilizable and precalibrated, may have a shorter lifetime, and need to be installed by the single-use reactor manufacturer. Furthermore, these sensors must be cheap, small, and modular, and if possible should include a standardized sensor port to facilitate the design, fabrication, and application for mechanically stable integration. Alternatively, well-established classical sensors could still be used if they include a novel sensor port or adapter that allows the connection to single-use systems.

Overall, whatever the strategy used for the online analysis of a bioprocess, the availability of in situ or automated at-line analytical techniques is of utmost importance for describing key broth variables. Therefore, new challenges for process monitoring are arising, including: (1) mining large amounts of data generated during a bioprocess to uncover hidden information contained in online signals from different sensor systems and offline data; (2) better models to describe relationships between intracellular properties and extracellular parameters; and (3) the extension of a range of current analytical techniques and the miniaturization of sensor systems.

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