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Measurement, Monitoring, Modelling and Control of Bioprocesses

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In general, volumes are edited by well-known guest editors. The series editor and publisher will, however, always be pleased to receive suggestions and supplementary information. Manuscripts are accepted in English.

In references, Advances in Biochemical Engineering/Biotechnology is abbreviated as *Adv. Biochem. Engin./Biotechnol.* and cited as a journal.

Carl-Fredrik Mandenius
Nigel J. Titchener-Hooker
Editors

Measurement, Monitoring, Modelling and Control of Bioprocesses

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Preface

In 1988, the European Federation of Biotechnology EFB founded a new Working Party with the target to deal with measurement and control of bioprocesses. This was an ambitious move. Now, some two decades later a myriad of new issues within this broad scope have arisen: bioprocess performance monitoring, fault detection, experimental design, modelling of bioreactors and bioprocesses, fighting "data drowning", detection and monitoring of impurities, faster product innovation together with the reconfiguration control and integrated design. There is also a growing need to foster these aspects in teaching and training within the relevant higher education programmes. The M^3C reflects those drivers for measurement, monitoring, modelling and control and is the brand name used by the European Federation of Biotechnology and the European Society of Biochemical Engineering Science. This collection of contributions on M^3C provides an update of a previous volume in this series and represents a state-of-the-art assessment of the different elements of M^3C. It brings together leading academics and industrial practitioners, from global institutions, to provide expert analysis and opinion in a pragmatic and useful format.

(i) The book begins with two chapters which provide overviews of the two original main areas of M^3C: measurement techniques and control methods.

Bernhard Sonnleitner provides an overview of the state-of-the-art of monitoring and measurement devices that are appropriate tools for development and operation of biotechnological production processes. In particular, he describes these tools from a user perspective, i.e. what can be measured and how the tools contribute to the regulatory requirements in industry and, importantly, concludes with what we still are lacking.

Marc Stanke and Bernd Hitzmann continue with an overview of state-of-the-art of control methods currently applied in biochemical engineering. In particular, they highlight closed-loop control techniques to construct hybrid

systems and of applications of soft sensors in combination with PID control. Gerald Striedner and Karl Bayer show how modern M^3C is applied to one of the most pertinent production systems in biotechnology, recombinant proteins. They provide an overview of the complications of monitoring and controlling the intrinsic physiology in *E. coli* when a heterologous protein production is initiated and how the current analytical tools for measurement and monitoring should be employed to carry out the control. They exemplify this with extensive experience from their own laboratory.

Sunil Chhatre develops the theme of ultra scale-down (USD). The material shows how a combination of USD mimics and mathematical models can be used to simulate the behaviour of bioprocesses. This is especially important for chromatographic separations where a range of miniaturised formats now exist. In the second chapter, Chhatre moves on to detail the characteristics of the different available techniques for high throughput chromatography scale down, the benefits and relative advantages.

(ii) The third M in M^3C refers to modelling. The complexity of biological systems brings a very strong motivation of using modelling methods in a production context. Two of the contributions are dedicated to this.

Krist Gernaey and colleagues discuss how mechanistic modelling, in their view, best can use mathematical modelling methods. They provide and exposé of current mechanistic modelling methods under the frame of a well-known process example—Baker's yeast production.

Jarka Glassey reviews the use of statistical methods for the modelling of bioprocesses. She describes the basics of a range chemometric approaches and illustrates the nature of the insights that can be gained from adoption of the non-traditional method of process modelling. In particular, she details the importance of data pre-processing as a step before the construction of statistical models and in so-doing provides a very practical update to this growing area of research and application.

Rui Oliveira and colleagues explore the basic tools for the design of bioprocess monitoring, optimisation and control algorithms that incorporate *a priori* knowledge of metabolic networks. The main advantage is that this ultimately enables the targeting of intracellular control variables such as metabolic reactions or metabolic pathways directly linked with productivity and product quality.

(iii) Process Analytical Technology PAT and Quality by Design (QbD) are crucially important features of the regulatory landscape and this book brings together key contributions to help in the understanding of how best industry can respond to these demands.

Anurag Rathore and colleagues discuss the basis behind Quality by Design (QbD) and also the concept of Process Analytical Technologies (PAT). They present a structured framework for data management and present a series of elegant case histories that demonstrate some of the features necessary for achieving PAT from a regulatory perspective.

Ian Marison and colleagues continue the PAT theme and show how these and other tools can be employed for monitoring and control of bioprocesses where biomass, substrates and products are the main critical process parameters. In particular, they highlight the opportunity of using biocalorimetry as PAT methods with a very interesting potential.

We hope that the content will not only educate, but also provide a valuable resource for practitioners in industry and for academics as we move forward in this rapidly evolving sector of biotechnology. The scope and impact of M^3C technologies are broad and critical. Our vision is that this text will provide a useful marker in time of the relevant developments and the basis for future debate.

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Automated Measurement and Monitoring of Bioprocesses: Key Elements of the M^{3}C Strategy

Bernhard Sonnleitner

Abstract The state-of-routine monitoring items established in the bioprocess industry as well as some important state-of-the-art methods are briefly described and the potential pitfalls discussed. Among those are physical and chemical variables such as temperature, pressure, weight, volume, mass and volumetric flow rates, pH, redox potential, gas partial pressures in the liquid and molar fractions in the gas phase, infrared spectral analysis of the liquid phase, and calorimetry over an entire reactor. Classical as well as new optical versions are addressed. Biomass and bio-activity monitoring (as opposed to "measurement") via turbidity, permittivity, in situ microscopy, and fluorescence are critically analyzed. Some new(er) instrumental analytical tools, interfaced to bioprocesses, are explained. Among those are chromatographic methods, mass spectrometry, flow and sequential injection analyses, field flow fractionation, capillary electrophoresis, and flow cytometry. This chapter surveys the principles of monitoring rather than compiling instruments.

Keywords Bio-activity · Biomass · Chemical variables · Physical variables · Products · Substrates

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1 Introduction

Successful process monitoring and control in industry depends heavily on proper and functioning measurement and monitoring techniques. Especially in the bioprocess industry, the demands on measurements and bioprocess analysis are great challenges for inventors, instrument suppliers, and industrial users for a number of reasons. One reason is that biological systems are very complex from an analytical perspective, with components of varying molecular size and concentration, all in a quite complicated and dynamically changing matrix. Another reason is the fact that most bioprocesses in research or industrial production are operated behind a sterile barrier and must provide some bio-active product with critical quality attributes. This makes the task of quantifying critical process variables directly in or at the process and (almost) in real time more demanding than measuring withdrawn samples in the laboratory. As a consequence, only a few sensors are routinely used and only a few instrumental analytical methods and instruments are adapted to and supported as process analyzers for bioprocess engineering [1–3]. However, the process analytical technology (PAT) initiative and the more recent *Guidance for Industry: Process Validation* of the Food and Drug Administration (FDA, USA) strongly support and favor these developments [4, 5]. This forms a central subset of the M^3C strategy. The papers cited above should be used to find references to older literature.

In the following, some technical terms will be used and may result in some confusion. For instance, the term "online" has recently often been used when results are made available in a short time after sampling and/or on a computer. Here, "online" is used in the sense of "fully automatic," meaning that no manual interaction by an operator is required to provide results on a process computer. "In situ" is used in the sense of "mounted and exposed to a representative volume element *inside*" the bioreactor. "In bypass" is used to indicate that a representative volume flux is withdrawn from the reactor and analyzed aside but close to the reactor. This sample may be returned to the reactor if the analyzer works noninvasively (i.e., does not affect the sample) and does not compromise the sterile barrier; however, the sample must probably be treated in some harmful way before analysis and therefore discarded thereafter. "In real time" is used in the sense that the result is available "quickly enough" that it can be used to affect the process, i.e., to close a control loop, meaning that delay or dead times are reasonably low with respect to the dynamics of a bioprocess. This time horizon is of course shorter for rapidly growing cells than for slowly growing ones; it should be as short as possible in very dynamic situations, for instance, when the physiological status of

a population changes, probably due to an overfeed of substrate. Exhaust gas is usually analyzed in the exiting gas stream, outside the sterile barrier (behind the off-gas filter) since all fermentation setups are open for the gas phase. Technical terms such as "at-line" or "inline" are often used ambiguously and will be avoided in this context.

Many in situ sensors deliver a continuous signal which is often gained in real time and noninvasively—this is the monitoring part—while interfaced analytical subsystems (such as chromatographs) deliver their results discretely and the time steps between available data can be quite long—this is the measurement part. This may be acceptable in slow bioprocesses but poses problems in highly dynamic processes; in such cases, mathematical models are needed. Generally, sensors and instruments with short, constant, and—very importantly—well-known response behavior should be preferred.

Many sensors can be calibrated in usual units. One should take care of the calibration status of the individual sensors, since systematic errors make the results false. However, most of those sensors cannot be calibrated or recalibrated after sterilization. This fact poses extra demands in terms of long-term stability, little drift, and minimized effects due to sterilization.

Several sensors and process analyzers produce "relative" signals rather than crisp concentration values. This is not a priori "bad" but has to be accounted for accordingly. Especially in production processes, during which the design space should not be exceeded, such signals can be compared automatically and in real time with the respective time trajectories of historical (e.g., reference or validation) processes and, thus, give instantaneous information about deviations from expected development, or are useful for fault detection. These relative signals are also quite valuable in R&D environments because they make "additional" information available that would otherwise be skipped: modeling and chemometric techniques can extract "hidden" information from such signals or can reveal otherwise unseen, yet useful correlations. Modeling is an essential element in bioprocess monitoring and state estimation [6–8]; however, this aspect will be left out here since Chaps. 2 and 6 focus on this.

The structure of this chapter tries to depict the present situation in online bioprocess monitoring: The state-of-routine section covers process variables that are generally monitored and often also kept under closed-loop control. This holds also for industrial production processes. Obviously, most of these variables are physical and chemical variables rather than biological ones [9]. The state-of-the-art section addresses all variables that can be accessed online, although they are, with few exceptions, acquired in academic laboratories and in industrial R&D laboratories and pilot plants only. A similar structure was used by Olsson et al. [10], who discussed several advantages and disadvantages of the various methods for academia and industry.

Variables are those properties of the system which vary in time, and whose dynamic properties are therefore determined by the values of the parameters [11]. The *parameters* of a system are those properties which are inherent to the system. What we can measure are the variables of a system; all bioprocesses are multivariate

systems and should, therefore, be monitored using multi-analyzer techniques [12, 13]. We can retrieve the parameters by appropriate evaluation of the variables, which is also known as the inverse problem or system identification problem. The entire chapter will use this definition in spite of the widespread misuse of the term "process parameter" in place of process variable.

2 State of Routine

The variables treated in this section are also quite important for nonbiological or chemical processes; they are not typical of or particular to bioprocesses. However, the respective commercially available sensors are robust, reliable, and quite mature.

2.1 Monitoring Physical Variables

Among these are sensors for agitator speed, aeration flux, weight of vessels, and probably electrical power consumption. They are not critical with respect to contamination risks because all these measurements take place outside the sterile barrier.

Gas flow rates can be measured as volumetric or mass flow rates. The latter are recommended because gas mass flow rates occur in the respective mass balances needed to evaluate process data, e.g., to determine a volumetric or specific oxygen consumption rate. The instruments at hand are thermoanemometers provided by various companies.

Some care must be taken when using balances or scales to determine the weights of vessels and to conclude from these weights the respective filling degrees or changes thereof; liquid feed or harvest mass flow rates can be accurately and precisely derived from the time trajectories. However, a proper force-neutral mechanical setup is required and must not be changed during the entire operation period.

Omnipresent is the monitoring of temperature and, although usually not at the very small scale, pressure. Respective sensors and amplifiers are available from various suppliers. However, these sensors, as well as those discussed in the following, are to be mounted in situ. They must not be prone to drifts or gain changes during sterilization, and their housing, installation method, and sterile sealing are critical with respect to contamination.

2.2 Monitoring Chemical Variables

When moving from physical to chemical variables, the sensors are more typical for biochemical processes, namely those that are specific or selective for protons, dissolved gases, and dissolved redox couples. Several alternative brands are available commercially.

The classical electrodes for pH, redox, and pCO_2 monitoring measure an electrical potential difference and, therefore, require good cabling and amplifiers with high input impedance. The classical electrodes for pO_2 monitoring measure an electrical current which is quite low; therefore, the demands with respect to electrical components are also high. Temperature compensation of the raw signals is often implemented in combination electrodes and the amplifiers. New versions of electrodes based on optical principles do not suffer from these electrical constraints.

2.2.1 pH

Many currently used pH electrodes are constructed from special glass. Today, such electrodes no longer suffer from a significant alkali error, they show very little drift over time, and their lifetime is usually limited by (unintended) mechanical damage. The electrodes are constructed as combination electrodes; i.e., they also contain the reference electrode in a single rod. The reference is usually an Ag/AgCl electrode dipped into a reference electrolyte and must be in electrical contact with the measuring solution. If the reference electrolyte is a low-viscosity liquid, it must be separated from the measuring solution by a diaphragm in order to minimize exchange of the two liquids but satisfy the electrical connection. These diaphragms are built of porous glass or ceramics and can clog, especially when proteins adsorb or salts precipitate. The combination electrode will then drift (significantly) due to the increasing electrical resistance of the diaphragm junction. This is sometimes counterbalanced by pressurization of the reference electrolyte chamber, thus forcing a small electrolyte efflux through and "cleaning" the diaphragm. Newer types of pH electrode have a hydrogel-solidified reference electrolyte and no longer need a diaphragm. Such electrodes are, in our experience, much more stable and show practically no drift over their (mechanically limited) lifetime.

These classical pH electrodes are potentiometric electrodes: they determine a potential difference. Hence, the amplifier must have very high input impedance and the cable connecting the electrode to the amplifier should be as short as possible and must be well shielded. Newer versions have the amplifier [plus some further intelligence such as calibration data, therefore also being called intelligent sensor management (ISM)] integrated on a built-in chip and transmit data in digital form, in some cases also via a wireless path. This is certainly a timely improvement compared with the critical transmission of analog raw data.

Specifically from food biotechnologists came the need to substitute glass—if a glass electrode breaks in a production batch, they had to dump the whole batch; imagine, if you were to eat a yoghurt containing broken glass. Suppliers were able to come up with an alternative, i.e., the proton-selective field-effect transistor (pH-FET), which is a derivative of the usual metal–oxide–semiconductor FET (MOSFET). The gate electrode made, e.g., of Ta_2O_5 is not electrically charged but exposed to the measuring solution, and so controls the current from source to drain.

The pH-FET also needs a reference electrode (with all the pitfalls discussed above) but is constructed from unbreakable material. However, such pH electrodes suffered from substantial drift during sterilization (up to 80 mV, corresponding to >1 pH unit) and have, therefore, been partially taken off the market.

Another valuable alternative is the optical pH sensor, in which single or more mixed and probably fluorescing pH indicator substances are immobilized in a thin polymer film and mounted on a transparent carrier (e.g., a viewing glass or optical window), which is exposed to the measuring solution. Optical fibers connected to the transparent carrier allow excitation of the indicator(s) and reading of the spectrum; depending on the indicator(s) used, the spectral data need to be converted into pH units [14–16]. The indicator(s) must be biocompatible and show sufficient long-term stability, especially against sterilization.

An indirect estimation of pH based on mid-infrared spectroscopy with a standard error of prediction >0.15 pH units was reported by Schenk et al. [17] for microbial cultivations.

2.2.2 Redox Potential

The physical construction of redox electrodes is very similar to the above-mentioned glass pH electrodes. However, the sensing element is a small ring or wire made of noble metal such as Pt or Au instead of the proton-sensitive glass. The noble metals sense the sum of all redox couples that are in solution, and the signal represents an average of all those dissolved components. Most often it is unclear what this means for aerobic cultivations, although sometimes one can apply the interpretation that the signal mirrors "the general availability of electrons" [18]. Although we normally do not know which compounds are captured by this signal, the time trajectory of the redox potential may have a very typical shape. This may allow discrimination between "good" and "bad" when comparing the actual trajectory with historical ones. In some way, the redox potential depicts a typical pattern (or signature) of qualitatively unknown bioreaction components. In anaerobic cultivations, however, the redox signal gives a very good indication of the degree of anaerobicity; strict anaerobes will not grow at redox potentials above approximately −300 mV.

2.2.3 Oxygen Partial Pressure, pO_2

A substantially important variable in aerobic cultivations is the oxygen partial pressure (pO_2, or DOT for dissolved oxygen tension). The availability of oxygen to the cells can be decisive for a range of effects from simple changes in metabolism, to altering the spectrum of (by-)products, to loss of energy and even cell death. Being aware of such effects and knowing the oxygen availability from a sensor signal we can (in principle) steer the development of a bioprocess by applying closed-loop control; the measured variable is the pO_2 (reflecting what the cells

experience), and the manipulated variables may be the stirring power or speed, aeration rate, pressure, or fresh gas composition (or combination thereof).

The classical pO_2 electrodes are membrane-covered amperometric electrodes, so-called Clark(-type) electrodes. The membrane separates the measuring solution from the electrode. The flux of oxygen through the membrane is diffusion controlled but decisive for the sensitivity and response time. This is also why the electrode reflects the partial pressure rather than the dissolved gas concentration. The membrane is also responsible for the selectivity towards oxygen, since it permits only gases and volatiles to diffuse. Membranes are usually made of silicon or Teflon, or are a sandwich of both. The inner electrode consists of an anode made from Ag and a cathode made from Pt, and the potential between them is usually tuned to between 600 and 700 mV, but kept constant. Under these conditions, oxygen is consumed (namely reduced) at the cathode, while four electrons per O_2 come from the anode, which converts to 4 Ag^+ (and finally, in a chloride-containing electrolyte, AgCl, which must be removed from time to time in order to keep the Ag accessible). The electron flux is low (typically in the range of nA) and must be amplified to yield a useful signal. The rate-limiting step is the diffusion through the membrane, which makes the electrode slow at low pO_2.

Optical alternatives have been commercially available for several years. They consist of an immobilized ruthenium or platinum complex that fluoresces in the absence of oxygen; this fluorescence, however, is quenched in the presence of oxygen. Oxygen is not consumed by these sensors, nor are parts of the sensor itself consumed (such as the Ag anode in the amperometric type). Furthermore, there is no more need to change the inner electrolyte of the electrode once in a while and, probably, the patch-holding electrode tip is mechanically more resistant than the membrane and needs less frequent exchange. The very thin patch of the immobilized complex, which must be biocompatible and sterilizable, can be glued onto a (glass) window, a shaking flask, a translucent plastic container, or directly on top of fiber optics; the patches are inside while the optics are outside the sterile barrier [19]. Contrary to the amperometric sensor, the signal is maximal at minimal pO_2, and therefore the signal-to-noise ratio is best under oxygen-limited conditions. We propose to use both amperometric and optical pO_2 sensors at the same time (mounted close to each other) to take full advantage of their different sensitivity regimes [20]. More detailed information about optical sensors can be found in the review by Lam and Kostov [21].

2.2.4 Carbon Dioxide Partial Pressure, pCO_2

pCO_2 electrodes are membrane-covered pH electrodes. The membrane separates the pH electrode from the measuring solution. The flux of carbon dioxide through the membrane is diffusion controlled, as in pO_2 electrodes. CO_2, an acidic gas, dissolves in the inner electrolyte buffer and provokes a pH drop which is detected by the pH electrode, whose signal is proportional to the (decadic) logarithm of pCO_2. This provides an elegant means to estimate the exponential character of a

cultivation at a glance: if the fully growth-associated CO_2 production happens to be exponential, the raw (logarithmic) signal of the pCO_2 electrode develops linearly with time; from its slope, one can directly derive the specific growth rate. The response time of pCO_2 electrodes is usually longer than that of pO_2 or pH electrodes, since the equilibration of the inner electrolyte buffer is slow. With time, this buffer becomes saturated, thus limiting the useful lifetime of the electrode. However, pH measurement is not selective for CO_2. This implies that all acidic or alkaline components that can diffuse through the membrane, e.g., H_2S, formic, acetic acid etc. or NH_3 in alkalophilic cultivations, must also contribute to the final signal; i.e., they exhibit cross-talk.

Of course, optical variants have also been described, even with low cost, for instance by Ge et al. [22]. The correlation between the signal and the dissolved CO_2 is not linear, but stability of at least 10 days is claimed.

2.2.5 Composition of the Gas Phase

Oxygen and carbon dioxide are the noninert components of the gas phase in a great majority of bioprocesses. Various analyzers of exhaust gas are on the market from different suppliers. Depending on the construction of the analyzer (and its price), signals represent the particle density per volume of the measuring cuvette and not a percentage, i.e., the molar ratio times 100: $n/V = p/R/T$.

If the analyzer cuvette (with constant volume) is ventilated against atmosphere, then the signal is directly affected by pressure changes, which must be accounted for. The measuring cuvettes are usually thermostated, so temperature variations are less critical.

Carbon dioxide is a greenhouse gas, and this effect is often exploited to quantify it via its infrared absorption. Water (vapor) is a greenhouse gas and absorbs IR radiation as well. Therefore, water should be eliminated prior to measurement (by a gas cooler and/or dryer) because water is always present in the exhaust gas of a bioprocess.

Oxygen is one of the few paramagnetic gases and certainly the only one which can be tolerated in bioprocesses. The respective analyzers are usually mechanically sensitive to water vapor, and therefore the gas should also be dried before measurement.

The sound velocity in a gas depends on its composition; it is proportional to $M^{-0.5}$, where M is the molar mass of the gas [23]. So, electroacoustic measurements are also valuable alternatives to determine CO_2. Magnetoacoustic instruments are the alternatives for oxygen.

If other gases such as H_2 or CH_4 or volatiles such as alcohols and aldehydes need to be determined too, mass spectrometry is obviously the most usual choice because it is so versatile. A relatively inexpensive instrument with electron impact ionization and a quadrupole mass filter can cover all the relevant gases. Magnetic-field mass filters are probably more stable. If greater sensitivity and excellent dynamics are required, chemical ionization techniques, e.g., PTR-MS instruments, are used [6, 24, and Chapter by Striedener and Bayer].

In most cases, such instruments are shared among several reactors. This is accomplished by multiplexing the analyzer's inlet using revolving selection valves connected to the individual exhaust gases. Up to 256 ports have been reported. It is necessary to purge theses lines well, in order to avoid long dead and delay times. A more complete survey of methods, techniques, and instrumentation is given by Pollard and Christensen [25].

Several interesting variables—from a technical as well as from a physiological point of view—can be derived from exhaust gas analyses. Most important is the physiological variable RQ, the respiratory quotient. It is defined as the quotient of CO_2 production rate over O_2 consumption rate in units of mol mol^{-1} and can be directly derived from gas composition data. In a first approximation, the RQ is the negative slope of the line connecting data points in a phase-plane plot of CO_2 content versus O_2 content (Fig. 1).

Strictly, this is true only if the RQ equals unity. If the RQ deviates from 1, the gas mass flow rates of fresh and exhaust gas differ; rather than measuring each flow rate individually, one can assume that all gas components except O_2 and CO_2 are inert (if appropriate) and calculate the exhaust gas flux from the respective inert gas balance and, after rearrangement, the RQ as

$$RQ = \frac{y_{CO_2}^{out} - y_{CO_2}^{in} - y_{O_2}^{in}\, y_{CO_2}^{out} + y_{O_2}^{out} y_{CO_2}^{in}}{y_{O_2}^{in} - y_{O_2}^{out} - y_{O_2}^{in}\, y_{CO_2}^{out} + y_{O_2}^{out}\, y_{CO_2}^{in}},$$

where y denotes the molar fractions of O_2 and CO_2 in the fresh (superscript "in") and exhaust (superscript "out") gas, respectively. Care must be taken that the gas analyzers are well calibrated because, otherwise, the quadratic terms (coming from the inert gas balance) can lead to dramatic error propagation. If gases other than O_2 and CO_2 are involved in the bioreaction, the term "inert" needs to be redefined appropriately of course.

Yet another error can be caused by chemisorption of CO_2 to HCO_3^- or even CO_3^{2-}, at neutral pH but specifically under alkaline process conditions. This reaction is almost negligible under acidic conditions, e.g., for cultivation of yeasts, fungi or lactic acid bacteria.

Knowing one gas mass flow rate explicitly, one can calculate the oxygen consumption (or uptake) rate (OUR) and the carbon dioxide production rate (CPR) of the entire plant from the gas analysis data. Further knowing also the working volume of the liquid phase, the respective gas transfer rates can be deduced and, knowing the biomass concentration as well, the specific gas turnover rates (q_{O2} and q_{CO2}) are easily calculated.

3 State-of-the-Art: Biomass, Substrates, and Products

Although the biomass is the (most) crucial variable in bioprocesses, online monitoring does not seem to be accepted as a general standard. The problem is posed by the interferences that are captured by many online techniques. The respective results

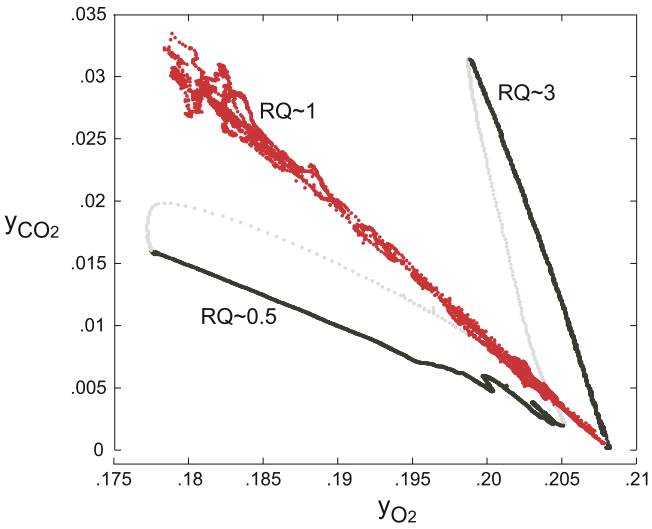

Fig. 1 Phase-plane plot of CO_2 versus O_2 content (as molar fraction, *y*) in the exhaust gas during a batch and a consecutive fed-batch cultivation of *Saccharomyces cerevisiae*. The time information is lost in a phase-plane plot. Three typical "straight lines"—almost linear domains—are visible, and the negative slopes estimate the RQ: the steepest domain was observed during oxido-reductive batch growth on glucose with concomitant ethanol excretion (RQ $\approx$ 3), the flattest domain characterizes the consecutive growth of the cells on ethanol (RQ $\approx$ 0.5), and the middle domain was recorded during the controlled glucose feed phase in the final fed-batch (RQ $\approx$ 1). Linear regression of the data domains must be applied with the objective of minimizing the orthogonal distances of data points from the regression line (where transition phases should be omitted since the RQ changes dynamically)

are often not trusted, even though good coincidence between actual but relative data with those of approved reference cultivations should be sufficient indication of good reproducibility (of the process). Obviously, many people rely on offline reference methods only. Madrid and Felice [26] compiled a broad comparison of all those methods and differentiated nicely what a method can and cannot detect.

3.1 Biomass

To my knowledge, no fully automatic version of the SOP for manual determination of cell dry weight has been commercialized to date. The sensors and instruments useful to quantify biomass are either indirect methods—and deliver estimates—or methods implementing counting of suspended particles—and these deliver a cell number concentration rather than a cell mass concentration.

3.1.1 Optical Density

Various sensors that measure the turbidity of a suspension have been commercially available for many years. Their major drawback is that they also "see" gas bubbles and particles as well as cells, e.g., precipitates in the suspension or particulate medium components. There are a few tricks reported to minimize the effect of gas bubbles: one can capture a volume aliquot in the optical path from time to time, let it degas through a vent hole, read the absorbance, and open the optical path again for fresh suspension to enter (Foxboro Cerex). Older instruments sucked a suspension aliquot out of the reactor and, after reading, pushed it back; however, contamination problems occurred (Fundalux). One can also protect the optical path from gas bubbles by surrounding this space (at the tip of the sensor) with a fine grid, the mesh size of which permits cells to pass but gas bubbles to be excluded (Komatsugawa). These sensors are also sensitive to the absorption of light due to the color of the medium. To circumvent this effect, most instruments exploit light sources (nowadays LEDs or lasers) with wavelengths in the near-infrared instead of visible range. Some sensors determine the attenuation of light in the forward direction (turbidimetry), while others measure light scattered at a 90° angle (nephelometry) and others the reflected light. Some prototypes may also analyze a combination of these. None of these sensors can discriminate between live and active versus dead and inactive cells or abiotic particles. The control goal to keep the optical density of a continuous culture constant is called a "turbidostat"; i.e., the *turbid*ity is forced to be pseud*ostat*ic.

3.1.2 Impedance (Permittivity)

Another type of sensor is based on the fact that all intact cells are enclosed by a cytoplasmic membrane which functions somehow as an electrical insulator. If the cells are exposed to an alternating electrical field, the charged species inside the cells are attracted to one or the other side of the field, but they cannot leave the cells. This mirrors an electrical capacitor, where the capacitance depends on the size and the contents of the membrane-enclosed volume and the frequency of the electrical field. Furthermore, the capacitance can be "short-circuited" by the conductivity (like a parallel resistor) of the medium (i.e., the freely moving charged species not enclosed by a membrane). The breakdown of the impedance ΔC (also known as β-dispersion) provides an estimate of the cellular volume, and the critical frequency at which this breakdown occurs provides an estimate of the size of the cells. Nowadays, there are instruments on the market that can resolve (or scan) a range of frequencies (from approximately 100 KHz to 20 MHz) and so deliver dielectric spectroscopy information. This method usually works better with large cells, for instance, CHO cell lines (e.g., [27]). It reaches its operating limits when the conductivity of the medium is high (or rapidly changing); changes in aeration also affect the signal. However, it estimates the intact biomass volume. Sarrafzadeh et al. [28, 29] were able to distinguish quite clearly between growing

and sporulating cells as well as mature spores of *Bacillus* during endotoxin production. Maskow et al. [30] monitored lipid storage in yeasts using this technique (scanning for the critical frequency). The operating scheme that keeps this signal (the so-called permittivity) constant in continuous culture mode is known as a permittistat [31].

Common to all these sensor types is that they need to be calibrated specifically for the biological system under investigation against at least one reference gold standard: both optical and electrical properties differ between biosystems.

3.1.3 In Situ Microscopy

One such gold standard, especially in animal cell cultivation, is the determination of cell number concentration. A manual procedure involves a desktop microscope, yet special microscopes have also been mounted and used in situ and fully automatically; actually, the objective, illumination source (e.g., a LED), and probably some mechanical actuators are inside the sterile barrier, and can be sterilized in situ, whereas the rest, including the camera and computer, are of course outside. The sample zone (in front of the objective) is on the order of 50 μm (and can probably be adjusted to obtain "optical dilution" to adapt the method to the actual cell density) for the observation of animal cells; the sample zone is usually open and thus permits the acquisition of sequences of images (with exposure times of 1 ms or less). Microbubbles can be quite easily detected by image evaluation software due to their size and almost perfectly spherical shape; because they occupy part of the measured volume, this must be taken into account during calculation of the cell number concentration.

Bluma et al. [32], Hoepfner et al. [33], Rudolph et al. [34], and Ulber et al. ([35] including other optical sensors) have compiled comprehensive surveys of the in situ instruments in use and also versions in bypass interfaced to the process via a flow injection system. Recently, they reported the use of in situ microscopy also for the monitoring of processes with immobilized enzymes and two liquid phases [36].

3.1.4 Online Flow Cytometry

Another technique (being more powerful than just counting; see Sect. 3.2.5) is counting cells with a flow cytometer (FCM). Such an instrument must be linked to the bioprocess via an interface (see Sect. 3.2.4) that is at least able to dilute the suspension (see also Sect. 3.3.1) and, probably, to stain the cells too. The first group to employ such a setup used a degassing unit in bypass to the reactor and, further, a sequential injection analysis (SIA)-type interface to first stain the cells, wash them in a membrane-covered microchamber, and eventually feed them to the flow cytometer [37, 38]. The microchamber was also used to dilute the cells appropriately in order to achieve a cell density of approximately 10^6 ml^{-1} (or less) for the FCM measurement. Around 10^5 events were then evaluated at an event rate

of 10^3 s^{-1}. The online counts were found to be "extremely accurate" for dilute suspensions (i.e., $\leq 10^7$ cells ml^{-1}) with respect to the offline gold standard; however, accuracy was limited due to errors caused by the dilution, which has to be increased with increasing cell density during the batch cultivation. Kinetics and growth dynamics could be derived from these datasets with high resolution (ca. every 15 min [39]). The FCM-determined cell number concentration has also been used to control continuous cultivation, in a so-called cytostat, in which the cell number concentration is the time-invariant variable in steady state [40].

3.2 Bio-Activity

As stated above, the biomass concentration is a variable of great importance. To be exact, it is the mass of living and metabolically active cells that has to be targeted, because this represents the amount of available biocatalyst in kinetic and balance equations.

3.2.1 Calorimetry

Calorimetry is a relatively simple method to derive information about the activity of a population, yet it is very seldom exploited. It has long been known that the specific heat production rate is closely—and almost constantly—correlated with the specific oxygen uptake rate; this is also known as the oxocaloric yield ($Y_{Q/O2}$), having a value of around 450 kJ mol^{-1} [41, 42]. The signals necessary to calculate the heat flux are usually available but not evaluated: in most bioreactors, the temperature of the biosuspension is well known because a temperature controller is employed; such controllers are often cascaded controllers which also require the temperature of the coolant as an input. If only the flow rate of the coolant is known (or at least kept constant) too, one can simply derive the heat flux from these data. The larger the scale of the reactor, the less important are errors added due to heat transfer to the ambient (i.e., the "more adiabatic" the reactor), but the technique is also reported for bench-scale bioreactors, with sensitivity as low as 50 mW l^{-1} [43]. This provides a simple, heat-based biomass and specific growth rate estimator for microbial cultures [44]. Of course, there are some systematic disturbances included in this result such as mechanical power dissipation to the liquid, friction converted to heat in bearings and sealing, or the evaporation loss of heat when dry air is used to aerate a fermenter. However, all these effects can be quantified in abiotic control experiments and, later, accounted for in the real fermentation processes (e.g., [45, 46]). Since this technique balances the entire reactor, one gets a direct estimate for the performance of a production lot.

3.2.2 Gas Balancing Techniques

A very similar situation holds for the gas balancing techniques. Gas analysis is quite straightforward (see Sect. 2.2.5), and the aeration rate is usually also well known. A simple gas balance over the reactor yields quantitative gas consumption and production rates (independent of the oxocaloric information). Knowing the working volume of the reactor as well allows one to derive the gas transfer rates that a reactor is able to achieve under given operating conditions very accurately, precisely, and simply. Since these all represent rate information, one can also deduce the specific growth rate of a growing culture from these signals (or soft sensors): provided the CO_2 production is strictly growth associated—an assumption that very often holds true—and chemisorption of CO_2 is not a great problem (due to a nonalkaline and well-controlled pH value), a linear regression of the natural logarithm of the rate versus time reveals the specific growth rate as the slope. All these calculations are simple arithmetic and should be implemented on process controllers or computers—for the time being, this information source is left unexploited (exceptions may confirm this rule). We also recommend integrating the CO_2 production rate over time (numerically) in order to calculate the carbon recovery in every bioprocess, provided the amounts or concentrations of other "important" carbon-containing reactants are known. If the carbon recovery does not match 100 ± a few percent, then there are actually "important" contributors (substrates or byproducts) that are simply not known; this fact—by itself—is "important," as it reveals a poor understanding of the process which, according to the PAT initiative, should be urgently overcome.

Gas, redox, charge, elemental, and proton balancing methods have been exploited for online data reconciliation, along with predictions derived from online infrared and dielectric spectroscopic datasets and offline calibrated models [47]. The data reconciliation algorithm could be directly implemented into the prediction algorithms of the online spectrometers.

3.2.3 In Situ Fluorescence Monitoring

Fluorescence monitoring at a distinct wavelength (couple: one specific excitation and one specific emission) or with scanning extensions (so-called two-dimensional fluorescence monitoring) is of course less expensive than FCM but yields a population average only. For many purposes, this may be sufficient and desirable, as one can follow growth, product formation, or physiological changes of a population extremely rapidly and noninvasively. The interpretation of the resulting signals deserves some care, however, as illustrated by the disaster of one pioneering fluorescence sensor, built specifically for NAD(P)H (both components that are essential for every living cell); however, the conclusion (and marketing argument) that such a sensor would monitor the biomass concentration was false. Indeed, every living cell contains NADH and NADPH, but depending on the balance state of growth, the ratio between the reduced and oxidized forms can

change very rapidly, in seconds to minutes for the entire population in a bioreactor. Since only the reduced forms fluoresce, the sensor signal reflects the (inner) redox state of a population rather than the biomass concentration. If this fact is not absolutely clear, the user of this sensor will eventually be completely misled. The commercial solution to this dilemma was withdrawal of the sensor from the market.

Meanwhile, there are other commercially available sensors that allow the monitoring of other fluorophores inside the cells, for instance, pyridoxins, flavines, or various fluorescent proteins [48–51]. Skibsted et al. [52] scanned *P. fluorescens* cultivations (270–550 nm excitation, 310–590 nm emission) and related the spectral information to other process variables using partial least-squares regression models. From that, they concluded that this sensor could also predict—with the model, of course—the states of nonfluorescent compounds such as nitrate and succinate. Haack et al. [53] estimated the cell mass concentration of baker's yeast grown in a defined medium from 2D fluorescence spectra including tryptophan, NAD(P)H, and riboflavin using a chemometric model. The method was also reported to be used online during downstream processing [54].

Since the in situ sensors always pick up the excitation light as scattered (or reflected) light, it is worth considering the use of fluorescent reporter proteins that have a maximal separation between excitation and emission wavelength, for instance, wild-type GFP (395/509 nm) rather than the enhanced variant (eGFP: 484/507 nm), in order to achieve a reasonable separation of the desired signal from the unwanted stray light band, a notorious disturbance.

3.2.4 Interfacing Axenic Bioprocesses to Process Analyzers

The techniques described thus far either exploit (heat or mass) balancing techniques or consist of in situ sensors that tolerate sterilization. Hence, no problem of compromising the sterile barrier is to be expected. The use of process analyzers, however, requires an interface to the process that must—in most cases of scientifically and industrially exploited processes with pure cultures—secure the axenic state of the process. In many cases, this interface will also have the task of defoaming and degassing the sample aliquot removed to feed the process analyzer, simply because this is practically always a volumetric dosage. Defoaming and degassing require some nonnegligible hydraulic residence time, independent of whether a small settler (the sample being sucked out of its core volume after an appropriate batch time for degassing) or a gas-permeable membrane (over which the sample flows) is used. An extremely decisive aspect is the transport of the sample through the interface into the process analyzer: the biocatalysts will continue to metabolize en route and can so falsify the results obtained after the finite transfer time, even if the analyzer is error free. In this context, the use of an autosampler (and a multifunctional analyzer) is questionable, since the connection to the bioprocess is quite long (see, e.g., [55], where the 1-mm capillary is 3 m long).

The simplest way to acquire a (continuous or—not recommended—intermittent) sample stream from a reactor is by using a one-way pump. If a sample shall be taken at discrete time intervals, a sampling valve will be the right choice; this can work without a pump if the reactor is (slightly) pressurized. If a valve is employed, this should (a) have a minimized dead volume, (b) seal at the reactor wall or reach into a turbulent reactor zone, (c) have a connection to a steam line for cleaning and hygienizing the valve's dead volume before and/or after sampling, and (d) probably have a connection to a sterile air line for drying after cleaning; these extra efforts help to avoid nonrepresentative results, carryover, and dilution of samples. Cells are not affected by this type of sample acquisition as long as the time required is (very) short, e.g., between a few 100 ms and a few seconds, depending on the cell density and specific substrate consumption rates. If it takes longer, substrates and, most probably first, oxygen will become limiting. However, cell properties can be determined in samples prepared in this way.

If, however, one is not interested in cell properties but in concentrations of dissolved reactants only, the option is either to remove or inactivate the cells during sample acquisition, in which case (micro)filtration, poisoning, heating or cooling right at the exit of the reactor can be technical solutions. Filters can be mounted in situ or in bypass. The in situ version needs a proper mount point in a turbulent space of the reactor in order to avoid fouling or even clogging of the filter (e.g., [56]). The bypass version has the advantage that the filter can be changed during cultivation, if necessary, but it has the disadvantage that the mean residence time of cells in contact with the liquid outside the (well-mixed) reactor is nonzero, thus giving rise to falsification of the subsequent analyses. A promising alternative—and one that is easy to miniaturize—could be dielectrophoretic cell retention, as described by Gastrock et al. [57].

Poisoning the removed biosuspension with cyanide or azide (or any other appropriate agent) in a double-lumen catheter stops only a portion of the metabolic actions of the cells: they are not completely inactivated, and one has to choose the poison appropriately. Heating will destroy all thermolabile molecules, including intracellular biomolecules (certainly most enzymes) and probably also extracellular components one might be interested in. Cooling is usually restricted by the solidification of the suspension at something like 2 °C, which might not be low enough to arrest significant metabolic reactions that could introduce systematic errors. If the suspension is mixed with prechilled solvent, such as methanol at –40 °C, one can reach much lower temperatures, albeit at the expense of dilution and probably extraction of intracellular components into the aqueous liquid.

3.2.5 Online Flow Cytometry

Flow cytometry (FCM) has only very recently been connected to bioprocesses in a fully automated manner [37, 38, 58]. FCM is the only technique that permits analysis of populations from a bioprocess at the single cell level [59]. Depending on the staining method applied, one can, therefore, evaluate what percentage of a

population is, e.g., highly active, what percentage is dormant but functional, and what percentage is dead. Viability probes can be selected from a wide variety of commercially available fluorescent dyes, depending on the instrument equipment available (lasers, filters, and detectors) and the focus of the analysis, for instance, membrane integrity, intracellular enzymatic activity, uptake of substrates, or conversion of substrates [60, 61]. Part of the dyes is membrane permeant and another part is membrane impermeant, which can be exploited for differentiation. Multiple staining could be used to monitor cell physiological responses of bacterial batch cultures and provided important physiological information at the single cell level [62].

An alternative is the exploitation of fluorescent reporter molecules produced by the cells, e.g., green fluorescent protein (GFP). For instance, this can be linked to a protein of interest and used to indirectly monitor the cell-specific production of the target product, as long as this is associated with the cells (intracellular or periplasmic). The method may involve a fusion protein [51] or linkage by an internal ribosome entry site [63]. Using online FCM, Broger et al. [58] found that, in a *Pichia pastoris* cultivation, only 75 % of the cells growing in the population were able to produce, with widely varying titers, whereas 25 % of all cells were absolute nonproducers. Such distinction at the single cell level and thus quantitative characterization of the performance of the production strain cannot be acquired with any other technique established to analyze products in a population-averaging manner (e.g., gel electrophoresis, blotting, ELISA, chromatography, mass spectrometry, etc.) nor with in situ online fluorescence sensors.

Cell cycle and synchronization studies are predestined to use FCM (e.g., [64, 65]): a great variety of nucleic acid (both DNA and RNA) dyes are readily available nowadays. They also permit quite sensitive analysis for contaminants accumulating in a bioprocess [66], specifically because several tens or hundreds of thousands of events can be screened in a short time.

Besides fluorescence events, FCM also detects light scattering events. The small-angle forward-scattered light is linked to the size of a particle, whereas side-scattered light is linked to the granularity or intracellular structure of a cell. Both characteristic properties may undergo significant changes during a bioprocess. A review of recent developments, including online applications of FCM, is given by Diaz et al. [67], highlighting features such as obtaining population distribution information at the single cell level, validating more accurate kinetic models, and using the data for process control and optimization.

3.2.6 Sizing of Particles and Solutes

After a decade or so of dormancy, field flow fractionation (FFF) techniques and apparatuses recently appeared on the market. However, FFF is mostly used in laboratories, not online during processes, although this may only be a matter of time. FFF is an elution/separation technique suitable for molecules with molecular weight of approximately >1,000 Da up to particle size of some 100 μm. A separating

external field force is applied perpendicular to a laminar liquid flow of sample in a carrier, forcing all analytes towards the "accumulation wall" of the flow channel and so causing different species to be placed in different stream lines. Small molecules and particles (less than ~ 1 μm) are separated due to their different diffusivities: the larger ones are less mobile and remain longer near to the accumulation wall, where the flow velocity is smallest; hence, they are eluted later—"normal mode". Particles larger than ~ 1 μm are, for geometric reasons, permanently exposed to the stream lines of higher velocity and therefore eluted more quickly—"steric mode". Useful fields are gravity, temperature, cross flow, and electrical charge (among others). The range of the (molecular) size of the analytes covered by FFF usually exceeds that which can be determined by classical laboratory analytical methods such as size-exclusion chromatography in a single run. Reports on investigated substances are widespread and cover applications such as the separation and characterization of proteins and enzymes, of viruses and mammalian cells, isolation of plasmid DNA, or the molecular weight and particle size distribution of polymers. Langwost et al. [68] have provided a comprehensive survey of various applications in biomonitoring. Although not online, Hawe et al. [69] investigated the aggregation of IgG with an asymmetrical-flow FFF.

3.3 Components Dissolved in the Medium

The biocatalysts consume substrates dissolved in the medium and produce products, many of which are excreted in soluble form into the medium. The masses or concentrations of these components are important state variables and need to be known. For many solutes online methods are available, as discussed in the following; however, the methods for estimating recombinant proteins are currently laboratory techniques that are not fully automatically coupled to the processes [70]; important exceptions are—at least potentially—fluorescence-tagged proteins [51, 63, 71–74].

3.3.1 Flow Injection Analysis

Flow injection analysis (FIA) is an extremely versatile tool to (a) automate (bio)chemical analyses, (b) combine various chemical or physical reaction steps into a sequence, and (c) dilute samples appropriately. Any kind of appropriate detector can be implemented at the end of the line. The automation may produce systematic errors if calibration is done improperly and/or recalibration is omitted. Frequent recalibration may be necessary if peristaltic pumps are used. FIA systems are usually constructed as mini- or even microfluidic assemblies, and all the difficult problems associated with this technology apply for FIA as well, specifically the formation and entrapment of gas bubbles in the system.

FIA was originally defined as "… information gathering from a concentration gradient formed from an injected, well-defined zone of a fluid, dispersed into a continuous unsegmented stream of a carrier …" [75]. Accordingly, basic components of any FIA equipment are a transport system consisting of tubing, pumps, valves, and a carrier stream into which a system (practically always an injection valve attached to an injection loop of defined, constant volume) injects a sample. Rehbock et al. [76] assign FIA a major key role in bioprocess monitoring. In contrast, a sequential injection analysis (SIA) system does not work continuously, since it must aspirate the necessary components sequentially and ejects them afterwards, and the flow may be segmented; however, this setup can save reagents and reduce waste. The principles are briefly sketched in Fig. 2.

A central element in the FIA is the injection valve used to load an injection loop (position as shown: sample is pumped through the loop and further to waste) and then expel this defined volume with carrier fluid after switching of the valve. The sample can be diluted (not shown) and mixed with reagent(s). The mixture is allowed to react for a time given by the flow rate and the volume of the residence time reactor, usually a long, thin tube (tubular microreactor), and finally directed to a flowthrough detector.

The SIA works with a precise bidirectional pump, usually a syringe pump. It first aspirates an appropriate amount of the sample and—in sequence, using the selection valve—the necessary reagents (in appropriate amounts), which are then expelled into a (micro)reactor to be mixed and react. Thereafter, the reaction mixture is pumped to a detector.

A fascinating property of FIA is the elegant implementation of the dilution step (Fig. 3). This is highly desirable for monitoring batch and probably also fed-batch processes: some components change their concentrations more than a few decades during a cultivation, but good resolution at low concentrations (e.g., while a substrate is being limiting) is highly desirable.

Whenever injection loops are implemented, it is mandatory to avoid the occurrence of gas bubbles in the sample stream, since they falsify the volumetric dosage. This error would, of course, be propagated by the dilution factor. Degassing vessels (i.e., sedimenters for the liquid phase) are simple to make but add a mixing device upstream of the dilution and/or analysis, whereas degassing membranes may not be as effective but they do not mix (they just disperse axially).

Downstream of the dilution section everything can be arranged to work in continuous flow mode, e.g., adding reagents ahead of static mixers, "inactive" tubes to achieve a necessary hydraulic residence time, flowthrough cartridges hosting immobilized (bio)catalysts, membrane reactors retaining cells but allowing to wash them, etc. Finally, there is a detector, appropriately selected for the final product to be quantified. Optical and electrochemical detectors qualify well, and biosensors are equally useful in this position since they need not be sterilized there [77]. However, any other instrument is welcome if appropriate: as described in Sect. 3.2.5, a flow cytometer is the final detector and certainly much more expensive than the preceding FIA. Vojinovic et al. [78] have compared several

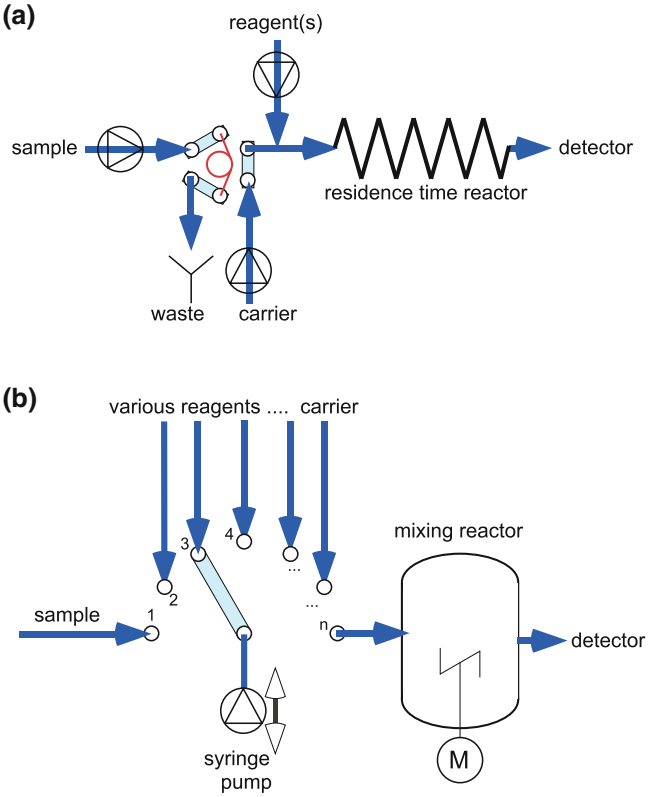

Fig. 2 Minimal schematic sketch of (**a**) flow injection analysis (FIA) and (**b**) sequential injection analysis (SIA) setups. Many extensions to these very basic setups are conceivable, such as a dilution section (Fig. 3)

variants of flow analytical approaches: continuous flow, segmented flow, flow injection, segmented injection, and bead injection.

Almeida et al. [79] exploited several FIA options for online analysis of cutinase activity in a single setup: sampling, dilution, clarification by a micro- or ultrafilter, and reaction. Bracewell et al. [80] linked an optical biosensor (dual channel to compensate for unspecific reactions) with an FIA to the bioprocess. Haouz and Stieg [81] reported the use of copolymerized glucose oxidase and lactate oxidase for simultaneous quantification of glucose and lactic acid on top of an oxygen electrode mounted in an FIA system, which could be used continuously for up to 3 months. With an FIA system containing enzymes immobilized in highly porous agarose, Nandakumar et al. [82] monitored intracellular β-galactosidase (as a reporter enzyme for cellular activities) with a cycle time of 13 min, and glucose plus cell density with a cycle time of 3 min.

An SIA was employed by Horstkotte et al. [83] for monitoring of sorbitol in mixed feed cultivations of *Pichia*. No crosstalk of methanol was observed, and the achievable frequency was quite high at 17 analyses per hour. An interesting trick

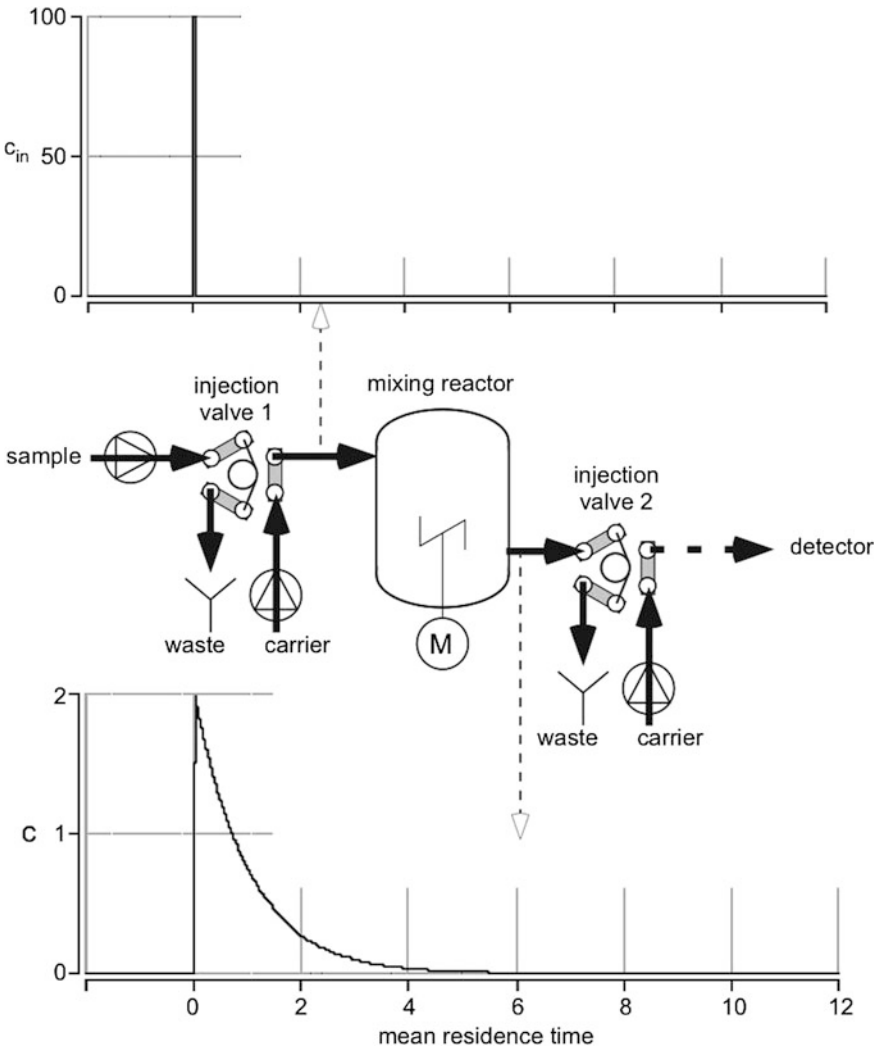

Fig. 3 Schematic of the dilution setup in an FIA: injection valve 1 is shown in the loop loading position. When it is switched (by a 60° rotation), the reactor is fed with the contents of the loop first and then with carrier fluid only (upper time course of c_{in}). Given that the reactor behaves as an ideally mixed continuous stirred tank, the concentration at the outlet (equal to that loaded into injection valve 2) follows the trajectory shown at the bottom for a ratio of loop volume to reactor volume of 0.02; in other words, a thousand-fold dilution can be achieved after roughly three mean residence times. It is a matter of timing when injection valve 2 is switched to determine the desired dilution used further downstream in the FIA

to eliminate gas bubbles, namely repetitive flushing with small volumes of acetonitrile, was applied. Sohn et al. [84] packed immobilized enzymes into an SIA, considering further miniaturization on microfluidic chips. In this work, glucose

oxidase and lactate dehydrogenase were immobilized onto magnetic nanoparticles, which were held in place with a permanent magnet.

3.3.2 Chromatography (GC and LC)

Either gas or liquid chromatographs (GC or LC) are standard equipment in analytical laboratories. However, process samples can be directly transferred to chromatographs provided that an appropriate interface makes the link, i.e., one that is safe for the process in not compromising its sterile barrier and safe for the instrument by preparing the sample accordingly, e.g., filtering cells off, diluting, or even derivatizing. Again, an FIA is a good choice here. A closer look at the operation of a chromatograph makes this clear: it is a special case of FIA, since there is a continuous, unsegmented flow of an inert carrier, either gas or liquid, into which a sample is injected; downstream of the injection, the only "reaction" that takes place is the separation of sample components according to the properties of the selected chromatographic column and carrier fluid. The final detector may be a simple flame ionization detector (FID) or a mass spectrometer (LC–MS), depending on the problem to be solved.

A gas chromatograph is certainly useful to separate gases or volatile compounds, taken either from the exhaust gas—which is least demanding for the interface—or from the culture supernatant—which requires an interface capable of at least filtering and degassing. Injection valves or pistons with a metering groove are more convenient than syringes. An interesting example is the combination of gas chromatography with pyrolytic methylation as the sample preparation procedure for determination of dihydroxyacetone [85].

Liquid chromatographs are used to analyze sugars, amino acids, and even proteins such as vaccines [86].

A recent report described automatic analysis of extracellular glucose, ethanol, glycerol, and acetic acid in 5-min intervals, with good consistency for three comparable batch cultivations of *S. cerevisiae* [87]. Warth et al. [88] reported use of online HPLC data to calculate volumetric and specific reaction rates (which are themselves, then, software sensors). Cos et al. [89] reviewed the importance of monitoring and control of methanol concentration during the induction and feed phase in *P. pastoris* cultivation, which should not be too low and be kept constant for the production of foreign proteins. Even very complex and demanding setups have been reported for online monitoring of starting materials and intermediates in bioprocesses, e.g., HPLC with diode array detection coupled to an electrospray tandem mass spectrometer (ESI–MS/MS [90]).

3.3.3 Capillary Electrophoresis

Capillary electrophoresis (CE) seems to be developing rapidly along with chromatographic methods because it can provide higher resolution and higher speed, works with smaller samples, and needs less carrier liquid (buffer). It is more

suitable for miniaturization using microfluidic components compared with chromatography (e.g., [91]). There is also no need for probably tedious column equilibration, and the columns themselves are less expensive. Sample preparation can be omitted, as shown during degradation studies of phenols with *Rhodococcus* [92]. However, monitoring of organic acids from lactic acid bacteria had to use a membrane interface to the bioreactor prior to loading the samples into CE [93]; the time resolution could be reduced to 2 min in this case.

CE comprises a system in which fluids are transported (by either micropumps or air pressure, or electroosmotically) while separation of dissolved components takes place in an electrical field. Since this ranges into the 10-kV domain, the materials used for construction must sustain this high voltage: good electrical insulators are needed, and PEEK, PTFE or glass are materials of choice.

The method is versatile and enables the separation of small ionic species (e.g., trace elements [94]) over organic acids up to (charged) biopolymers. The variability (or heterogeneity) of the substances that can be separated in one run is usually broader than in chromatographic techniques. Applicable detectors are either electrochemical or optical, e.g., UV/Vis or laser-induced fluorescence detectors. The degree of downsizing (miniaturization) is nowadays limited by the sensitivity of the detectors.

3.3.4 Mass Spectrometry

Mass spectrometers may be the detectors at the end of the analytical line (for instance as a LC–MS chain) or may be the analytical instrument of choice per se.

If more than two gasses, or gasses other than O_2 and CO_2, should be analyzed, or if one needs to follow aroma components or simply volatiles, a mass spectrometer would be the ideal instrument (see also Sect. 2.2.5). A capillary inlet is appropriate for this goal. A mass spectrometer is essential even for CO_2 (and other gases or volatiles) when its isotopic distribution is of interest; for instance, in the case of tracing the fate of a ^{13}C-labeled substrate, the mass spectrometer can distinguish between $^{12}CO_2$ and $^{13}CO_2$, whereas other instruments cannot. This may be of significant importance in metabolomic, regulomic, or fluxomic studies [95, 96].

Sampling directly from the gassed biosuspension is possible and has been done using membrane interfaces in which the membrane serves as (a) a sterile barrier, (b) a pressure barrier, and (c) a selective gate to avoid or permit entry of various species. However, one has to order a new mass spectrometer (at least the high-vacuum parts) if the membrane happens to fail, which is not attractive for production facilities. Chemical ionization MS (CIMS) was used by Custer et al. [24] to follow many volatiles produced during a *B. subtilis* cultivation, namely acetaldehyde, ethanol, acetone, butanol, acetoin, diacetyl, and isoprene, with simultaneous gas chromatography as reference. This technique is also being applied in the food industry, for instance, laser-ionization time-of-flight mass spectroscopy in

very rapid coffee roasting processes [97] or proton transfer reaction mass spectroscopy (PTR-MS) for analysis of odorous volatile organic compounds [98].

Matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-tof–MS) has even been reported as "intact cell mass spectrometry, ICM," being not online but "rapid" [99]. With rapid cell pretreatment, viability could be estimated from mass spectral data, and discrimination among cell lines with different recombinant proteins or different productivities could be achieved.

3.3.5 Infrared Spectroscopy

Most molecules can be excited with infrared light to perform typical molecular vibrations. This is exploited in infrared (IR) spectroscopy. The far IR is not very informative since water—the solvent of most bioprocesses—absorbs massively in this range and most of the primary or fundamental molecular vibrations are excited in the mid-infrared (MIR), which lies in the wavenumber range from 200 to 4,000 cm^{-1}. The respective absorption bands are relatively narrow and can, in the case of pure compounds, be attributed to typical chemical bonds such as –C–O–C–, >C = O, –CHO, –O–H, or –S–H groups and so on. At higher wavenumbers (4,000–13,000 cm^{-1}), the near-infrared (NIR), vibrational combinations or simply overtones are excited. In this region, the absorption bands are much broader due to the smaller energy separations of the excited states. NIR and MIR are used in process monitoring. This is much simpler for the case of chemical processes with fewer components at higher concentrations—as already established by the pharmaceutical industry in production processes (e.g., [100–102])—when compared with biological processes: the various absorption bands of many components must necessarily overlap, which makes the (qualitative) identification procedure quite cumbersome. Some components in low concentration will contribute to a minute absorption on top of another band from a major component, and, therefore, deconvolution of the spectral information and estimation of concentrations therefrom are not easy. In bioprocesses, we always work with multicomponent solutions, and therefore we need multivariate evaluation algorithms, typically chemometric procedures (see Chaps. 2 and 9). Furthermore, near-infrared spectra of mycelial biomass appear to be influenced by morphological changes of the cells, too [103].

Calibration models are typically built from spectrometric analyses of solutions of pure substances or (synthetic) mixtures of a few components in known concentrations. Spiking of spent culture supernatants with known amounts of one single component is a good method to test the calibration model: if the model predicts a change in only this single concentration while the predictions for the other components remain constant, the calibration model reflects an analytically crisp fact. If, however, other predictions move too, although the concentrations of those components have not been changed, the calibration model is likely to have learnt a nonanalytic correlation, most often a biological one; for instance, when substrate concentration is high, the product concentration is low, and vice versa.

Exactly this may happen when spent culture supernatants are used for calibration purposes rather than well-designed synthetic mixtures. An exciting and good example of a successful test by spiking substances directly into a batch culture of *S. cerevisiae* is reported in Schenk et al. [17, 104]; such a test is likely to be performed in academia only and not in producing industry.

Two great advantages of IR process spectroscopy are the high speed—there is practically no delay, and highly dynamic systems can be analyzed—and the absolutely noninvasive and nondestructive character of the analysis—IR intensities are usually very low and will not harm cells or biomolecules. A significant disadvantage is the interference from gas bubbles in (highly) gassed reactors. Probe fouling and vibrational effects caused by agitation are also mentioned as drawbacks of in situ IR probes [105].

The MIR range allows determination of components in aqueous solution at much lower concentrations than the NIR range. MIR has a higher degree of spectral resolution than NIR. The combination of the Fourier-transform (FT) method with attenuated total reflection (ATR) probes (see below) renders MIR monitoring ideal for analysis of complex mixtures in aqueous solutions [106]. Although fiber optics are available (and constantly improving), their strong attenuation of light intensity makes the use of so-called conduits the method of choice (or waveguides, an assembly consisting of blackened tubes, linked over junctions and mirrors). However, if one changes the geometry only slightly, one has to recalibrate; otherwise, stability over 3 years is reported [107]. NIR instruments need a better signal-to-noise ratio than MIR since "only" broadband overtones are analyzed. The use of fiber optics is more advanced in the NIR range (e.g., [108]). The sensor itself is nowadays mostly an ATR tip. The IR beam is totally reflected a few or more times at the interface of the crystal tip to the measuring solution; the evanescent field or wave interacts with the solutes and is changed in intensity before it is guided back to the detector in the instrument. ATR crystals are ideally made of diamond (with a high refractive index). Transflective- and transmissive-mode sensors have also been described [109]. McGovern et al. [110] analyzed metabolites in complex industrial media by FT-IR, Raman spectroscopy, and pyrolysis mass spectrometry. Lee [111] estimated glucose, phenylalanine, and organic acids by Raman spectroscopy in *E. coli* cultivations online; the observed estimation accuracy was found to be limited by errors in the physical model for the system and the limited stability of the optical window material.

Examples of monitored components were recently summarized by Landgrebe et al. [112], including sugars such as glucose and fructose, alcohols such as methanol, ethanol, and glycerol, the amino acid glutamine, organic acids such as acetic and lactic acid, element sources such as ammonia and phosphate, and products such as biomass itself, antibodies or clavulinic acid. With a stop-and-go strategy to let cells settle on the ATR surface, Jarute et al. [113] monitored PHB in an ATR flow through cell after sample preparation using an SIA. Kansiz et al. [114] also used an SIA in combination with FT-IR spectroscopy to follow acetone–butanol fermentations and could perform 30 analyte determinations per hour.

Sensors monitoring optical density are sometimes also considered as NIR probes, since the wavelength range used in many instruments is on the order of 900–1,100 nm; however, these sensors determine the intensity of just one wavelength peak and are not spectrometers (e.g., [88]).

3.3.6 Orphaned Sensors and Methods

Several types of sensors or analyzers have not yet been discussed here but are the subject of and are referred to in other papers. Some of these seem to have been a short-lived hype and have faded away in the recent scientific literature and/or have not made their way to commercialization for process monitoring.

One example is biosensors. These cannot be used inside the sterile barrier (in situ) because they do not withstand proper sterilization. However, their use in a process analyzer operated in bypass mode (and with the sample not returned to the process but discarded) is feasible [115]. In spite of extensive studies in the past, many authors have come to the conclusion that implementation of biosensors is strongly hindered by their limited stability. Improvements are reported for a few enzymes, but the results are obviously not very satisfactory: Vojinovic et al. [116] stated in this context that operational stability allowing "up to 8 h continuous lactate conversion" with a lactate oxidase-based sensor and virtually no activity loss had been achieved. This is definitely less than the usual duration of a (batch) production process. In another work [117], they investigated immobilized glucose (GO), alcohol (AO), lactate (LO), galactose (GalO), and L-amino acid oxidases (LAAO) together with horseradish peroxidase (HPR) and found that shelf-life was as high as 6 months for GO/HRP, AO/HRP, and LAAO/HRP. After 1,400 and 8,000 FIA injections, respectively, GalO and LAAO had lost half of their original activity. Katrlik et al. [118] employed sensors made with *Gluconobacter oxydans* for 1,3-propanediol measurements, but those analyses were made manually after sampling.

Another example is electronic noses (arrays of gas sensor elements) and electronic tongues (arrays of potentiometric or voltammetric elements). Although such arrays have been used to characterize biologically important aspects such as the metabolic burden of recombinant organisms or the indirect estimation of product concentration [119, 120], the number of such publications has decreased significantly in the last decade. The background is probably that (a) one does not know (exactly) what the sensor array elements do or could sense, (b) the limited stability of the (gas sensor) array elements, and (c) the evaluation of the high-dimensional datasets (with probably more than 1,000 array elements = dimensions) requires multivariate regression models (chemometrics) which are not easy to build and to validate. However, recently, an electronic nose was used to distinguish various physiological forms of microbes, e.g., vegetatively growing and sporulating/sporulated *Bacilli* [121], and electronic tongues have been used to monitor methane [122] or to discriminate between species that can often occur as unwanted food contaminants [123].

Prototype instruments of an ATP analyzer are described as a useful PAT tool [124]. However, to my knowledge, not many analyzers have been sold and are in use.

Practically all immunoassays such as surface-based fluoroimmunoassays are postprocess analyses and not online, despite being described as "during cell culture" (e.g., [125]); a similar statement holds true for protein and DNA chip technologies [126].

4 Conclusions

Few new sensors and process analyzers have invaded production facilities in the last decade, in spite of the PAT initiative of the FDA published 8 years ago. However, a considerable improvement is obvious in the R&D process domain and in academia over this time, although automation of laboratory techniques and use of automated *laboratory* analyzers (as opposed to *process* analyzers) has proceeded at a much faster pace and, obviously, brings much greater return on investment, because this market is larger and healthcare oriented rather than technically focused. The (commercially producing) biotechnology and pharmaceutical industry seems to be conservative and very reluctant to implement these process analyzer tools, probably because the potential benefits are not routinely investigated, evaluated, and demonstrated, probably because of the short-sighted, shareholder-oriented manager attitudes resulting in never-ending personnel and budget cuts.

References

1. Sonnleitner B (2000) Instrumentation of biotechnological processes. Adv Biochem Eng/Biotechnol 66:1–64
2. Schuegerl K (2001) Progress in monitoring, modeling and control of bioprocesses during the last 20 years. J Biotechnol 85(2):149–173
3. Carloni A, Turner APF (2011) Bioprocess monitoring. Encycl Ind Biotechnol 2:766–783
4. Junker BH, Wang HY (2006) Bioprocess monitoring and computer control: key roots of the current PAT initiative. Biotechnol Bioeng 95(2):226–261
5. Duennebier G, Tups H (2007) FDA PAT Initiative—Eine Anwendersicht zu technischen Möglichkeiten und aktueller industrieller Umsetzung. Chem Ing Tech 79(12):2019–2028
6. Clementschitsch F, Bayer K (2006) Improvement of bioprocess monitoring: development of novel concepts. Microb Cell Fact 5:19
7. Julien C, Whitford W (2007) Bioreactor monitoring, modeling, and simulation. BioProc Int 5(1):10–17
8. Glassey J, Gernaey KV, Clemens C, Schulz TW, Oliveira R, Striedner G, Mandenius CF (2011) Process analytical technology (PAT) for biopharmaceuticals. Biotechnol J 6:369–377
9. Vojinovic V, Cabral JMS, Fonseca LP (2006) Real-time bioprocess monitoring. Part I: in situ sensors. Sens Actuator B 114(2):1083–1091
10. Olsson L, Schulze U, Nielsen J (1998) On-line bioprocess monitoring—an academic discipline or an industrial tool? Trends Anal Chem 17(2):88–95

11. Kell DB, Sonnleitner B (1995) GMP—good modelling practice. Trends Biotechnol 13:481–492
12. Cimander C, Bachinger T, Mandenius CF (2003) Integration of distributed multi-analyzer monitoring and control in bioprocessing based on a real-time expert system. J Biotechnol 103(3):237–248
13. Cimander C, Mandenius CF (2002) Online monitoring of a bioprocess based on a multi-analyser system and multivariate statistical process modelling. J Chem Technol Biotechnol 77(10):1157–1168
14. Harms P, Kostov Y, Rao G (2002) Bioprocess monitoring. Curr Opin Biotechnol 13(2):124–127
15. Badugu R, Kostov Y, Rao G, Tolosa L (2008) Development and application of an excitation ratio metric optical pH sensor for bioprocess monitoring. Biotechnol Prog 24(6):1393–1401
16. Kermis HR, Kostov Y, Harms P, Rao G (2002) Dual excitation ratiometric fluorescent pH sensor for noninvasive bioprocess monitoring: development and application. Biotechnol Prog 18(5):1047–1053
17. Schenk J, Marison IW, von Stockar U (2008) pH prediction and control in bioprocesses using mid-infrared spectroscopy. Biotechnol Bioeng 100(1):82–93
18. Kjaergaard L (1977) The redox potential: its use and control in biotechnology. Adv Biochem Eng 7:131–150
19. Funke M, Diederichs S, Kensy F, Mueller C, Buechs J (2009) The baffled microtiter plate: increased oxygen transfer and improved online monitoring in small scale fermentations. Biotechnol Bioeng 103(6):1118–1128
20. Henes B, Sonnleitner B (2007) Controlled fed-batch by tracking the maximal culture capacity. J Biotechnol 132(2):118–126
21. Lam H, Kostov Y (2009) Optical instrumentation for bioprocess monitoring. Adv Biochem Eng/Biotechnol 116:1–28
22. Ge X, Kostov Y, Rao G (2005) Low-cost noninvasive optical CO_2 sensing system for fermentation and cell culture. Biotechnol Bioeng 89(3):329–334
23. Granstedt F (2005) Modelling of an electroacoustic gas sensor. Sens Actuat B 104(2):308–311
24. Custer TG, Wagner WP, Kato S, Bierbaum VM, Fall R (2003) Potential of on-line CIMS for bioprocess monitoring. Biotechnol Prog 19(4):1355–1364
25. Pollard D, Christensen J (2010) Vent gas analysis. Encycl Ind Biotechnol 7:4759–4773
26. Madrid RE, Felice CJ (2005) Microbial biomass estimation. Crit Rev Biotechnol 25(3):97–112
27. Ansorge S, Esteban G, Schmid G (2010) On-line monitoring of responses to nutrient feed additions by multi-frequency permittivity measurements in fed-batch cultivations of CHO cells. Cytotechnology 62(2):121–132
28. Sarrafzadeh MH, Belloy L, Esteban G, Navarro JM, Ghommidh C (2005) Dielectric monitoring of growth and sporulation of *Bacillus thuringiensis*. Biotechnol Lett 27(7):511–517
29. Sarrafzadeh MH, Guiraud JP, Lagneau C, Gaven B, Carron A, Navarro JM (2005) Growth, sporulation, delta-endotoxins synthesis, and toxicity during culture of *Bacillus thuringiensis* H14. Curr Microbiol 51(2):75–81
30. Maskow T, Roellich A, Fetzer I, Ackermann JU, Harms H (2008) On-line monitoring of lipid storage in yeasts using impedance spectroscopy. J Biotechnol 135(1):64–70
31. Markx GH, Davey CL, Kell DB (1991) The permittistat: a novel type of turbidostat. J Gen Microbiol 137:735–743
32. Bluma A, Hoepfner T, Lindner P, Rehbock C, Beutel S, Riechers D, Hitzmann B, Scheper T (2010) In-situ imaging sensors for bioprocess monitoring: state of the art. Anal Bioanal Chem 398(6):2429–2438
33. Hoepfner T, Bluma A, Rudolph G, Lindner P, Scheper T (2010) A review of non-invasive optical-based image analysis systems for continuous bioprocess monitoring. Bioproc Biosys Eng 33(2):247–256

34. Rudolph G, Brueckerhoff T, Bluma A, Korb G, Scheper T (2007) Optische Inline-Messverfahren zur Zellzahl- und Zellgroessenbestimmung in der Bioprozesstechnik. Chem Ing Tech 79(1–2):42–51
35. Ulber R, Frerichs JG, Beutel S (2003) Optical sensor systems for bioprocess monitoring. Anal Bioanal Chem 376(3):342–348
36. Prediger A, Bluma A, Hoepfner T, Lindner P, Beutel S, Mueller J, Hilterhaus L, Liese A, Scheper T (2011) In-situ-Mikroskopie zur Online-Ueberwachung von Enzymtraegern und Zweiphasenprozessen. Chem Ing Tech 83(6):884–887
37. Zhao R, Natarajan A, Srienc F (1999) A flow injection flow cytometry system for on-line monitoring of bioreactors. Biotechnol Bioeng 62(5):609–617
38. Abu-Absi NR, Zamamiri A, Kacmar J, Balogh SJ, Srienc F (2003) Automated flow cytometry for acquisition of time-dependent population data. Cytometry Part A 51(A):87–96
39. Sitton G, Srienc F (2008) Mammalian cell culture scale-up and fed-batch control using automated flow cytometry. J Biotechnol 135(2):174–180
40. Sitton G, Srienc F (2008) Growth dynamics of mammalian cells monitored with automated cell cycle staining and flow cytometry. Cytometry Part A 73(A):538–545
41. Birou B, Marison IW, von Stockar U (1987) Calorimetric investigation of aerobic fermentations. Biotechnol Bioeng 30(5):650–660
42. von Stockar U, Birou B (1989) The heat generated by yeast cultures with a mixed metabolism in the transition between respiration and fermentation. Biotechnol Bioeng 34(1):86–101
43. Schubert T, Breuer U, Harms H, Maskow T (2007) Calorimetric bioprocess monitoring by small modifications to a standard bench-scale bioreactor. J Biotechnol 130(1):24–31
44. Sivaprakasam S, Schuler MM, Hama A, Hughes KM, Marison IW (2011) Biocalorimetry as a process analytical technology process analyser; robust in-line monitoring and control of aerobic fed-batch cultures of crabtree-negative yeast cells. J Therm Anal Calorim 104(1):75–85
45. Voisard D, Pugeaud P, Kumar AR, Jenny K, Jayaraman K, Marison IW, von Stockar U (2002) Development of a large-scale biocalorimeter to monitor and control bioprocesses. Biotechnol Bioeng 80(2):125–138
46. von Stockar U, Marison IW (1989) The use of calorimetry in biotechnology. Adv Biochem Eng/Biotechnol 40:93–136
47. Dabros M, Amrhein M, Bonvin D, Marison IW, von Stockar U (2009) Data reconciliation of concentration estimates from mid-infrared and dielectric spectral measurements for improved on-line monitoring of bioprocesses. Biotechnol Prog 25(2):578–588
48. Staerk E, Hitzmann B, Schuegerl K, Scheper T, Fuchs C, Koester D, Maerkl H (2002) A useful tool for non-invasive bioprocess monitoring. Adv Biochem Eng/Biotechnol 74:21–38
49. Anton F, Lindemann C, Hitzmann B, Reardon KF, Scheper T (2010) Fluorescence techniques for bioprocess monitoring. Encycl Ind Biotechnol 4:2482–2491
50. Reischer H, Schotola I, Striedner G, Poetschacher F, Bayer K (2004) Evaluation of the GFP signal and its aptitude for novel on-line monitoring strategies of recombinant fermentation processes. J Biotechnol 108(2):115–125
51. Broger T, Odermatt RP, Ledergerber P, Sonnleitner B (2009) Exploiting fluorescent reporter molecules for process analytical technology (PAT). Chimia 63(3):171–173
52. Skibsted E, Lindemann C, Roca C, Olsson L (2001) On-line bioprocess monitoring with a multi-wavelength fluorescence sensor using multivariate calibration. J Biotechnol 88(1):47–57
53. Haack MB, Eliasson A, Olsson L (2004) On-line cell mass monitoring of *Saccharomyces cerevisiae* cultivations by multi-wavelength fluorescence. J Biotechnol 114(1–2):199–208
54. Harbeck C, Hitzmann B, Lindemann C, Sosnitza P, Faurie R, Scheper T (2001) Verwendung der 2D-Fluoreszenzspektroskopie zur On-line-Ueberwachung der chromatographischen Melasseentzuckerung. Chem Ing Tech 73(7):861–864

55. Derfus GE, Abramzon D, Tung M, Chang D, Kiss R, Amanullah A (2009) Cell culture monitoring via an auto-sampler and an integrated multi-functional off-line analyzer. Biotechnol Prog 26(1):284–292
56. Martens S, Borchert SO, Faber BW, Cornelissen G, Luttmann R (2011) Fully automated production of potential malaria vaccines with *Pichia pastoris* in integrated processing. Eng Life Sci 11(4):429–435
57. Gastrock G, Lemke K, Metze J (2001) Sampling and monitoring in bioprocessing using microtechniques. J Biotechnol 82(2):123–135
58. Broger T, Odermatt RP, Huber P, Sonnleitner B (2011) Real-time on-line flow cytometry for bioprocess monitoring. J Biotechnol 154:240–247
59. Hewitt CJ, Nebe-von-Caron G (2004) The application of multi-parameter flow cytometry to monitor individual microbial cell physiological state. Adv Biochem Eng/Biotechnol 89:197–223
60. Straeuber H, Mueller S (2010) Viability states of bacteria—specific mechanisms of selected probes. Cytometry Part A 77(7):623–634
61. Mueller S, Nebe-von-Caron G (2010) Functional single-cell analyses: flow cytometry and cell sorting of microbial populations and communities. FEMS Microbiol Rev 34:554–587
62. Lopes da Silva T, Piekova L, Mileu J, Roseiro JC (2009) A comparative study using the dual staining flow cytometric protocol applied to *Lactobacillus rhamnosus* and *Bacillus licheniformis* batch cultures. Enzyme Microb Technol 45(2):134–138
63. Freimark D, Jerome V, Freitag R (2010) A GFP-based method facilitates clonal selection of transfected CHO cells. Biotechnol J 5(1):24–31
64. Muench T, Sonnleitner B, Fiechter A (1992) New insights into the synchronization mechanism with forced synchronous cultures of *Saccharomyces cerevisiae*. J Biotechnol 24:299–314
65. Fritsch M, Starruss J, Loesche A, Mueller S, Bley T (2005) Cell cycle synchronization of *Cupriavidus necator* by continuous phasing measured via flow cytometry. Biotechnol Bioeng 92(5):635–642
66. Marx A, Hewitt CJ, Grewal R, Scheer S, Vandre K, Pfefferle W, Kossmann B, Ottersbach P, Beimfohr C, Snaidr J, Auge C, Reuss M (2003) Anwendungen der Zytometrie in der Biotechnologie. Chem Ing Tech 75(5):608–614
67. Diaz M, Herrero M, Garcia LA, Quiros C (2010) Application of flow cytometry to industrial microbial bioprocesses. Biochem Eng J 48(3):385–407
68. Langwost B, Kresbach GM, Scheper T, Ehrat M, Widmer HM (1995) Field-flow fractionation—an analytical tool for many applications. Eur Congr Biotechnol 7 (Nice, F: poster)
69. Hawe A, Friess W, Sutter M, Jiskoot W (2008) Online fluorescent dye detection method for the characterization of immunoglobulin G aggregation by size exclusion chromatography and asymmetrical flow field flow fractionation. Anal Biochem 378(2):115–122
70. Baker KN, Rendall MH, Patel A, Boyd P, Hoare M, Freedman RB, James DC (2002) Rapid monitoring of recombinant protein products: a comparison of current technologies. Trends Biotechnol 20(4):149–156
71. Cha HJ, Dalal NG, Bentley WE (2004) In vivo monitoring of intracellular expression of human interleukin-2 using green fluorescent protein fusion partner in *Pichia pastoris*. Biotechnol Lett 26(14):1157–1162
72. Kaiser C, Pototzki T, Ellert A, Luttmann R (2008) Applications of PAT—process analytical technology in recombinant protein processes with *Escherichia coli*. Eng Life Sci 8(2):132–138
73. Peckham GD, Bugos RC, Su WW (2006) Purification of GFP fusion proteins from transgenic plant cell cultures. Protein Expr Purif 49(2):183–189
74. Zhuang R, Zhang Y, Zhang R, Song C, Yang K, Yang A, Jin B (2008) Purification of GFP fusion proteins with high purity and yield by monoclonal antibody-coupled affinity column chromatography. Protein Expr Purif 59(1):138–143
75. Ruzicka J, Hansen EH (1981) Flow injection analysis. Wiley, New York

76. Rehbock C, Beutel S, Brueckerhoff T, Hitzmann B, Riechers D, Rudolph G, Stahl F, Scheper T, Friehs K (2008) Bioprozessanalytik. Chem Ing Tech 80(3):267–286
77. Endres C, Haake C, Landgrebe D, Beutel S, Stahl F, Hitzmann B, Scheper T, Friehs K (2009) Moderne Bioprozessanalytik—eine kurze Uebersicht. Biospektrum 15(6):662–664
78. Vojinovic V, Cabral JMS, Fonseca L (2007) Ex situ bioprocess monitoring techniques. Chem Ind Chem Eng Q 13(2):103–116
79. Almeida C (2004) Flow injection analysis system for on-line cutinase activity assay. Anal Chim Acta 502(1):115–124
80. Bracewell D, Gill A, Hoare M (2002) An in-line flow injection optical biosensor for real-time bioprocess monitoring. Food Bioprod Proc 80(2):71–77
81. Haouz A, Stieg S (2002) Continuous monitoring of D-glucose and L-lactate by flow injection analysis. Enzyme Microb Technol 30(1):129–133
82. Nandakumar MP, Palsson E, Gustavsson PE, Larsson PO, Mattiasson B (2000) Superporous agarose monoliths as mini-reactors in flow injection systems. On-line monitoring of metabolites and intracellular enzymes in microbial cultivation processes. Bioseparation 9(4):193–202
83. Horstkotte B, Arnau C, Valero F, Elsholz O, Cerda V (2008) Monitoring of sorbitol in *Pichia pastoris* cultivation applying sequential injection analysis. Biochem Eng J 42(1):77–83
84. Sohn OJ, Kim CK, Rhee JI (2008) Immobilization of glucose oxidase and lactate dehydrogenase onto magnetic nanoparticles for bioprocess monitoring system. Biotechnol Bioproc Eng 13(6):716–723
85. Lili W, Jie Q, Zhongce H, Yuguo Z, Wei H (2006) Determination of dihydroxyacetone and glycerol in fermentation broth by pyrolytic methylation/gas chromatography. Anal Chim Acta 557(1–2):262–266
86. Fricke J, Pohlmann K, Tatge F, Lang F, Faber B, Luttmann R (2011) A multi-bioreactor system for optimal production of malaria vaccines with *Pichia pastoris*. Biotechnol J 6(4):437–451
87. Tohmola N, Ahtinen J, Pitkaenen JP, Parviainen V, Joenvaeaerae S, Hautamaeki M, Lindroos P, Maekinen J, Renkonen R (2011) On-line high performance liquid chromatography measurements of extracellular metabolites in an aerobic batch yeast (*Saccharomyces cerevisiae*) culture. Biotechnol Bioproc Eng 16(2):264–272
88. Warth B, Rajkai G, Mandenius CF (2010) Evaluation of software sensors for on-line estimation of culture conditions in an *Escherichia coli* cultivation expressing a recombinant protein. J Biotechnol 147(1):37–45
89. Cos O, Ramon R, Montesinos JL, Valero F (2006) Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review. Microb Cell Fact 5:17
90. Plum A, Rehorek A (2005) Strategies for continuous on-line high performance liquid chromatography coupled with diode array detection and electrospray tandem mass spectrometry for process monitoring of sulfonated azo dyes and their intermediates in anaerobic-aerobic bioreactors. J Chrom A 1084(1–2):119–133
91. Buettgenbach S, Michalzik M, Wilke R (2006) New approaches to online bioprocess monitoring. Eng Life Sci 6(5):449–454
92. Kulp M, Vassiljeva I, Vilu R, Kaljurand M (2002) Monitoring of the degradation of phenols by *Rhodococcus* bacteria by using micellar electrokinetic chromatography. J Separat Sci 25(15–17):1129–1135
93. Ehala S, Vassiljeva I, Kuldvee R, Vilu R, Kaljurand M (2001) On-line coupling of a miniaturized bioreactor with capillary electrophoresis, via a membrane interface, for monitoring the production of organic acids by microorganisms. Fresenius J Anal Chem 371(2):168–173
94. Tahkoniemi H, Helmja K, Menert A, Kaljurand M (2006) Fermentation reactor coupled with capillary electrophoresis for on-line bioprocess monitoring. J Pharm Biomed Anal 41(5):1585–1591

95. Yang TH, Wittmann C, Heinzle E (2006) Respirometric ^{13}C flux analysis, Part I: design, construction and validation of a novel multiple reactor system using on-line membrane inlet mass spectrometry. Metabol Eng 8(5):417–431
96. Yang TH, Wittmann C, Heinzle E (2006) Respirometric ^{13}C flux analysis—Part II: in vivo flux estimation of lysine-producing *Corynebacterium glutamicum*. Metabol Eng 8(5):432–446
97. Dorfner R, Ferge T, Yeretzian C, Kettrup A, Zimmermann R (2004) Laser mass spectrometry as on-line sensor for industrial process analysis: process control of coffee roasting. Anal Chem 76(5):1386–1402
98. Biasioli F, Gasperi F, Yeretzian C, Maerk TD (2011) PTR-MS monitoring of VOCs and BVOCs in food science and technology. Trends Anal Chem 30(7):968–977
99. Feng HT, Wong NSC, Sim LC, Wati L, Ho Y, Lee MM (2010) Rapid characterization of high/low producer CHO cells using matrix-assisted laser desorption/ionization time-of-flight. Rap Comm Mass Spectrom 24:1226–1230
100. Wiss J, Zilian A (2003) Online spectroscopic investigations (FTIR/Raman) of industrial reactions: synthesis of tributyltin azide and hydrogenation of chloronitrobenzene. Org Process Res Dev 7(6):1059–1066
101. Wiss J, Laenzlinger M, Wermuth M (2005) Safety improvement of a Grignard reaction using on-line NIR monitoring. Org Process Res Dev 9(2):365–371
102. Burgbacher J, Wiss J (2008) Industrial applications of online monitoring of drying processes of drug substances using NIR. Org Process Res Dev 12(2):235–242
103. Vaidyanathan S, White S, Harvey LM, McNeil B (2003) Influence of morphology on the near-infrared spectra of mycelial biomass and its implications in bioprocess monitoring. Biotechnol Bioeng 82(6):715–724
104. Schenk J, Marison IW, von Stockar U (2007) Simplified Fourier-transform mid-infrared spectroscopy calibration based on a spectra library for the on-line monitoring of bioprocesses. Anal Chim Acta 591(1):132–140
105. Scarff M, Arnold SA, Harvey LM, McNeil B (2006) Near infrared spectroscopy for bioprocess monitoring and control: current status and future trends. Crit Rev Biotechnol 26(1):17–39
106. Roychoudhury P, Harvey LM, McNeil B (2006) The potential of mid infrared spectroscopy (MIRS) for real time bioprocess monitoring. Anal Chim Acta 571(2):159–166
107. Rhiel M, Ducommun P, Bolzonella I, Marison I, von Stockar U (2002) Real-time in situ monitoring of freely suspended and immobilized cell cultures based on mid-infrared spectroscopic measurements. Biotechnol Bioeng 77(2):174–185
108. Arnold SA, Crowley J, Woods N, Harvey LM, McNeil B (2003) In-situ near infrared spectroscopy to monitor key analytes in mammalian cell cultivation. Biotechnol Bioeng 84(1):13–19
109. Vaidyanathan S, Arnold A, Matheson L, Mohan P, Macaloney G, McNeil B, Harvey LM (2000) Critical evaluation of models developed for monitoring an industrial submerged bioprocess for antibiotic production using near-infrared spectroscopy. Biotechnol Prog 16(6):1098–1105
110. McGovern AC, Broadhurst D, Taylor J, Kaderbhai N, Winson MK, Small DA, Rowland JJ, Kell DB, Goodacre R (2002) Monitoring of complex industrial bioprocesses for metabolite concentrations using modern spectroscopies and machine learning: application to gibberellic acid production. Biotechnol Bioeng 78(5):527–538
111. Lee H (2004) In situ bioprocess monitoring of *Escherichia coli* bioreactions using Raman spectroscopy. Vibrat Spectrosc 35(1–2):131–137
112. Landgrebe D, Haake C, Hoepfner T, Beutel S, Hitzmann B, Scheper T, Rhiel M, Reardon KF (2010) On-line infrared spectroscopy for bioprocess monitoring. Appl Microbiol Biotechnol 88(1):11–22
113. Jarute G, Kainz A, Schroll G, Baena JR, Lendl B (2004) On-line determination of the intracellular poly (β-hydroxybutyric acid) content in transformed *Escherichia coli* and

glucose during PHB production using stopped-flow attenuated total reflection FT-IR spectrometry. Anal Chem 76(21):6353–6358
114. Kansiz M, Gapes JR, McNaughton D, Lendl B, Schuster KC (2001) Mid-infrared spectroscopy coupled to sequential injection analysis for the on-line monitoring of the acetone-butanol fermentation process. Anal Chim Acta 438(1–2):175–186
115. Bilitewski U (2005) Biosensors for bioprocess monitoring. Compr Anal Chem XLIV: 539–578
116. Vojinovic V, Bertin L, Cabral JMS, Fonseca LP (2006) Horseradish peroxidase combined with oxidase enzymes a valuable bioanalytical tool: lactate oxidase—a case study. Eng Life Sci 6(2):181–186
117. Vojinovic V, Esteves F, Cabral J, Fonseca L (2006) Bienzymatic analytical microreactors for glucose, lactate, ethanol, galactose and L-amino acid monitoring in cell culture media. Anal Chim Acta 565(2):240–249
118. Katrlik J, Vostiar I, Sefcovicova J, Tkac J, Mastihuba V, Valach M, Stefuca V, Gemeiner P (2007) A novel microbial biosensor based on cells of *Gluconobacter oxydans* for the selective determination of 1,3-propanediol in the presence of glycerol and its application to bioprocess monitoring. Anal Bioanal Chem 388(1):287–295
119. Bachinger T, Riese U, Eriksson RK, Mandenius CF (2000) Electronic nose for estimation of product concentration in mammalian cell cultivation. Bioproc Eng 23(6):637–642
120. Bachinger T, Mandenius CF, Striedner G, Clementschitsch F, Durrschmid E, Cserjan-Puschmann M, Doblhoff-Dier O, Bayer K (2001) Non-invasive detection of the metabolic burden on recombinant microorganisms during fermentation processes. J Chem Technol Biotechnol 76(8):885–889
121. Clemente JJ, Monteiro SMS, Carrondo MJT, Cunha AE (2008) Predicting sporulation events in a bioreactor using an electronic nose. Biotechnol Bioeng 101(3):545–552
122. Buczkowska A, Witkowska E, Gorski L, Zamojska A, Szewczyk, Krzysztof W, Wroblewski W, Ciosek P (2010) The monitoring of methane fermentation in sequencing batch bioreactor with flow-through array of miniaturized solid state electrodes. Talanta 81(4–5):1387–1392
123. Soederstroem C, Rudnitskaya A, Legin A, Krantz-Ruelcker C (2005) Differentiation of four *Aspergillus* species and one *Zygosaccharomyces* with two electronic tongues based on different measurement techniques. J Biotechnol 119(3):300–308
124. Schmidt J, Mueller U, Wallimann C, Mathes S, Probst C, Andretta C, Leist C, Graf-Hausner U (2008) Monitoring ATP status in the metabolism of production cell lines. BioProc Int 6:46–54
125. Szmacinski H, Smith DS, Hanson MA, Kostov Y, Lakowicz JR, Rao G (2008) A novel method for monitoring monoclonal antibody production during cell culture. Biotechnol Bioeng 100(3):448–457
126. Becker T, Hitzmann B, Muffler K, Poertner R, Reardon KF, Stahl F, Ulber R (2007) Future aspects of bioprocess monitoring. Adv Biochem Eng/Biotechnol 105:249–293

Adv Biochem Eng Biotechnol (2013) 132: 35–63
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Automatic Control of Bioprocesses

Marc Stanke and Bernd Hitzmann

Abstract In this chapter, different approaches for open-loop and closed-loop control applied in bioprocess automation are discussed. Although in recent years many contributions dealing with closed-loop control have been published, only a minority were actually applied in real bioprocesses, the majority being simulations. As a result of the diversity of bioprocess requirements, a single control algorithm cannot be applied in all cases; rather, different approaches are necessary. Most publications combine different closed-loop control techniques to construct hybrid systems. These systems are supposed to combine the advantages of each approach into a well-performing control strategy. The majority of applications are soft sensors in combination with a proportional–integral–derivative (PID) controller. The fact that soft sensors have become this importance for control purposes demonstrates the lack of direct measurements or their large additional expense for robust and reliable online measurement systems. The importance of model predictive control is increasing; however, reliable and robust process models are required, as well as very powerful computers to address the computational needs. The lack of theoretical bioprocess models is compensated by hybrid systems combining theoretical models, fuzzy logic, and/or artificial neural network methodology. Although many authors suggest a possible transfer of their presented control application to other bioprocesses, the algorithms are mostly specialized to certain organisms or certain cultivation conditions as well as to a specific measurement system.

Keywords Automation · Bioprocess · Closed-loop control · Fuzzy logic · Neural network

Abbreviations and Nomenclature

ANN	Artificial neural network
CER	Carbon dioxide evolution rate
C_{feed}	Substrate concentration in feed flow
CPR	Carbon dioxide production rate
DO	Dissolved oxygen
$e(t)$	Control deviation at time t

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EKF	Extended Kalman filter
FIA	Flow injection analysis
$F_0(t)$	Feeding rate
$F_b(t)$	Feeding rate due to feedback part
GC	Gas chromatography
HCDC	High-cell-density cultivation
K	PID controller parameter matrix
K_d	PID controller parameter, derivative part
K_i	PID controller parameter, integral part
$K_L a$	Oxygen transfer coefficient
K_p	PID controller parameter, proportional part
LLM	Local linear model
LoLiMoT	Local linear model tree
m	Substrate consumption due to cell maintenance
MIMO	Multiple-input multiple-output
MISO	Multiple-input single-output
MPC	Model predictive controller
NMPC	Nonlinear model predictive controller
ORP	Oxidation–reduction potential
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
PID	Proportional–integral–derivative
r_{DO}	Specific oxygen consumption rate
$r_{DO}X_t$	Oxygen consumption rate
SISO	Single-input single-output
t	Time
$u(t)$	Control action at time t
V	Cultivation volume
V_0	Initial volume
V_{head}	Volume of head space
X	Biomass
$x(t)$,x	Input values (measurement)
X_0	Initial biomass
Y_{XS}	Yield factor for biomass formation
μ	Specific growth rate
$\mu_B(X)$	Membership function of a fuzzy logic controller
μ_{sp}	Set-point of specific growth rate
τ	Integration variable

Contents

1 Introduction

Due to competition, industry tends to increase the degree of automation in production processes. Only an automated system is never tired and always attentive, will act reliable, and therefore can provide optimal process operation. It can react quickly to changes in raw material quality as well as changes in environmental conditions. As a result, energy and material input can be decreased and process safety and product yield and quality can be increased. This applies, of course, also for bioprocesses. The operation of these processes is usually carried out in three successive steps:

Upstreaming (filling, sterilization, and mixing)
Cultivation/enzyme reaction (growth of cells, bioconversion, and production)
Downstreaming (harvesting, separation, concentrating, and crystallization)

Each step demands a high degree of automation. In the first step, standard automatic sequence control units are available. The quality of raw materials is of special importance for the subsequent steps. The automation in the second step is more complicated, since complex transport processes are combined with a multitude of dynamic biochemical reactions during cultivation. Therefore, one has to deal with a complex, nonlinear, multiparameter, time-variant system. Little detailed comprehensive knowledge is available. The microorganisms used for the synthesis of the product have many inherent closed-loop systems of their own, which can only be manipulated indirectly through environmental conditions by physical and chemical variables. Frequently, open-loop control systems are

employed to control cultivation processes. In order to realize closed-loop control, reliable system measurements are vital. However, the application of closed-loop control is still rare, due to many reasons:

In many cases important process variables can be determined online only with excessive effort. They become available delayed by a dead time as well as lag elements and also discontinuously. Most bioprocesses are batch or fed-batch processes; therefore, one has to deal with a transient (not stationary) process, where the automation task is to provide an optimal environment for the micro-organism. The typical goals of automation of bioprocesses are to:

- Compensate failure of any kind
- Minimize energy and raw materials
- Maximize yield and product quality
- Guarantee safe operation
- Prevent substrate, overflow metabolite, or product inhibition
- Ensure well-directed induction and repression of enzyme production
- Prevent high shear stress
- Present an optimal environment for the organism for growth as well as production

With the help of standard control algorithms, some of these goals can already be achieved. Basic bioreactor equipment often includes control algorithms for the volume, temperature, pH, dissolved oxygen, and addition of antifoam agents. However, these basic controllers are not always sufficient for special applications.

In this chapter, state-of-the-art bioprocess automation and recent progress are discussed. An overview of the discussed application is presented in Table 1.

2 Controller Design

2.1 Direct Measurements

Especially for closed-loop control purposes, measurements are fundamental. For bioprocesses, in situ measurements such as temperature, pH, dissolved oxygen concentration (DO), optical density, and pressure and at-line measurements such as the exhaust gas composition are performed most frequently and can be used as input variables for a controller [1, 2]. At-line measurements based on spectro-photometric, mass-spectrometric, HPLC, GC, and flow injection analysis (FIA) systems are applied less frequently for online measurements and even less often as input variables for a controller [1–3]. Due to the fact that direct measurements of important variables such as growth rate, substrate uptake rate, and carbon dioxide production rate are missing, soft sensors have been established to enable a kind of indirect measurement that can provide access to relevant variables using different techniques.

Table 1 Outline of the employed algorithms, the measured values, and the controlled processes presented in this chapter

Measured value	Actuating variable	Controlled process	Remarks	Employed algorithm	References
Glucose	Feeding rate	Yeast	–	On/off control	Lindgren et al. [11]
Glucose	Feeding rate	*S. cerevisiae*	EKF	SISO PID	Arndt and Hitzmann [14]
Glucose	Feeding rate	*S. cerevisiae*	EKF + ring buffer	SISO PID	Klockow et al. [17]
Glucose	Feeding rate	*E. coli*	EKF	SISO PID	Arndt et al. [15]
Glucose	Feeding rate	*E. coli*	EKF	SISO PID	Kleist et al. [16]
Glucose	Feeding rate	*E. coli*	EKF + Smith predictor	SISO PID	Roever and Slavov [18]
Glucose	Feeding rate	*E. coli*	EKF + Smith predictor	SISO PID	Tsonyo and Roeva [19]
NIR	Methanol feeding rate	*Pichia pastoris*	Two-point calibration	SISO PID	Schenk et al. [72]
DO, nitrate	DO, nitrate	WWT	Simulation studies	MISO PID	Wahab et al. [20]
OTR, CPR	Feeding rate	HCDC	Simulation studies	MISO PID	Chung et al. [25]
OTR, CPR	Feeding rate	HCDC	Simulation studies	MISO PID	Ranjan and Gomes [26]
OUR, CPR, pH	Feeding rate	*E. coli*	–	MISO PID	Jenzsch et al. [7]
Temperature, DO	Temperature, feeding rate	HCDC *E. coli*	Calorimetric model	MISO PID cascade	Biener et al. [21]
Temperature, DO	Temperature, feeding rate	HCDC *S. cerevisiae*	Calorimetric model	MISO PID cascade	Biener et al. [22]
Temperature, DO	DO, feeding rate	*S. cerevisiae*	EKF	MISO PID cascade	Soons et al. [23]
DO OUR, CPR, DO, V	Feeding rate	Fungal cultivation	Industrial plant	MISO PID cascade	Bodizs et al. [24]
Ethanol, DO	Feeding rate	*S. cerevisiae*	–	RTS controller	Renard et al. [41]
Ethanol, DO	Feeding rate	*S. cerevisiae*	Simulation studies	RTS controller	Renard and Vande Wouwer [42]
Ethanol, DO	Feeding rate	*S. cerevisiae*	Industrial scale	RTS controller	Dewasme [43]
Ethanol, DO	Feeding rate	*S. cerevisiae*	–	RTS controller	Cannizzaro et al. [44]
Ethanol, DO	Feeding rate	*E. coli*	–	RTS controller	Valentinotti et al. [45]
Ethanol, DO	Feeding rate	*E. coli*	Pilot plant	RTS controller	Hocalar and Türker [46]
pH, ORP, DO	Recycle flow rate air flow	Nitrification process	–	Fuzzy logic	Ruano et al. [47]

(continued)

Table 1 (continued)

Measured value	Actuating variable	Controlled process	Remarks	Employed algorithm	References
pH, temperature	pH, temperature	Penicillin production	Type 2 fuzzy logic simulation studies	Fuzzy logic	Cosenza and Galluzzo [52]
Temperature	Temperature	Batch reactor	Simulation studies	Hybrid fuzzy logic	Causa et al. [48]
DO	DO	Wastewater treatment process	Simulation studies	TS fuzzy logic	Belchior et al. [54]
O_2, CO_2, feeding rate, temperature, pH	Feeding rate	*S. cerevisiae*	ANN soft sensor	Fuzzy logic	Karakuzo et al. [55]
DO	Feeding rate	*S. cerevisiae*	On-line adapting neural network	ANN	Gadkar et al. [56]
pH, temperature	pH, temperature	Penicillin production	–	NMPC	Ashoori et al. [57]
Substrate	Feeding rate	*E. coli*	Simulation studies	NMPC	Santos et al. [59]
Volume base feed, dry biomass	Feeding rate	Antibiotic production simulation	NMPC EKF online trajectory planning (OT)	NMPC	Kawohl et al. [10]
Feeding rate, cell recycle rate, flash recycle rate	Feeding rate	Fuel-ethanol production	ANN model, simulation study	NMPC	Meleiro et al. [64]
DO, temperature	Temperature, feeding rate	*E. coli*	–	Probing feeding	Velut et al. [65]
DO, temperature	Temperature, feeding rate	*E. coli*	–	Probing feeding	Velut et al. [66]
DO, temperature	Temperature, feeding rate	*E. coli*	Pilot plant	Probing feeding	Xue and Fan [67]
OUR, CPR	Feeding rate	Microbial growth	Simulation studies	Extremum seeking	Dochain et al. [68]
OUR, CPR	Feeding rate	Microbial growth	Simulation studies	Extremum seeking	Cougnon et al. [69]
OUR, CPR	Feeding rate	*S. cerevisiae*	Simulation studies	Extremum seeking	Dewasme et al. [70]
Fluorescence	Feeding rate	*S. cerevisiae*	–	Heuristic procedures	Hantelmann et al. [71]

2.2 Soft Sensors

Economist and business consultant Peter Drucker once said: "If you can't measure it, you can't manage it." Although he did not mean bioprocesses, it can also be applied here. Keeping that in mind, soft sensors will here be introduced to "manage the immeasurable." Soft sensors or virtual sensors are employed to calculate variables from one or more of the directly measured variables. Commonly employed indirect measurements based on theoretical models are oxygen uptake rate (OUR), oxygen transfer rate (OTR), carbon dioxide production rate (CPR), and the respiration coefficient, which are calculated from exhaust gas measurements and the aeration rate. The identification of critical needs to successfully develop state-of-the-art soft sensors is presented by Luttmann et al. [4]. They particularly discuss soft sensor methods for bioprocess engineering and pharmaceutical applications.

Data-driven soft sensors use chemometric models for the estimation of process variables [2, 3, 5]. An example is the calculation of the glucose concentration and dry cell mass concentration from fluorescence data [6]. When using these data-driven approaches, one has to be careful not to leave the calibration range. Therefore, theoretical model-based soft sensors usually have a broader range of application. An important class of these soft sensors is based on state observers.

2.2.1 State Observer

A state observer uses a dynamic theoretical model (state model) of the process to estimate process variables (state variables). Using available measurements, the state observer corrects the estimated state variable in such a way that its values will converge to the true process values. For the implementation of a state observer, detailed knowledge of the process is necessary. The advantage of state observers is the determination of immeasurable process variables which can be used for process automation.

An example is given by Jenzsch et al. [7]. They estimated the biomass using a mass-balance-based state observer by employing the relation between OUR, CPR, and base consumption. One kind of standard state observer is the Luenberger observer [8] and the Kalman filter. A special class of Kalman filter is discussed in the next section.

2.2.2 Extended Kalman Filter

To smooth noisy measurement signals as well as to estimate immeasurable process variables, different Kalman filters have been applied for controller implementation. For this purpose, process knowledge is required in the form of a dynamic state model, a measurement model, and known measurement noise. The main idea of

the Kalman filter [9] is minimization of the error covariance of the estimation of the state variables. Therefore, not only the process model, but also the estimated error covariance differential equations have to be integrated online. If a nonlinear state model is used, the filter is called an extended Kalman filter (EKF). A continuous–discrete EKF uses a continuous nonlinear state model and a discrete measurement model. The differential equations are integrated as long as no new measurement value is available. If a new measurement is available, the filter equation is applied. As a result, the estimation error covariance is minimized and the estimated values of the state variables are adjusted to the measurements. By using a Kalman filter, the time-varying characteristics of cultivation processes can be implemented in a control algorithm.

A general overview of specialized state observers is given by Kawohl et al. [10], who compared different optimization-based state estimation algorithms to judge their estimation quality. The Bayesian maximum, an a posteriori-based constrained extended Kalman filter, the moving-horizon state estimation, and the classical unconstrained extended Kalman filter are compared through Monte Carlo simulation experiments. The authors conclude that the moving-horizon state estimation shows greater potential for state estimation in small systems. For higher-order systems, the adjustments of the filter parameters as well as the numerical optimizations were more difficult.

2.3 Control Action

The control action (actuating variable) is the resulting action that the controller performs corresponding to the control law, e.g., setting the substrate flow rate to the appropriate value. Figure 1 shows the principle of a closed-loop controller. The control action is the result of the feedback given by the process measurement, which might be further processed by a soft sensor, and the control algorithm. The overall goal is to minimize the deviation between the set-point and the controlled variable.

The next sections discuss a wide variety of state-of-the-art control applications for bioprocess automation. First, PID-based controllers in combination with different soft sensors are presented, and then model linearization approaches are discussed. This is followed by fuzzy logic- and artificial neural network-based controllers, a model predictive controller, as well as combinations of the three latter methods. Lastly, probing feeding, extremum-seeking control, and a heuristic control strategy are discussed.

A very basic approach is presented by Lindgren et al. [11] and Kriz et al. [12] based on a real-time in situ SIRE® biosensor system combined with a two-step controller (on/off control) for yeast cultivation at different biomass concentrations. Their controller could manage a set-point of 10 mM glucose for 60 min with standard deviation of 0.99 mM at biomass concentration up to 80 gL^{-1}.

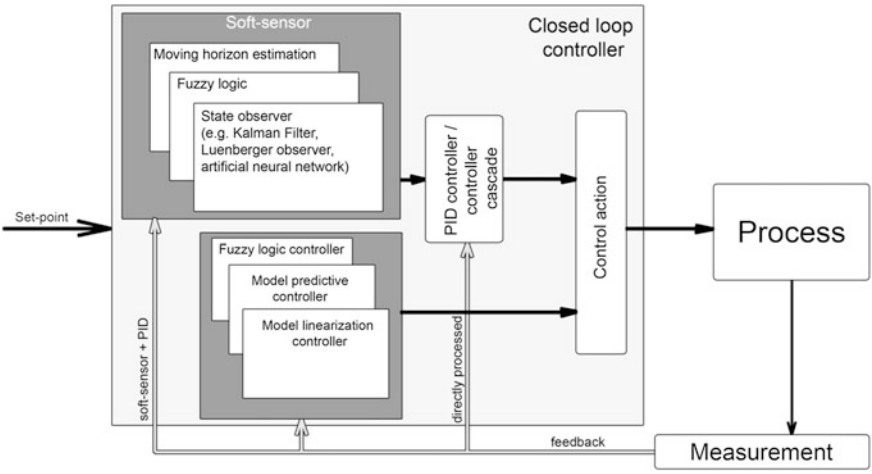

Fig. 1 General schematic of different (alternative) closed-loop control schemes

3 State-of-the-Art Control Algorithm

The most common closed-loop control algorithm is the PID controller. Here, $e(t)$, the difference between the controlled variable and the set-point at time t, is used to calculate the control action. Equation (1) is the general form of a PID controller, with K_p as the proportional gain, K_i the integral gain, K_d the derivative gain parameter, and $u(t)$ as the control action.

$$u(t) = K_\mathrm{p} e(t) + K_\mathrm{i} \int_0^t e(\tau)\mathrm{d}\tau + K_\mathrm{d} \frac{\mathrm{d}}{\mathrm{d}t} e(t). \tag{1}$$

Basic PID control is an algorithm responding to current changes with constant parameters, in which the knowledge of the process is presented. A special form of the PID controller is the PI controller, which lacks the derivative part (i.e., $K_\mathrm{d} = 0$), leading to a steadier system in the presence of noisy measurement data. This type of controller can be found most frequently.

3.1 Open Loop–Closed Loop Controller

The simplest control action for a cultivation process is open-loop control. One can rearrange typical mass balance equations describing a cultivation process in a stirred tank reactor to obtain, e.g., Eq. (2), representing the feeding law for substrate. This is basically an assumption of a known, constant substrate consumption rate as well as a predetermined constant specific growth rate μ_sp, which is smaller than the maximal specific growth rate.

$$F_0(t) = \left(\frac{\mu_{sp}}{Y_{XS}} + m\right)\frac{V_0 X_0}{C_{feed}} e^{\mu_{sp}(t-t_0)} \tag{2}$$

$$X_0 = X(t=0) \qquad V_0 = V(t=0)$$

Using such a controller, the cultivation feed is performed following a predefined trajectory. Knowledge of the inoculum size as well as the specific growth rate and the yield factors is obviously needed to perform this kind of open-loop control, which is called feed forward control. Advantages and disadvantages of open-loop control are presented by Gnoth et al. [13]. In case of disturbances (e.g., wrong pH value as a result of a failure), the system's behavior will differ from the prediction. In this case, the feed is predicted incorrectly and the result of the cultivation is not as designated, resulting in a waste of resources. To prevent this, the plainest closed-loop control approach is to add a feedback to the feed forward term for regulatory action. These feed forward/feedback controllers are usually a class of single-input single-output (SISO) controller. The control law Eq. (4) is the sum of the feed forward part F_0, which is the estimation of the approximately needed feed rate Eq. (2) at time t, and the feedback part F_b, in this case delivered by a PI algorithm Eq. (3).

$$F_b(t) = K_P e(t) + K_i \int_0^t e(\tau)\mathrm{d}\tau \tag{3}$$

$$u(t) = F_b(t) + F_0(t) \tag{4}$$

3.2 PID Control Based on Soft Sensors Measurements

Controllers with more than one input value are classified as multiple-input single-output (MISO) or multiple-input multiple-output (MIMO) and are represented as multiloop PID or cascade PI controllers. MIMO systems couple their input values according to the interaction and are therefore able to map higher complexities of the controlled process. They are supposed to be more accurate in their control action than SISO systems. The structures of both controllers (SISO and MIMO) are shown in Fig. 2.

Applications of these controller types for bioprocess automation are discussed in the following sections.

3.2.1 Single-Input Single-Output Control

The combination of a Kalman filter, whose measurements came from a glucose FIA system, and a PI controller is presented by Arndt and Hitzmann [14]. The system was applied to *S. cerevisiae* cultivation to control the glucose concentration. The Kalman

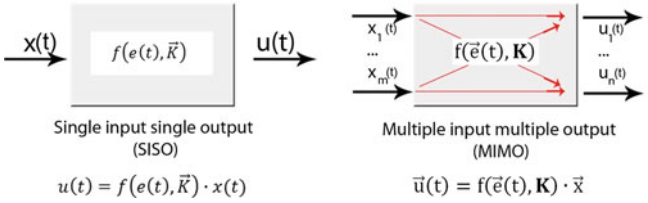

Fig. 2 Scheme of a SISO controller compared with a MIMO controller

filter estimates the glucose and biomass concentration, the volume of culture broth, and the maximal specific growth rate. The predicted values were used for a PI feedback controller with set-points of 0.08 and 0.05 gL^{-1}. The controller established well-defined growth conditions over several hours. The authors demonstrated that, in contrast to the higher set-point, no ethanol was produced at the lower set-point. A similar approach was applied by Arndt et al. [15] for *E. coli* cultivation to produce phytase. They discussed the response of the controller during a failure of the glucose FIA measurement. After the process analyzer was fixed, the online glucose measurement returned after 0.2 h to the set-point of 0.2 gL^{-1} and in a total of 0.4 h to the intended performance. Control strategies based on DO set-points between 5 and 10 % did not result in higher yield of phytase, as shown by Kleist et al. [16].

A set-point substrate controller for the glucose concentration in baker yeast fermentation was presented by Klockow et al. [17]. They compensated the dead time of 6 min caused by the FIA measurement system using an extended Kalman filter in combination with a ring buffer, where the estimated variables as well as the pumping rate are stored. If a new measurement value from the FIA system is sent to the Kalman filter, the dead time is considered by taking the historical process variable data of that time point out of the ring buffer and the ordinary filtering is carried out followed by simulation up to the current time. Since during the control phase the relative standard deviation of the measured values and the set-point were 2.9 and 4.4 % for the set-point of 0.07 and 0.5 gL^{-1}, respectively, the authors concluded that the control was successful.

Roever and Slavov [18, 19] presented a closed-loop control for the application of *E. coli* cultivation. They also used measurements from a glucose FIA system for substrate control of the bioprocess. They tested three different approaches by using: FIA measurements with a PI controller, measurements processed by an EKF and a PI controller, as well as measurements processed by an EKF combined with a Smith predictor and a PI controller. The latter was used to compensate the dead time of the FIA measurements. The authors claim satisfactory control of glucose concentration and emphasize the superiority of the control employing an EKF, resulting in higher biomass yields.

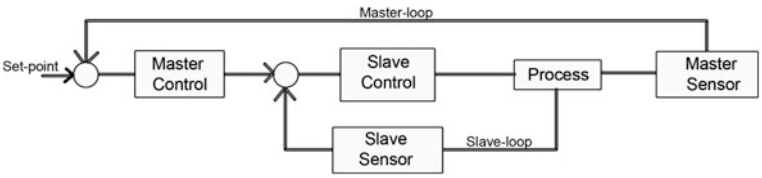

Fig. 3 Scheme of a cascade process control system

3.2.2 Multiple-Input Single/Multiple-Output Control

Wahab et al. [20] applied a multiple-input single-output controller for DO and nitrate control in a wastewater treatment process (WWT). They carried out extensive simulation studies on a nonlinear model to demonstrate the superior performance concerning set-point tracking and disturbance robustness.

Another MISO approach is given by Jenzsch et al. [7], representing a nonlinear adaptive controller based on multiple inputs (oxygen uptake rate, carbon dioxide production rate, and base consumption) to estimate the specific growth rate. They compare the results of their generic model control with the control performance employing only a PI controller. The generic model control shows better performance due to the model-based feedforward part and online adjusted control parameters obtained from the state estimation.

Cascade PI/PID controllers are employed to increase the precision of the PI principle, mostly for nonlinear control problems. Typically a so-called slave (inner) control loop is nested within a master (outer) control loop, as shown in Fig. 3.

Biener et al. [21] used this principle to precisely control the temperature in the reactor, considering a controller cascade for the reactor jacket and the reactor interior. The temperature inside the reactor is the main target of this control. Therefore, it is the outer cascade circle that is called the master loop. The temperature of the reactor jacket is used for the inner, slave loop. The state observer for the process control uses a heat balance equation that calculates the specific growth rate from the heat flow supposed to result from cell metabolism. Based on the specific growth rate estimation, they formulate a control law for the substrate feed rate. This controller design was employed in high-cell-density cultivation (HCDC) of *E. coli* producing green fluorescent protein. The authors suggested that the method is advantageous for HCDC because of the high heat flow due to the high cell density and describe a gain of sensitivity with increasing biomass. The specific growth rate can be controlled just below the critical growth rate where overflow metabolites occur. The authors demonstrate that no other measurement is necessary except DO concentration to guarantee an aerobic milieu. The heat flow generated by the cells and therefore the specific growth rate can be estimated reliably. Since the method only uses easily and quickly measurable process variables, they suggest its potential application in standard industrial bioprocesses. They recently applied the described method to a *Saccharomyces cerevisiae*

cultivation [22], demonstrating the transfer of the technique to another organism. However, this method is applied to standard cell growth in 15 and 30-L reactors. The method is not applicable after, e.g., product induction, due to the changing heat balance while the cells change their metabolism.

Soons et al. [23] applied the cascade principle to precisely control the dissolved oxygen using the oxygen in the reactor headspace (slave loop) and the dissolved oxygen in the medium (master loop). They employ a closed-loop control based on the DO concentration. With the simplification that the oxygen uptake rate is proportional to the OTR, the specific growth rate of the cultivation can be held at a constant level by controlling the DO concentration. This is shown in Eq. (5). According to Fig. 3, the closed-loop control of the dissolved oxygen is carried out through the cascade control. The outer loop (Eq. 7) compares the measured DO (DO_{sensor}) with the set-point. The result is handed to the inner loop (Eq. 8), calculating the controller output through the difference between headspace and medium.

$$\mathrm{OUR} = \mathrm{OTR} = k_{\mathrm{L}}a\left(\mathrm{O}_{2,\mathrm{head}} - \mathrm{DO}\right) \tag{5}$$

$$\frac{\mathrm{dO}_{2,\mathrm{head}}}{\mathrm{d}t} = \frac{F_{0_2}}{V_{\mathrm{head}}}\left(\mathrm{O}_{2,\mathrm{in}} - \mathrm{O}_{2,\mathrm{head}}\right) - \mathrm{OTR} \tag{6}$$

$$\mathrm{O}_{2,\mathrm{a}} = K_{\mathrm{p}}\left(\mathrm{DO}_{\mathrm{set}} - \mathrm{DO}(t)_{\mathrm{sensor}}\right) + K_{\mathrm{i}}\int_0^t \left(\mathrm{DO}_{\mathrm{set}} - \mathrm{DO}(\tau)_{\mathrm{sensor}}\right)\mathrm{d}\tau \tag{7}$$

$$\mathrm{O}_{2,\mathrm{in}} = K_{\mathrm{p}}\left(\mathrm{O}(t)_{2,\mathrm{a}} - \mathrm{O}(t)_{2,\mathrm{head}}\right) + K_{\mathrm{i}}\int_0^t \left(\mathrm{O}(\tau)_{2,\mathrm{a}} - \mathrm{O}(\tau)_{2,\mathrm{head}}\right)\mathrm{d}\tau \tag{8}$$

The cascade is realized by using the result of the PI control action of Eq. (7) in the control action of Eq. (8). This provides more flexible and sophisticated control compared with if only one PI controller were to be employed, because not only the transport of oxygen between the gas flow and the medium is considered, but also the transport from the headspace of the reactor into the medium. Further, Soons et al. use a Kalman filter to calculate the specific growth rate from the oxygen consumption provided from the DO control cascade. They demonstrated the implementation of a stable and robust closed-loop controller for specific growth rate control. The method does not need an online model and therefore lacks a complex implementation. They show through simulations and fed-batch experiments that the controller is robust against disturbances and able to maintain the specific growth rate of vaccine-producing *Bordetella pertussis* at a constant level of $\mu = 0.05\ \mathrm{h}^{-1}$.

Bodizs et al. [24] observed that a simple PI controller for DO does not perform well enough in their system and therefore employed a cascade controller. Since they implemented the controller for an established reactor, they used a set of available multiple inputs, namely OUR, CPR, DO, and volume. The process is a 2,700-L fed-batch filamentous fungal fermentation. The control is applied to

substitute a predetermined feeding strategy that is employed to guarantee no limitation of oxygen. The dissolved oxygen consumption rate $r_{DO}X_t$ is measured via a soft sensor using the approximation of Eq. (9), whereas β is a proportionality factor and CER is the carbon dioxide evolution rate, which is assumed to be approximately the carbon dioxide transfer rate.

$$r_{DO}X_t = \beta \frac{OUR + CER}{V} \tag{9}$$

The master-loop controls the specific consumption rate r_{DO}. The slave loop controller is applied to control the feed rate based on OUR and CPR measurements. It is possible to efficiently control the specific consumption rate of oxygen. The main advantage is that it is not limited to a specific strain of microorganisms and applicable for a wide variety of fungal fermentations.

Another application for MIMO-PID bioprocess control is a multiloop PID feedback controller for HCDC control applied by Chung et al. [25], coupling the OTR and the CPR. They compared the method with a model predictive controller and presented better results for their MIMO-PID using simulation studies. The controller can compensate for disturbances in the exhaust gas measurement data. Ranjan and Gomes [26] also applied a cascade MIMO, showing a performance enhancement compared with a normal PI controller.

For all these previously described applications, the parameters of the PID controller must be determined. Different approaches to determine the PID parameters are discussed in the next section.

3.2.3 PID Tuning

The obviously crucial part for all PID control-based approaches is the determination of the corresponding PID parameter values. Changes in the process dynamic will most likely lead to suboptimal control actions. Ideally, a tuned controller should show a minimum of oscillation and lead the system quickly and reliably to the set-point.

Tuning methods can be divided into two groups: parametric model and nonparametric. Parametric methods use either model or experimental data to determine the controller parameters and are mostly described as offline tuning methods, though online approaches have also been tested. Nonparametric methods only partially use models such as critical states and are suitable for online use as well as for implementation without previous extensive plant studies. Wahab et al. [20] compared four nonparametric methods for multivariable PID tuning, introducing one on their own and comparing it with established methods from Davison [27], Penttinen–Koivo [28], and Maciejowski [29]. Soons et al. [23] used a parametric tuning algorithm proposed by Bastin and Dochain [30], guaranteeing stable behavior and fast convergence towards the set-point. Other parametric approaches

to tune a PID controller are described in many publications [19, 20, 31–33] where the authors use, e.g., genetic algorithms to obtain the optimal parameters.

Online estimation of control parameters is described by Bastin and Dochain [30] as well as by Perrier et al. [34]. This is used in various control strategies [35–38], where the upper bound of the estimation error is minimized online and the resulting parameters are considered to be the optimal ones. Another approach in this direction is given by Kansha et al. [39], introducing a self-tuning PID design applying just-in-time learning. This algorithm compares a given database with the state of the process online and adjusts the gain parameter according to the obtained results, performing self-tuning derived from the Lyapunov method [40] to guarantee convergence of the given gain parameters.

3.3 Model Linearization-Based Control

Due to the inherent complexity, nonlinearity, and nonstationarity of bioprocesses, Renard et al. [41] proposed a so-called RTS control scheme with Youla parameterization to overcome these problems. They develop their control approach for *S. cerevisiae* cultivation, controlling the ethanol concentration to a nonzero value. For substrate concentrations higher than the critical substrate concentration S_{crit}, the occurrence of overflow metabolism is assumed. Since S_{crit} for yeast fermentations is 0.1 gL^{-1}, the authors suggest respirofermentative conditions and a quasisteady state of the substrate concentration (considering no accumulation of substrate and instantaneous consumption, as long as the process does not deviate dramatically from the predetermined operation conditions). They obtain a model for the relation between the feed F_{in} and the measured ethanol concentration as well as a discrete time transfer function mapping the feeding rate to the ethanol concentration, which is linearized for the purpose of control law application. The controller considers cell growth as an unstable exponential disturbance. This control method is based only on online measurement of ethanol. For the yield coefficient, a rough estimation (e.g., from literature) seems sufficient. They identify the state between fermentative and respirative operation as another way to control the specific growth rate close to the critical value when overflow metabolism occurs. They initially employed the controller in laboratory-scale fermentation [41] with a set-point of 0.7 gL^{-1} ethanol concentration, achieving very small errors in the controlled variable. Later in the cultivation, they observe an accumulation of ethanol, considering the limitation of oxygen. This is considered in a subsequent investigation [42], leading to a redesign in the controller scheme employing a feedforward term and determination of the OTR from exhaust gas measurements. The performance of the new proposed controller is evaluated via simulation studies of the process with offline data. The same control algorithm was later [43] employed in an industrial fermentation process. The authors claim 40 % increased productivity using their algorithm compared with the previously used open-loop control.

Cannizzaro et al. [44] and Valentinotti et al. [45] describe a linearization approach capturing the main macroscopic processes, exponential substrate uptake, and very low ethanol production at laboratory scale. They suggest it as another way to control the specific growth rate close to the critical value when overflow metabolism occurs. They maintain the overflow metabolite concentration (ethanol and acetate for yeast and *E. coli*, respectively) and view cell growth as a perturbation to the system. They introduce an adaptive control strategy for the unstable exponential disturbance and are able to hold the ethanol concentration at 0.7 gL^{-1} while the biomass grows exponentially at a specific growth rate of 0.1 h^{-1}, despite only the overflow metabolite being obtained online. Hocalar and Türker [46] present an upscaling of this control approach to a 25-m^3 airlift reactor with online ethanol, CO_2, and O_2 measurements. They present good results for ethanol control around 0.7 gL^{-1} and high biomass concentration of up to 75 gL^{-1} with mean specific growth rate of 0.1 h^{-1}.

3.4 Fuzzy Logic-Based Control

Fuzzy logic uses linguistic expressions to handle uncertainties. It does not need a mathematical model but rule-based process knowledge of an expert operator. In fuzzy control the control action is executed by a predefined rule basis, using rather imprecise linguistic expressions. The expansion of crisp, true/false-based logic to a vague, partially true linguistic concept is required, because operators are more familiar with this. In fuzzy logic, process variables such as pH and temperature whose values can be very *high, low*, or *middle* are formulated in expressions. Then, fuzzy sets are used to represent these linguistic values. A classical set such as $A = \{x \mid 5 < x < 7,\ x \in \Re\}$ contains all the real numbers between 5 and 7. An extended version of this classical, crisp set is a fuzzy set. The fuzzy set B is defined as a set of ordered pairs $B = \{(x, \mu_B(x)) \mid x \in X, \mu_B: X \rightarrow [0, 1]\}$, where X contains all elements of measurement values that can occur, and $\mu_B(x)$ is the membership function of x in B that maps each element of X to a membership value. An element belongs to a set to a certain degree, which is expressed by the membership function, which can be a value between 0 and 1 (e.g., "the temperature in the reactor is high to a degree of 0.7"). The membership function can be of any shape. Triangual, Gaussian, or sigmoidal functions are often used. To demonstrate the operation of a fuzzy controller, a simplified example is presented in Fig. 4 with just two rules:

Rule 1: If substrate is *low* OR DO is *high* then feeding rate is *high*;
Rule 2: If substrate is *high* AND DO is *low* then feeding rate is *low*.

The control action is calculated in three steps: (1) fuzzification, (2) inference, and (3) defuzzification. In the fuzzification step the membership function is used to calculate the membership value of the measurements for the corresponding linguistic term. In the inference step the fuzzy operators (here OR and AND) are

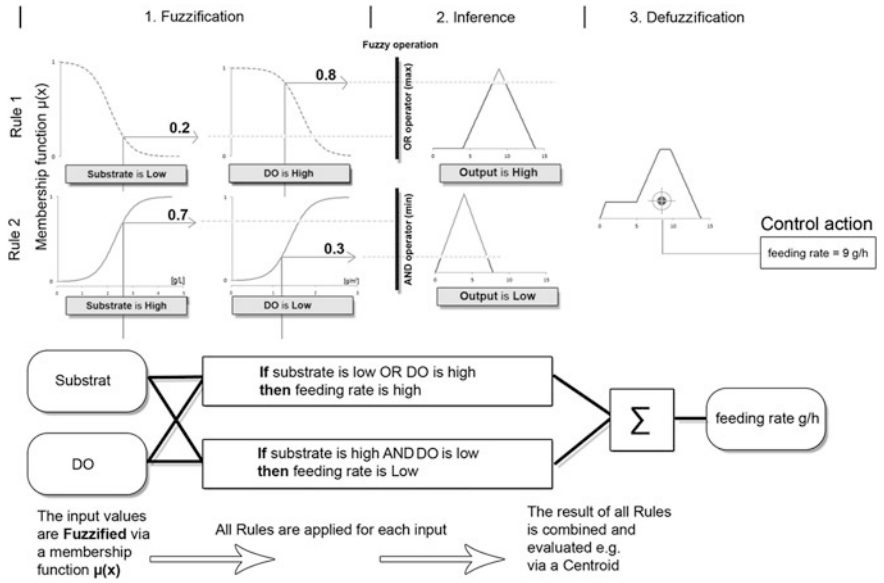

Fig. 4 Basic fuzzy logic scheme for two input values, two rules, and one output value

applied, which is equivalent to the maximum and minimum determination of the membership values of a rule, respectively. For each rule the so-obtained value is used as the upper limit value for the conclusion ("then" part) of the rule. During the defuzzification, the output fuzzy sets of all rules are aggregated to one output fuzzy set as shown in Fig. 4. The centroid of the output fuzzy set is calculated as the value for the actuating variable of the controller.

A fuzzy controller using nine rules was implemented by Ruano et al. [47] for a biological nitrification process in a pilot plant with wastewater from a full-scale plant. Instead of using an expensive nitrogen sensor, they employed several pH, oxidation–reduction potential (ORP), and DO sensors. Their fuzzy controller comprises two independent controllers: the nitrification as well as the denitrification process controller. The former works as a supervisory control of the aeration control system, whereas the latter modifies the internal recycle flow rate from the aerobic to the anoxic reactor. The authors demonstrated that using low-cost sensors in combination with their fuzzy controller leads to a minimized energy consumption of the process.

For the temperature control of a batch reactor, Causa et al. [48] compared different versions of a hybrid fuzzy predictive controller. Two on/off input valves and a discrete-position mixing valve were used as controlled variables. The authors concluded that the hybrid fuzzy predictive control in combination with an optimization algorithm based on a genetic algorithm gives similar performance to that of a typical hybrid predictive control strategy but a significant saving with

respect to computation time. Compared with a nonlinear optimization algorithm [49–51], the genetic algorithms make a time saving of approximately 25 %.

A nonlinear fuzzy controller was presented by Cosenza and Galluzzo [52] for the control of pH and temperature during a penicillin production process. In their application the authors used the so-called type 2 fuzzy set, where uncertainty in the membership function is also implemented. If no uncertainty is present, the membership function is as described above, which is called type 1. In simulations, the performance of the type 2 fuzzy controller was compared with an ordinary (type 1) fuzzy controller as well as a PID controller. It was concluded by the authors that, due to the nonlinearities and uncertainty of the process, the PID controller cannot be compared with the fuzzy controller equitable. The best results were obtained with the type 2 fuzzy controller. When increasing the measurement noise level, the difference between type 1 and type 2 becomes more evident.

A special controller based on fuzzy logic has been developed by Takagi and Sugeno [53]. The difference from the abovementioned fuzzy logic systems is that, in the conclusion part, a function is defined with the input values as arguments. The conclusion of the whole rule system is the sum of the function values weighted by the corresponding membership functions. Belchior et al. [54] implemented an adaptive Takagi–Sugeno (TS) fuzzy control algorithm for DO of an activated sludge wastewater treatment process, where the parameters of the conclusion were adapted online. The controller was constructed by using the Lyapunov synthesis approach with a parameter projection algorithm. Parallel to the adaptive control algorithm, the authors implemented a supervisory fuzzy control with a smooth switching scheme between supervisory and nonsupervisory modes. In simulations, they could demonstrate that the error obtained by the fuzzy controller was less than 2 %, whereas a PI controller produced peaks greater than 10 %.

3.5 Artificial Neural Network-Based Control

Artificial neural networks copy the functionality as well as the structure of biological neural networks by using a mathematical model. In such networks, a possibly very complex input vector (e.g., a visual and/or acoustical signal) can be transferred via various neurons to condense information. Figure 5 presents a basic artificial neural network (ANN) with four inputs in the input layer, five neurons in the hidden layer, and two outputs in the output layer. The structure of an ANN can vary in the number of layers, the connections of the layers, and the number of neurons in each layer, depending on the complexity the network is supposed to map. Even more outputs than available inputs can be generated. To determine whether an artificial neuron will “transmit” a signal, all its weighted input values are employed as arguments of a transfer function, which can be, e.g., a sigmoidal function for smooth transitions or a step function for on/off behavior.

Corresponding to a biological neural network, an artificial neural network needs to be trained for pattern recognition or decision making. During training the

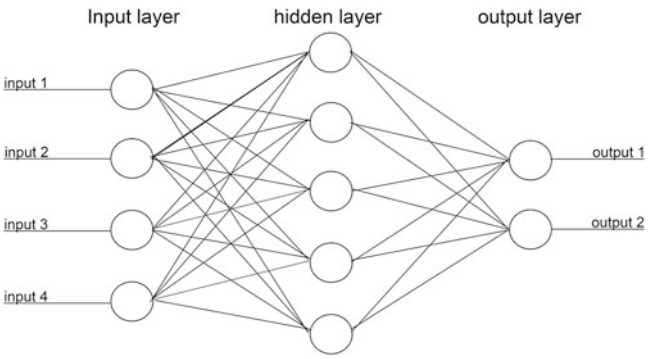

Fig. 5 Basic artificial neural network with four inputs in the input layer, five neurons in the hidden layer, and two outputs in the output layer

parameters, here called weighting factors, are calculated by an optimization algorithm. The weighting factors are used to weight each input of a neuron. The sum of the weighted inputs is used as an argument of the activation function to calculate the output of the neuron. A vast amount of different training data is necessary to build the training sets for a certain problem. Greater variety in the training data leads to better prediction performance in unknown scenarios and prevents that the training data are only memorized.

Karakuzo et al. [55] presented an ANN soft sensor with fuzzy controller for fed-batch fermentations of baker's yeast. The performance of the controller was compared with that of a controller using a theoretical model-based estimation of the specific growth rate. As input to the network, the exhaust gas O_2 and CO_2 concentrations, the feeding rate, as well as the temperature and pH were used (five input neurons). The neural network consists of six neurons in the hidden layer and one output neuron to estimate the specific growth rate. For globally robust training data of their ANN, cultivation datasets under a lot of different process conditions were necessary: The authors generated a training dataset containing 360 patterns and an evaluation dataset with the same number of patterns from cultivation data. The results of the model predictive controller they used for comparison gave satisfactory estimation for the specific growth rate, however only under fixed inoculum sizes. The potential of their ANN becomes obvious during the change of inoculum sizes. The ANN continues to generate reliable estimations for the specific growth rate. They also applied a fuzzy logic controller for air flow and feeding control based on the ANN soft sensor specific growth rate estimation. They performed simulation studies with this controller setup, leading to acceptable results for large-scale applications.

Gadkar et al. [56] presented an online adaptive neural network as a soft sensor that estimates the substrate, ethanol, and biomass concentrations based on dissolved oxygen measurements (one input neuron, three output neurons) during *S. cerevisiae* fermentation. Their neural network had three hidden layers with ten, eight, and four neurons, respectively. They discuss the performance with and

without online adaption of the weights in the layers of the ANN. Based on the estimation and a mass balance equation, the feed rate was calculated by the controller to maintain the glucose concentration at the desired set-point. During controlled substrate fermentations with concentrations between 0.8 and 1.0 gL^{-1} and specific growth rate around 0.2 h^{-1}, the functional efficiency of the control algorithm was demonstrated. The calculation time for the weight adaption is 1–2 s, allowing online implementation. They admit the need from a priori offline data, mirroring different cultivation behaviors for training purposes, although in industrial plants such information is usually available. Furthermore, they carried out simulation studies with more than one measured variable and concluded that more than one measured variable will significantly increase the precision of the control. Especially the online adaption of the weighting factors of the ANN seems promising, leading to a broader range of applications even outside the training domain.

3.6 Model Predictive Control

In the model predictive control (MPC) strategy a dynamic model of a process is applied to simulate the future evolution of the process depending on possible simulated values of the controlled variable. Typically the future evolution will only be calculated up to a predefined prediction horizon. Using an optimization algorithm the best value of the controlled variable is calculated using a cost function. Due to the fact that a differential equation system must be solved online, MPC is computationally demanding. Therefore, for MPC a state estimator as well as a controller is required.

Improved understanding of penicillin formation mechanisms, morphological features, and the role of mycelia in the synthesis led Ashoori et al. [57] to implement a detailed unstructured model of penicillin production in a fed-batch fermenter. This model was used to implement a nonlinear MPC (NMPC) to control the feed rate to increase penicillin formation. As the controller input they apply online measurements of pH and temperature. They propose a novel cost function, applying the inverse of the product rather than the common quadratic relation. This is implemented to avoid ordinary differential equation solver problems where it is not possible to guarantee the efficiency of set-point tracking. They compare the control performance with a regular autotuned PID controller and identify the NMPC as superior with higher process yields. The NMPC controls the acid as well as the base flow, and the cooling water system. Due to the more sophisticated model, the control achieves better performance than in a previous work by Birol et al. [58]. To address the computational cost of this more detailed model, they propose the application of a locally linear model tree (LoLiMoT) to simplify the original nonlinear model, as described in the next section.

Certainly due to the high computational power that needs to be provided for a MPC, many NMPC approaches are still only proven by simulation but have not yet

been applied to real processes. Santos et al. [59] are working on simulated *E. coli* NMPC-controlled cultivations. They assume the measurement of the substrate concentration and keep the specific growth rate at the maximum oxidative capacity as well as inhibiting product formation. They applied a special NMPC scheme named min–max-based robustness consideration. Another new NMPC method as well as a comparative performance assessment were presented by Kawohl et al. [10]. They compared the performance of NMPC and NMPC–EKF for input signal prediction to a method called online trajectory planning (OT). OT is basically an NMPC in which the estimation horizon is extended to the end of the cultivation. If the system is strongly disturbed, this method has certain advantages for the estimation in order to return to optimal productivity, albeit at the cost of computational power. The experiments were carried out through Monte Carlo simulations, simulating experiments through disturbance scenarios. The aim of the experiments was to maintain the optimal productivity of the product penicillin. The authors show the potential of this closed-loop control by improving the mean productivity by 25 % for the MPC and 28 % for the OT method compared with open-loop control, where these methods especially increase the minimum productivity due to disturbances.

3.6.1 ANN–Fuzzy Hybrid-Based Estimation for NMPC Control

A possibility to decrease the complexity of nonlinear models in control algorithms such as MPC is provided by locally linear models, which are applied in a hybrid structure by combining the abilities of a neural network and fuzzy logic. The basic structure is displayed in Fig. 6. Each neuron in the hidden layer consists of a membership function and a local linear model (LLM). The arguments of the membership function are the input value x_i. The function value itself indicates the validity of the corresponding LLM, which is in fact a multilinear regression model. The estimate of this model type is the sum of the LLM output weighted by the normalized membership function.

The algorithm was successfully applied by Ashoori et al. [57] to generate a neuro–fuzzy model to replace equations in a mass balance model for penicillin formation. The authors assessed the resulting computational costs as very acceptable for a real-time process. They showed results which are comparable to results generated by the whole model. Although the method is rarely applied for biotechnological applications, it provides opportunities to overcome frequently mentioned computational limits.

Simulation studies for optimal model training, parameter identification, and comparisons between closed-loop performance are presented by Xu et al. [60–62]. Among others, they employed the LOLIMOT algorithm to achieve optimal parameters for the membership function as well as for the LLM. The LOLIMOT algorithm is an incremental tree-based learning algorithm. A detailed description can be found in Nelles [63]. The algorithm adds consecutive locally linear model neurons and thereby optimizes the error of calibration. Obviously, a large number

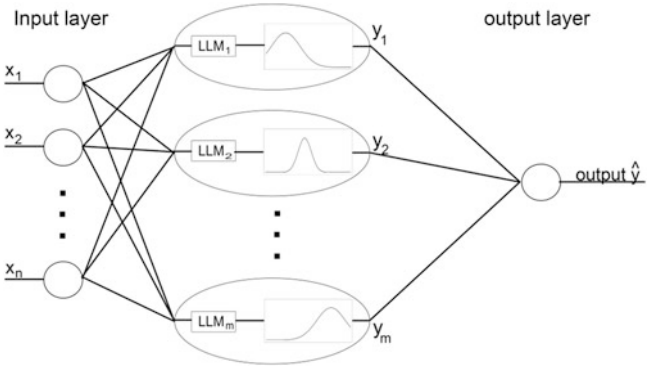

Fig. 6 Basic structure of a fuzzy–ANN local linear model

of neurons will describe a trajectory best, but will possibly not decrease the required computational power.

3.6.2 ANN-Based Estimation for NMPC

Meleiro et al. [64] presented results of a MPC strategy of a fuel-ethanol fermentation process using simulations. A neural network was applied as the internal model for the controller. The authors used an optimization algorithm to determine the neural network structure as well as the shape of their activation functions, guiding the parsimonious network architecture. The inputs were the feed flow rate, cell recycle rate, and flash recycle rate; the output were the biomass, substrate, and product concentrations. The authors presented results demonstrating successful control of the biomass, substrate, and ethanol concentrations with set-points varying between 37 and 32 gL^{-1}, 10 and 3 gL^{-1}, and 45 and 40 gL^{-1}, respectively.

3.7 Probing Feeding Controller Strategy

Velut et al. [65] presented a probing feeding strategy for *E. coli* fermentations, operating close to the maximum oxygen transfer rate capacity. The principle of the probing feeding strategy is to superimpose a short glucose pulse onto the glucose feeding flow and evaluate the response in the dissolved oxygen signal. If the dissolved oxygen level decreases, the feed rate is increased, due to the determined capacity. If no response can be detected, the feeding rate is decreased. The technique is combined with DO control, which is performed by adjusting the stirrer speed and the temperature control to decrease the oxygen demand when the reactor is at its maximum oxygen transfer capacity. They present the performance of the combined controller in an *E. coli* cultivation. Due to the probing

feeding control, no acetate was produced. The dissolved oxygen concentration was adjusted to a set point of 30 % over 22 h while decreasing the temperature from 36 to 25 °C.

For the probing feeding strategy, Velut et al. [66] examined the effect of reactor scale as well as the influence of different medium types. They applied 1.5-min glucose pulses for laboratory-scale fermenters and longer pulses of 3 min for large-scale fermenters to compensate for their slower response. The probing feeding strategy showed good results independent of the medium used. However, the use of a complex medium leads to complications in the interpretation of the pulse response. They determine that the pulsed feeding does not harm the productivity and propose an optimized predetermined feeding trajectory with additional superimposed pulses only for monitoring purposes.

This control strategy was also employed by Xue and Fan [67] at laboratory and pilot plant (500 L) scale for a recombinant *E. coli* strain producing human-like collagen. For the laboratory-scale experiments, they obtained similar results to previous optimized studies with 69.1 gL^{-1} dry cell weight and 13.1 gL^{-1} human-like collagen. Compared with previous experiments, they observe a reduction of the resulting dry cell weight when applied to the pilot plant. They assume that this results from the different oxygen transfer capacity. However, the collagen production of 9.6 gL^{-1} was a satisfying result. They therefore present a successful application of the probing feeding strategy in a pilot-plant-scale fermentation process.

3.8 Extremum-Seeking Control

Extremum-seeking control is a gradient method to determine online unknown parameters through the analysis of measurement results as a response to a periodical excitation signal called dither. Dochain et al. [68] present a survey on two important classes of extremum-seeking control: the perturbation-based and the model-based methods. They investigate the applicability to processes and reaction systems using theoretical models and show the theoretical efficiency of this closed-loop control algorithm. Cougnon et al. [69] carried out numerical simulation studies on a fed-batch process model to illustrate their performance for closed-loop control of bioprocesses. They present an adaptive extremum-seeking controller. The controller drives the system to an unknown desired set-point in order to maximize biomass production. In this contribution the authors assume that the primary carbon source is measurable.

Dewasme et al. [70] note that model-based controls are subject to high uncertainty. Therefore, they present a model-free extremum-seeking strategy in a simulation study on *S. cerevisiae.* They present simulations where the tracking of the critical substrate level (border between fermentative and reparative metabolism) is correctly performed by two different gradient estimation procedures. The input variables for the algorithm are OUR and CPR. The actuating variable is the

feeding rate. When parameter uncertainties and noise disturbances occur, they determined that a recursive least-squares formulation was simple to implement and improved the resulting set-point tracking.

3.9 Control Based on A Heuristic Procedure

Spectroscopic measurements provide a wide range of information based on the interaction of electromagnetic radiation with matter. At the same time, this abundance of information is the reason why it is often difficult to interpret. Advanced mathematical tools such as partial least squares or principle component analysis are employed to overcome this information overload. Hantelmann et al. [71] present a new method to monitor and control *S. cerevisiae* cultivations by two-dimensional (2D) in situ fluorescence spectroscopy. They introduce a chemometric model that is derived from multivariate data analysis. The glucose feeding rate is thereby controlled, predicting the metabolic state directly from the fluorescence intensities. The glucose concentration was held between 0.4 and 0.5 g L^{-1} over 11 h, completely avoiding ethanol formation. They point out that the BioView® technology that they used for cultivation control is suitable for industrial environments.

Schenk et al. [72] present a soft sensor based on mid-infrared spectroscopy. They introduce a simple and fast method to calibrate the instrument for *Pichia pastoris* fermentations. For this purpose, they assume that only the substrate concentration will change significantly during the cultivation and that the absorbance is proportional to the concentration. The control action is performed using a PI controller. They propose that, in some cases such as *Pichia pastoris*, a multivariate calibration procedure is not necessary and the measurement of one compound of interest is sufficient (methanol in the case of *P. pastoris*). The calibration is performed in situ using two points: one spectrum at the beginning without carbon source, and one with carbon source. They carried out six cultivations in the range of 0.8–15 gL^{-1} to demonstrate the performance of the control system. The standard error of prediction over all cultivations was 0.12 gL^{-1}. They point out that long-term baseline instability had an influence on the accuracy, which could be addressed using linear correction of the signal. Even though the method was designed for the special case of *P. pastoris*, the authors mention a possible application for other microorganisms.

4 Conclusions

In this chapter, different approaches of open-loop and closed-loop control for bioprocess automation are discussed. As a result of the diversity of bioprocess requirements, a single control algorithm cannot be applied in all cases; rather,

different approaches are necessary. During the last 6 years (2006–2012), 97 contributions dealing with closed-loop control for fermentation processes have been published (according to Science Direct, found by "closed loop control" AND fermentation OR cultivation). However, only a minority of up to 30 applications were actually applied to real bioprocesses; the majority were based on theoretical applications using simulated processes.

For closed-loop control applications, in the majority of cases, a soft sensor is combined with a PID controller to determine the feeding rate of substrate or the specific growth rate. This approach can be combined with a forward loop, to reduce the problems generated by the dynamics of the bioprocess. The reason why soft sensors have attained this level of importance for control purposes is the lack of direct measurements or their large additional expense for robust and reliable online measurement systems.

Model predictive control has been successfully applied in other application fields, and its importance will increase in bioprocess automation as well. However, reliable and robust process models are required, as well as very powerful computers to address the computational demands. The lack of theoretical bioprocess models is compensated by hybrid systems combining theoretical models, fuzzy logic, and/or artificial neural network methodology. These systems are supposed to combine the advantages of each approach into a well-performing control strategy.

Some applications involve control of fermentations at their oxidative maximal capacity such as the probing feeding approach or control based on the metabolic state, tolerating small amounts of overflow metabolism. Again, both approaches demonstrate the ability to control the system without direct measurements of important process variables such as oxidative capacity or the metabolic state of microorganisms.

Although many authors suggest a possible transfer of their presented control application to other bioprocesses, the algorithms are mostly specialized to certain organisms or certain cultivation condition as well as to a specific measurement system. The effort required to adapt the algorithm and the required measurement system to a specific application is still very large. Therefore, in the near future, closed-loop control of the feeding rate or growth rate will remain a challenge.

Furthermore, as Max Planck said, "A new scientific truth does not triumph by convincing its opponents and making them see the light, but rather because its opponents eventually die, and a new generation grows up that is familiar with it."

References

1. Becker T, Hitzmann B, Muffler K, Pörtner R, Reardon K, Stahl F, Ulber R (2007) Future aspects of bioprocess monitoring. In: Ulber R, Sell D (eds) Advances in biochemical engineering/biotechnology vol 105. Springer, Berlin, pp 249–293. doi:10.1007/10_2006_036
2. Navrátil M, Norberg A, Lembrén L, Mandenius C-F (2005) On-line multi-analyzer monitoring of biomass, glucose and acetate for growth rate control of a vibrio cholerae fed-batch cultivation. J Biotechnol 115(1):67–79. doi:10.1016/j.jbiotec.2004.07.013

3. Warth B, Rajkai G, Mandenius CF (2010) Evaluation of software sensors for on-line estimation of culture conditions in an *Escherichia coli* cultivation expressing a recombinant protein. J Biotechnol 147(1):37–45. doi:10.1016/j.jbiotec.2010.02.023
4. Luttmann R, Bracewell DG, Cornelissen G, Gernaey KV, Glassey J, Hass VC, Kaiser C, Preusse C, Striedner G, Mandenius C-F (2012) Soft sensors in bioprocessing: a status report and recommendations. Biotechnol J doi:10.1002/biot.201100506
5. Kadlec P, Gabrys B, Strandt S (2009) Data-driven soft sensors in the process industry. Comput Chem Eng 33(4):795–814. doi:10.1016/j.compchemeng.2008.12.012
6. Jain G, Jayaraman G, Kökpinar Ö, Rinas U, Hitzmann B (2011) On-line monitoring of recombinant bacterial cultures using multi-wavelength fluorescence spectroscopy. Biochem Eng J 58–59:133–139. doi:10.1016/j.bej.2011.09.005
7. Jenzsch M, Simutis R, Lübbert A (2006) Generic model control of the specific growth rate in recombinant *Escherichia coli* cultivations. J Biotechnol 122(4):483–493
8. Hulhoven X, Wouwer AV, Bogaerts P (2006) Hybrid extended Luenberger-asymptotic observer for bioprocess state estimation. Chem Eng Sci 61(21):7151–7160. doi:10.1016/j.ces.2006.06.018
9. Kalman RE (1960) A new approach to linear filtering and prediction problems. Trans ASME J Basic Eng (82 (Series D)):35–45. doi:citeulike-article-id:347166
10. Kawohl M, Heine T, King R (2007) Model based estimation and optimal control of fed-batch fermentation processes for the production of antibiotics. Chem Eng Process Process Intensif 46(11):1223–1241
11. Lidgren L, Lilja O, Krook M, Kriz D (2006) Automatic fermentation control based on a real-time in situ SIRE® biosensor regulated glucose feed. Biosens Bioelectron 21(10):2010–2013
12. Kriz D, Berggren C, Johansson A, Ansell RJ (1998) SIRE-technology. Part I. Amperometric biosensor based on flow injection of the recognition element and differential measurements. Instrum Sci Technol 26(1):45–57. doi:10.1080/10739149808002089
13. Gnoth S, Jenzsch M, Simutis R, Lübbert A (2007) Process analytical technology (PAT): batch-to-batch reproducibility of fermentation processes by robust process operational design and control. J Biotechnol 132(2):180–186
14. Arndt M, Hitzmann B (2004) Kalman filter based glucose control at small set points during fed-batch cultivation of *Saccharomyces cerevisiae*. Biotechnol Prog 20(1):377–383. doi:10.1021/bp034156p
15. Arndt M, Kleist S, Miksch G, Friehs K, Flaschel E, Trierweiler J, Hitzmann B (2005) A feedforward feedback substrate controller based on a Kalman filter for a fed-batch cultivation of *Escherichia coli* producing phytase. Comput Chem Eng 29(5):1113–1120
16. Kleist S, Miksch G, Hitzmann B, Arndt M, Friehs K, Flaschel E (2003) Optimization of the extracellular production of a bacterial phytase with *Escherichia coli* by using different fed-batch fermentation strategies. Appl Microbiol Biotechnol 61(5):456–462. doi:10.1007/s00253-003-1229-3
17. Klockow C, Hüll D, Hitzmann B (2008) Model based substrate set point control of yeast cultivation processes based on FIA measurements. Anal Chim Acta 623(1):30–37. doi:10.1016/j.aca.2008.06.011
18. Roeva O, Slavov T, Dimov I, Dimova S, Kolkovska N (2008) fed-batch cultivation control based on genetic algorithm PID controller tuning numerical methods and applications, vol 6046. Lecture Notes in Computer Science. Springer, Berlin, pp 289–296. doi:10.1007/978-3-642-18466-6_34
19. Tsonyo S, Roeva O (2011) Genetic algorithm tuning of PID controller in smith predictor for glucose concentration control. Int J BIO Autom 15(2):101–114
20. Wahab NA, Katebi R, Balderud J (2009) Multivariable PID control design for activated sludge process with nitrification and denitrification. Biochem Eng J 45(3):239–248
21. Biener R, Steinkämper A, Hofmann J (2010) Calorimetric control for high cell density cultivation of a recombinant *Escherichia coli* strain. J Biotechnol 146(1–2):45–53
22. Biener R, Steinkämper A, Horn T (2012) Calorimetric control of the specific growth rate during fed-batch cultures of *Saccharomyces cerevisiae*. J Biotechnol 160(3–4):195–201

23. Soons ZITA, Voogt JA, van Straten G, van Boxtel AJB (2006) Constant specific growth rate in fed-batch cultivation of *Bordetella pertussis* using adaptive control. J Biotechnol 125(2):252–268
24. Bodizs L, Titica M, Faria N, Srinivasan B, Dochain D, Bonvin D (2007) Oxygen control for an industrial pilot-scale fed-batch filamentous fungal fermentation. J Process Control 17(7):595–606
25. Chung YC, Chien IL, Chang DM (2006) Multiple-model control strategy for a fed-batch high cell-density culture processing. J Process Control 16(1):9–26
26. Ranjan AP, Gomes J (2009) Simultaneous dissolved oxygen and glucose regulation in fed-batch methionine production using decoupled input output linearizing control. J Process Control 19(4):664–677
27. Davison E (1976) Multivariable tuning regulators: the feedforward and robust control of a general servomechanism problem. IEEE Trans Autom Control 21(1):35–47. doi:10.1109/tac.1976.1101126
28. Penttinen J, Koivo HN (1980) Multivariable tuning regulators for unknown systems. Automatica 16(4):393–398
29. Maciejowski JM (1989) Multivariable feedback design. Addison-Wiley, Upper Saddle River
30. Bastin G, Dochain D (1990) On-line estimation and adaptive control of bioreactors, vol 1. Elsevier, Amsterdam
31. Roeva O, Slavov T, Dimov I, Dimova S, Kolkovska N (2011) Fed-batch cultivation control based on genetic algorithm PID controller tuning numerical methods and applications. vol 6046. Lecture Notes in Computer Science. Springer, Berlin, pp 289–296. doi:10.1007/978-3-642-18466-6_34
32. Roeva O (2008) Improvement of genetic algorithm performance for identification of cultivation process models. Paper presented at the proceedings of the 9th WSEAS international conference on evolutionary computing, Sofia, Bulgaria
33. Roeva O (2005) Genetic algorithms for a parameter estimation of a fermentation process model: a comparison. Bioautomation 3:19–28
34. Perrier M, de Azevedo SF, Ferreira EC, Dochain D (2000) Tuning of observer-based estimators: theory and application to the on-line estimation of kinetic parameters. Control Eng Pract 8(4):377–388
35. Mazouni D, Ignatova M, Harmand J (2004) A simple mass balance model for biological sequencing batch reactors used for carbon and nitrogen removal. Automatic systems for building the infrastructure in developing countries, vol IFAC-DECOM04. Bansko, Bulgaria
36. Lyubenova V, Ignatova M, Novak M, Patarinska T (2007) Reaction rates estimators of fed-batch process for poly- b-hydroxybutyrate (PHB) production by mixed culture. Biotechnol BioE 21(1):113–116
37. Ignatova M, Lyubenova V (2007) Control of class bioprocesses using on-line information of intermediate metabolite production and con-sumption rates. Acta universitatis cibiniensis Series E: Food Technol 11:3–16
38. Ignatova M, Lyubenova V (2007) Adaptive control of fed-batch process for poly-b hydroxybutyrate production by mixed culture. Acad Sci 60(5):517–524
39. Kansha Y, Jia L, Chiu MS (2008) Self-tuning PID controllers based on the Lyapunov approach. Chem Eng Sci 63(10):2732–2740
40. Chang WD, Hwang RC, Hsieh JG (2002) A self-tuning PID control for a class of nonlinear systems based on the Lyapunov approach. J Process Control 12(2):233–242. doi:10.1016/s0959-1524(01)00041-5
41. Renard F, Vande Wouwer A, Valentinotti S, Dumur D (2006) A practical robust control scheme for yeast fed-batch cultures—an experimental validation. J Process Control 16(8):855–864. doi:10.1016/j.jprocont.2006.02.003
42. Renard F, Vande Wouwer A (2008) Robust adaptive control of yeast fed-batch cultures. Comput Chem Eng 32(6):1238–1248. doi:10.1016/j.compchemeng.2007.05.008

43. Dewasme L, Richelle A, Dehottay P, Georges P, Remy M, Bogaerts P, Vande Wouwer A (2010) Linear robust control of *S. cerevisiae* fed-batch cultures at different scales. Biochem Eng J 53(1):26–37. doi:10.1016/j.bej.2009.10.001
44. Cannizzaro C, Valentinotti S, von Stockar U (2004) Control of yeast fed-batch process through regulation of extracellular ethanol concentration. Bioprocess Biosyst Eng 26(6): 377–383. doi:10.1007/s00449-004-0384-y
45. Valentinotti S, Srinivasan B, Holmberg U, Bonvin D, Cannizzaro C, Rhiel M, von Stockar U (2003) Optimal operation of fed-batch fermentations via adaptive control of overflow metabolite. Control Eng Pract 11(6):665–674. doi:10.1016/s0967-0661(02)00172-7
46. Hocalar A, Tüker M (2010) Model based control of minimal overflow metabolite in technical scale fed-batch yeast fermentation. Biochem Eng J 51(1):64–71
47. Ruano MV, Ribes J, Seco A, Ferrer J (2012) An advanced control strategy for biological nutrient removal in continuous systems based on pH and ORP sensors. Chem Eng J 183: 212–221. doi:10.1016/j.cej.2011.12.064
48. Causa J, Karer G, Núnez A, Sáez D, Skrjanc I, Zupancic B (2008) Hybrid fuzzy predictive control based on genetic algorithms for the temperature control of a batch reactor. Comput Chem Eng 32(12):3254–3263
49. Potocnik B, Music G, Zupancic B (2004) Model predictive control systems with discrete inputs. In: Electrotechnical conference, 2004. MELECON 2004. Proceedings of the 12th IEEE mediterranean, 12–15 May 2004, pp 383–386 vol 381.doi:10.1109/melcon.2004.1346886
50. Karer G, Mušič G, Škrjanc I, Zupančič B (2007) Hybrid fuzzy modelling for model predictive control. J Intell Robot Syst 50(3):297–319. doi:10.1007/s10846-007-9166-5
51. Karer G, Musšič G, Škrjanc I, Zupančič B (2007) Hybrid fuzzy model-based predictive control of temperature in a batch reactor. Comput Chem Eng 31(12):1552–1564
52. Cosenza B, Galluzzo M (2011) Nonlinear fuzzy control of a fed-batch reactor for penicillin production. Comput Chem Eng 36:273–281
53. Takagi T, Sugeno M (1985) Fuzzy identification of systems and its applications to modeling and control. IEEE Trans Syst Man Cybernet 15(1):116–132
54. Belchior CAC, Araujo RAM, Landeck JAC (2011) Dissolved oxygen control of the activated sludge wastewater treatment process using stable adaptive fuzzy control. Comput Chem Eng 37:152–162
55. Karakuzu C, Türker M, Öztürk S (2006) Modelling, on-line state estimation and fuzzy control of production scale fed-batch baker's yeast fermentation. Control Eng Pract 14(8):959–974
56. Gadkar KG, Mehra S, Gomes J (2005) On-line adaptation of neural networks for bioprocess control. Comput Chem Eng 29(5):1047–1057
57. Ashoori A, Moshiri B, Khaki-Sedigh A, Bakhtiari MR (2009) Optimal control of a nonlinear fed-batch fermentation process using model predictive approach. J Process Control 19(7):1162–1173
58. Birol G, Ündey C, Cinar A (2002) A modular simulation package for fed-batch fermentation: penicillin production. Comput Chem Eng 26(11):1553–1565
59. Santos LO, Dewasme L, Coutinho D, Wouwer AV (2011) Nonlinear model predictive control of fed-batch cultures of micro-organisms exhibiting overflow metabolism: assessment and robustness. Comput Chem Eng 39:143–151
60. Xu Z, Zhao J, Qian J, Zhu Y (2009) Nonlinear MPC using an identified LPV model. Ind Eng Chem Res 48(6):3043–3051. doi:10.1021/ie801057q
61. Gong Z (2009) A multistage system of microbial fed-batch fermentation and its parameter identification. Math Comput Simul 80(9):1903–1910
62. Lawryńczuk M (2011) Online set-point optimisation cooperating with predictive control of a yeast fermentation process: a neural network approach. Eng Appl Artif Intell 24(6):968–982. doi:10.1016/j.engappai.2011.04.007
63. Nelles O (2001) Nonlinear system identification. Springer, Berlin

64. Meleiro LAC, Von Zuben FJ, Filho RM (2009) Constructive learning neural network applied to identification and control of a fuel-ethanol fermentation process. Eng Appl Artif Intell 22(2):201–215. doi:10.1016/j.engappai.2008.06.001
65. Velut S, de Marco L, Hagander P (2007) Bioreactor control using a probing feeding strategy and mid-ranging control. Control Eng Pract 15(2):135–147
66. Velut S, Castan A, Short KA, Axelsson JP, Hagander P, Zditosky BA, Rysenga CW, De Maré L, Haglund J (2007) Influence of bioreactor scale and complex medium on probing control of glucose feeding in cultivations of recombinant strains of *Escherichia coli*. Biotechnol Bioeng 97(4):816–824. doi:10.1002/bit.21294
67. Xue WJ, Fan DD (2011) Fed-batch production of human-like collagen with recombinant *Escherichia coli* using feed-up DO-transient control. Huaxue Gongcheng/Chem Eng (China) 39(10):6–10
68. Dochain D, Perrier M, Guay M (2011) Extremum seeking control and its application to process and reaction systems: a survey. Math Comput Simul 82(3):369–380. doi:10.1016/j.matcom.2010.10.022
69. Cougnon P, Dochain D, Guay M, Perrier M (2011) On-line optimization of fedbatch bioreactors by adaptive extremum seeking control. J Process Control 21(10):1526–1532. doi:10.1016/j.jprocont.2011.05.004
70. Dewasme L, Srinivasan B, Perrier M, Vande Wouwer A (2011) Extremum-seeking algorithm design for fed-batch cultures of microorganisms with overflow metabolism. J Process Control 21(7):1092–1104. doi:10.1016/j.jprocont.2011.05.002
71. Hantelmann K, Kollecker M, Hüll D, Hitzmann B, Scheper T (2006) Two-dimensional fluorescence spectroscopy: a novel approach for controlling fed-batch cultivations. J Biotechnol 121(3):410–417. doi:10.1016/j.jbiotec.2005.07.016
72. Schenk J, Marison IW, von Stockar U (2007) A simple method to monitor and control methanol feeding of *Pichia pastoris* fermentations using mid-IR spectroscopy. J Biotechnol 128(2):344–353. doi:10.1016/j.jbiotec.2006.09.015

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An Advanced Monitoring Platform for Rational Design of Recombinant Processes

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Abstract Bioprocess engineering is an application-oriented science in an interdisciplinary environment, and a meaningful combination of different scientific disciplines is the only way to meet the challenges of bioprocess complexity. Setting up a reasoned process monitoring platform is the first step in an iterative procedure aiming at process and systems understanding, being the key to rational and innovative bioprocess design. This chapter describes a comprehensive process monitoring platform and how the resulting knowledge is translated into new strategies in process and/or host cell design.

Keywords Chemometrics · Host and process design · Prediction · Process monitoring · Recombinant protein production

Abbreviations

ANN	Artificial neural networks
amu	Atomic mass unit
CER	Carbon dioxide evolution rate
CRP	Catabolite regulation protein
CDM	Cell dry mass
cAMP	Cyclic adenosine monophosphate
DC	Dead cells
EMA	European medicines agency
GFP	Green fluorescent protein
ppGpp	Guanosine tetraphosphate
HCDC	High-cell-density cultivation
ICH	International conference on harmonisation
IPTG	Isopropyl β-D-thiogalactopyranoside
MVDA	Multivariate data analysis
MS	Mass spectrometry
NIR	Near-infrared

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OUR	Oxygen uptake rate
ppmV	Parts per million volume
pptV	Parts per trillion volume
PLS	Partial least squares
PCN	Plasmid copy number
PTR-MS	Proton transfer reaction mass spectrometry
QbD	Quality by design
RBF-NN	Radial basis function neural network
RT-PCR	Real-time polymerase chain reaction
RQ	Respiration quotient
RMSEP	Root-mean-square error of prediction
SOD	Superoxide dismutase
TCN	Total cell number
FDA	US food and drug administration
VOC	Volatile organic compound

Contents

1 Introduction

Recombinant biopharmaceutical production processes require qualified process and systems understanding for rational host and process design. As in other industrial processes, yield and product quality are the most important targets in biotechnological production. Due to tight regulatory requirements for manufacturing of

biopharmaceuticals for human use, additional demanding targets with respect to product homogeneity, batch-to-batch reproducibility, and process stability must also be met. Because of this, the pharmaceutical industry sticks to already well-established process designs, control strategies, and expression systems. Also, the approval procedure for new strategies, expression systems, or even changes to existing processes is time and cost intensive.

The state-of-the-art approach in quality assurance is based on comprehensive testing of final product quality, i.e., the so-called quality-by-testing approach. As a consequence, the quality and specification of the final product can only be assured by running the entire process under tight control of a limited number of state variables and by evaluation of the process performance by extensive testing of the product quality afterwards. A resulting problem is that systematic ongoing process optimization and rational process design as practiced in many other industries are widely ruled out. In comparison with standards established in other production industries, bioprocess monitoring and control are less well developed.

The recent guidelines for pharmaceutical development by the International Conference on Harmonisation, e.g., ICH Q8 [1], strongly emphasize the provision of comprehensive understanding of product and manufacturing processes for regulatory inspections. Greater understanding of pharmaceutical manufacturing creates a basis for more flexible regulatory approaches. The information gained from pharmaceutical development studies and manufacturing experience provides scientific understanding for the establishment of the design space, specifications, and manufacturing controls (see also the chapter on QbD and PAT by Rathore et al.).

To realize this flexibility, the applicant should demonstrate enhanced knowledge of product performance over a range of material attributes, manufacturing process options, and process parameters. Such understanding can be gained by application of, for example, formal experimental designs, PAT, and/or prior knowledge. Appropriate use of quality risk management principles can be helpful in prioritizing additional pharmaceutical development studies to collect such knowledge.

This chapter describes how improved process understanding can be accomplished in recombinant protein production processes with *E. coli* by M3C technology. It is shown how iterative process and systems development using an advanced online and offline process monitoring platform has been achieved, new concepts for process control have been introduced, and strategies for host cell design have been successfully put into practice.

1.1 Bioprocess Monitoring

Historically, process monitoring methods employed in bioprocessing were directly transferred from chemical engineering. Although this approach was very efficient, upstream bioprocessing is still viewed as a black box and far behind the state of the art in chemical industry, where real-time measurements of quality properties and

important process variables as well as automatic process control techniques are well established [2]. The main reasons for this difference are that cell-based processes are far more complex than pure chemical processes, that real-time access to physiologically relevant variables is not available, and that proteins as products are large molecules that are extremely sensitive to the manufacturing process. In spite of significant progress in sensor and analyzer technologies, only a few systems have been qualified for bioprocess monitoring [3–5]. The main challenge in this context is to perform measurements under common bioprocess conditions in a sterile environment with living cells as a solid phase in a gas–liquid mixture. Currently, routine online data acquisition is focused on classical variables such as pH, pO_2, pCO_2, rpm, air flow, stirrer speed, temperature, flow rates, and analysis of O_2/CO_2 in exhaust gas (cf. the chapter by Sonnleitner). Exhaust gas analysis can be used to calculate the oxygen uptake rate (OUR), the carbon dioxide evolution rate (CER), and the respiration quotient (RQ), but none of the state variables provide metabolism-related information [6]. State-of-the-art online monitoring tools, such as infrared spectroscopy [7–9], fluorescence spectroscopy [10–13], and dielectric spectroscopy [14, 15], reflect changes in the process, but signals cannot be directly assigned to biological process variables and host cell physiology [16].

More advanced methods include the application of analyzer technologies connected to the bioreactor via accurate interfaces, such as high-performance liquid chromatography [17, 18], gas chromatography [19], mass spectrometry [20], or flow cytometry [21, 22]. By following such strategies, timely information on metabolites, intermediate compounds, or end-products of metabolism excreted to the medium can be obtained. Each of these at-line technologies requires a sampling procedure, sample preparation steps, and a certain time to run the analysis. In this sense, such applications represent the link to offline analysis.

Quantitative and qualitative data on intracellular components definitely yield the highest level of physiology-related information, but are not accessible via direct online measurements [23]. To gain this essential process information, sample preparation steps and biochemical analysis are required.

Setting up an offline measurement platform is a nontrivial task as the set of selected techniques (i) must cover the wide diversity of all molecules of interest and (ii) should allow high sampling frequencies combined with low sample volumes, and (iii) cost–time demands must not exceed justifiable quantities. For more or less all molecule classes in a cellular system, various analytical methods are available but only a limited number meet the above-mentioned criteria. Hence, careful selection and combination of methods is imperative. Directly related to the analytical method and the analyte is the sampling strategy. Accurate sampling and quenching procedures are of highest priority to guarantee representative samples, and the sampling frequency must allow for adaptation to the expected kinetics of molecules of interest. Progress in this field has been strongly supported by systems-biology-based approaches and the development of -omics techniques [24, 25].

The shortcomings in real-time access to physiology-relevant variables can be circumvented by application of chemometric-based approaches. As mentioned,

bioprocess monitoring is heavily impaired by the lack of direct readable online and in situ sensor equipment. However, there has been significant progress in online signal acquisition and sensor technology as well as expansive development in the field of offline biochemical and molecular-biology-based analytical equipment. Therefore, the objective and the opportunity are to close the gap between the broad spectrum of bioanalytical offline methods and online devices. This can be done by the application of mathematical models, multivariate data analysis (MVDA), and computer science to set up "soft sensors" [26]. These sensors can be based on calculations utilizing online-accessible process variables or on correlations between online and offline datasets. Such soft sensors trained on historical datasets can be used to predict complex variables from previously unseen datasets by statistical modeling, such as artificial neural networks (ANNs) or partial least squares (PLS) (cf. chapters by Glassey and by Gernaey). In this context it is important to note that the prediction is only valid for datasets within the previously trained solution space; data cannot be extrapolated.

Moreover, the availability of these complex variables enables the implementation of control strategies that were previously not realizable. In addition to the improved observability of the process, the availability of these complex variables provides clues for the design of new control strategies.

1.2 Recombinant Protein Expression in E. coli Systems

For recombinant organisms the complexity of monitoring is even more pronounced because there are additional variables that have a strong impact on process performance. Genetic engineering techniques offer possibilities to modulate growth and product formation independently from each other, and also provide increased and subtle opportunities to develop process monitoring and control. From a pure control perspective, the genetic features of recombinant host–vector systems should allow improved controllability compared with nonrecombinant production strains, such as inducible promoter systems allowing for control of the recombinant protein synthesis rate. However, current development of recombinant protein production processes is widely empirically driven with restricted predictability.

In *E. coli* the expression of recombinant proteins is generally achieved by placing the foreign gene into a multicopy plasmid vector under control of a constitutive or inducible promoter. In the early use of industrial recombinant processes, only weak expression systems were available and even high-copy-number plasmids did not enable full exploitation of the metabolic potential of the host cell.

Subsequently, genetic-based optimization strategies led to the development of very strong and inducible expression systems such as the T7 system [27]. However, these strong vector systems could not be used efficiently because the product formation period is limited by metabolic breakdown of cells triggered by too high recombinant gene expression rates [28–31]. Hence, the potential of the cell factories

cannot be fully exploited, resulting in too low yield and noncompliant product quality.

During evolution, cellular systems have developed a multitude of mechanisms to cope with a broad range of stress situations, and a complex regulatory network guarantees optimal response reactions [32]. Stress triggered by recombinant gene expression originates in the cell, and the recombinant system is not or only partly controlled by the cellular regulatory network. In consequence, countermeasures of the cellular stress response system are not adequate and often inefficient [30, 31]. The main challenge in host cell design is that understanding of the biology of protein expression is still fragmentary and incomplete.

2 A Process Monitoring Platform for Recombinant Protein Production

The configuration of the established platform for monitoring of recombinant protein production processes with *E. coli* as the host cell is designed according to the demands of these processes. Beside routine monitoring of physical (e.g., agitator speed, flow rates, and temperature) and chemical variables (e.g., pH, pO_2, and CO_2 and O_2 in the off-gas), a series of state-of-the-art bioprocess monitoring devices are implemented in the established online monitoring platform. Online techniques are combined with accurate offline methods to allow for monitoring and measurement of cell growth and cell viability, quantification of recombinant compounds, and characterization of the cellular response to recombinant gene expression. This section describes how we developed such a platform for recombinant protein expression in *E. coli*.

2.1 Monitoring of Biomass and Growth

Biomass, viability, and growth kinetics are among the most critical bioprocess variables and therefore attractive monitoring targets for the process monitoring platform. The classical offline methods for determination of biomass are optical density measurements and gravimetric cell dry mass quantification. In face of the importance of these variables, development of methods for in situ measurement was a focus of the research [33–35]. Despite all these efforts and achievements, real-time measurement remains challenging.

Optical density probes are the most commonly used in situ devices for online biomass estimation. The TruCell™ cell growth monitor (Finesse Solutions AG, Bonaduz, Switzerland), an optical transmission sensor that enables in situ monitoring of fermentation processes, was selected from among the available optical probes.

Recombinant protein production exposes cells to high stress levels and can cause cell death in the course of the production process. As the employed NIR technology does not give any information on cell viability, dielectric spectroscopy was selected as an alternative. This technique makes use of the electrical properties of cells exposed to a radiofrequency electrical field. The nonconducting nature of the cell plasma membrane allows a buildup of charge, so that cells with intact plasma membranes act as capacitors. The resulting capacitance is proportional to the membrane enclosed volume and thereby to the concentration of viable cells [36, 37]. The ABER biomass monitor BM214 M allowing for capacity measurements at two frequencies is employed (Aber Instruments Ltd., Aberystwyth, UK). Although the capacity signal shows a strong correlation with the cell dry mass (CDM), a direct calibration of capacity versus CDM is not feasible as there are changes in cell size in the course of the process [38].

Flow cytometry is used (i) to gain offline information on total cell number (TCN) via ratiometric counting and (ii) to determine the percentage of dead cells (DC) by discrimination of dead cells via staining with propidium [38].

In addition to this set of methods, additional information on cell growth is derived from measurement of base consumption during the process. During growth of bacteria, acids are produced, and there is a close correlation of cell growth with release of acidic metabolites and thereby also with the amount of base needed to maintain the pH at a constant level. In case of using NH_4OH for pH control, for each uptake of NH_4^+ a proton is excreted, which in turn triggers the addition of NH_4OH. By considering the buffer capacity of the nutrient medium, a close correlation between NH_4OH consumption and growth can be derived.

2.2 Monitoring of Metabolites and Cell Compounds

The composition of the extracellular medium changes continuously during the process. There are decreasing concentrations of media compounds but also changes in the concentrations of cellular metabolites secreted by the cells.

HPLC methods (offline mode) are used to quantify concentrations of sugars, amino acids, and metabolites such as acetate and ethanol in the fermentation broth medium [39].

In addition, a method for online measurement of volatile organic compounds (VOC) is implemented [40]. The commercially available method of proton transfer reaction mass spectrometry (PTR-MS; Ionicon Analytik GmbH, Innsbruck, Austria) [41, 42] is a highly sensitive chemical ionization technique that enables soft ionization without fractionation and measurements of individual VOCs ranging from pptV (10^{-12} [vol/vol]) to ppmV (10^{-6} [vol/vol]) levels. VOCs are ionized via proton transfer reactions from H_3O^+ ions and are detected by MS at 1 atomic mass unit (amu) higher (M^{+1}) than the relative molecular weight (M_r) of the neutral compounds. This device allows real-time VOC quantification and noninvasive sampling without any sample preparation steps.

To overcome the limitation that dielectric spectroscopy and optical transmission cannot deliver information on intracellular components, 2D multiwavelength fluorescence spectroscopy has been used as a complementary monitoring system. Therefore, the BioView® spectrofluorometer (DELTA; Light & Optics, Lyngby, Denmark), which is specially designed for online measurements [10, 13], was implemented in our process monitoring platform. The scan of one complete excitation–emission matrix (150 excitation–emission wavelength pairs) takes about 90 s, which enables almost continuous measurement. Within a cell, fluorescent components such as tyrosine, tryptophan, riboflavin, and pyridoxine are present, and their fluorescence depends to a certain degree on the cellular state. Although wavelength combinations can be associated with these fluorescent compounds, direct calibration of fluorescence signals versus process variables was not possible [38].

2.3 Monitoring of Products from the Recombinant System

When recombinant cellular systems are used, quantification and monitoring of expressed products are of high priority. In particular, there are effects triggered by the recombinant system which are significant. Hence, a target-oriented offline monitoring approach must aim at qualification and quantification of recombinant components and simultaneously allow for measurement of the dynamics of the cellular responses triggered by the recombinant gene expression.

One-dimensional sodium dodecyl sulfate (SDS) gel technology is often employed for semiquantitative product analysis, for determination of the solubility distribution (ratio of soluble to insoluble recombinant protein), and in combination with Western blotting, for identification of target proteins and fragments thereof [43].

Enzyme-linked immunosorbent assay (ELISA) technology was used for quantification of green fluorescent protein (GFP) and human superoxide dismutase (SOD), two model proteins used to evaluate the platform. Protein-specific properties such as the autofluorescence of GFP or the enzyme activity of SOD can also be exploited to gain information on protein quantity and quality [44–46]. Formation of isoforms of the recombinant target proteins, e.g., due to addition of acetyl residues or phosphoryl groups or false processing during synthesis, is another important aspect of protein quality. To obtain this information, product samples are separated by narrow-range 2D electrophoresis gels and analyzed by MS after excision and digestion. Figure 1 shows isoforms of SOD produced with *E. coli* HMS174(DE3)(pET11aSOD) [47, 48].

The plasmid copy number representing the gene dosage is calculated from plasmid and chromosomal DNA according to Breuer et al. [49].

Real-time PCR (RT-PCR) is used to quantify messenger RNAs (mRNAs) for recombinant product genes and T7 RNA polymerase [50].

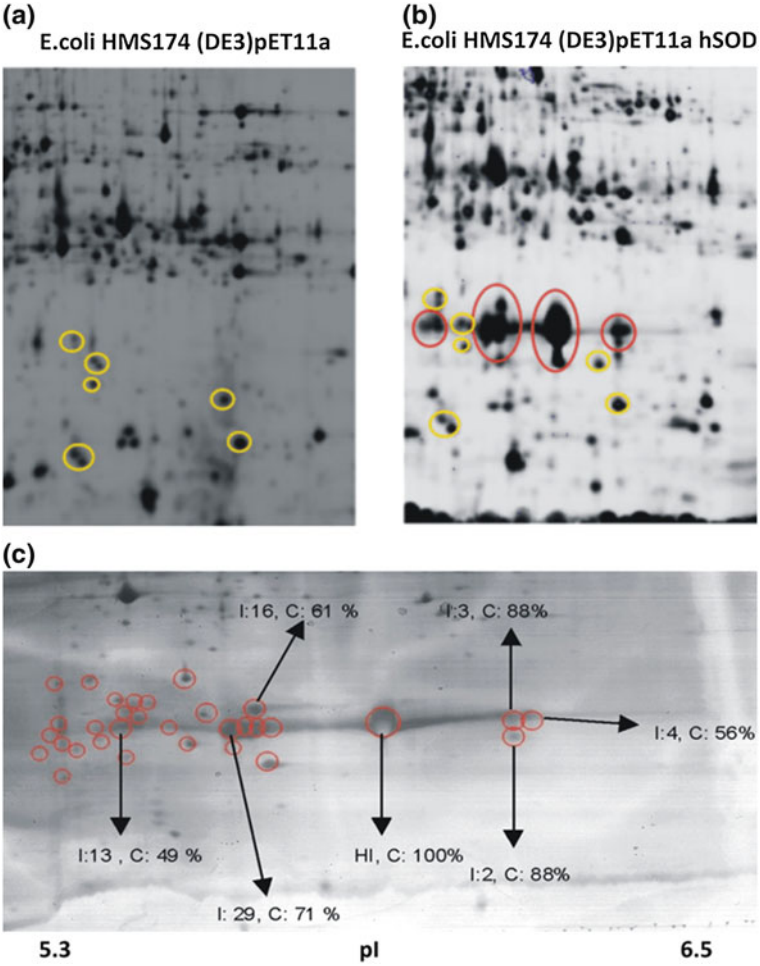

Fig. 1 Spot patterns of samples (150′ past induction) from a cultivation of *E. coli* HMS174 (DE3)pET11a (mock strain) (**a**) and from a cultivation of *E. coli* HMS174 (DE3)pET11a SOD (**b**) on gels separating proteins in the pH range of 3–10 on 18 cm. **c** Image of a 2D narrow range gel in the pH range of 5.3–6.5 on 24 cm (same sample as used in **b**). *Red circles* indicate spots of presumable SOD isoforms which were picked for MS identification. *Arrows* indicate the identified SOD isoforms

Determination of plasmid-containing cells is done by counting colony-forming units (cfu) after 24 h of cultivation on LB agar plates containing antibiotics depending on the resistance marker used for clone screening (100 mg/mL ampicillin or 50 mg/mL kanamycin). The same technique is also used to determine the genetic stability of integrated cartridges in plasmid-free expression systems.

2.4 Monitoring of Cellular Response to Recombinant Gene Expression

2.4.1 Nucleotides

Monitoring of nucleotides such as guanosine tetraphosphate (ppGpp), the central molecule of the stringent response system, and cyclic adenosine monophosphate (cAMP), a signal molecule involved in metabolite repression, is of great interest. Hence, a method for quantification of these molecules was developed, providing the ppGpp stress response to recombinant gene expression [51]. Monitoring of concentration levels of these molecules yields valuable information on cellular state and product formation. As shown in Fig. 2a, recombinant protein is accumulated in the cells after induction for approximately 7 h. After this period, the metabolism became overloaded and cells were no longer able to maintain growth and product formation. The level of ppGpp shows a similar course, with a significant increase after induction clearly indicating the high stress level in the cells. In Fig. 2b, cAMP and quantitative plasmid data from a plasmid production process are shown. The course of cAMP is very similar to the trend of specific plasmid content in the cells [52]. This finding strengthens the hypothesis that plasmids affect host metabolism through the perturbation of the cAMP–CRP complex, as postulated by Ricci et al. [29].

2.4.2 Proteome and Transcriptome Analysis

Since recombinant gene expression affects the host cell metabolism on a genome-wide scale, it is advisable to extend the investigations on this level. In terms of a systems-biology-based approach, changes to the transcriptome and proteome pattern can provide host and recombinant protein-specific information and valuable clues for metabolic engineering targets to cope with physiological bottlenecks of the host metabolism.

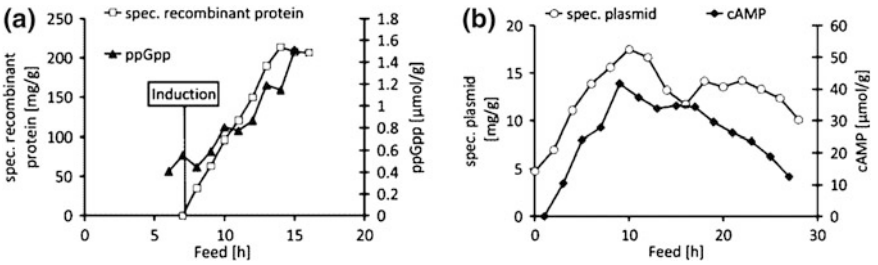

Fig. 2 **a** Courses of ppGpp and specific recombinant protein per gram cell dry mass in carbon-limited exponential fed-batch cultivation (growth rate 0.1 h^{-1}) of *E. coli* HMS174(DE3)(pET30a-SOD). **b** Courses of cAMP and specific content of plasmid per gram cell dry mass in carbon-limited exponential fed-batch cultivation (growth rate 0.1 h^{-1}) of *E. coli* JM108(pMCP-1)

The protein pattern and expression levels of cellular proteins during recombinant gene expression were monitored by 2D electrophoresis [47, 48, 50].

To monitor transcription profiles in the course of experiments, custom-made DNA microarrays for *E. coli* K-12 or *E. coli* BL21 were designed (Eurofins/MWG/Operon, Ebersberg, Germany). Based on sequence data of both strains and information on genes of recombinant compounds (DE3 lysogen, T7 RNA polymerase, plasmid-related genes), oligo probe sets were assembled on the microarray [53]. The array design (accession numbers A-MARS-11 for the BL21 array and A-MARS-12 for the K-12 array) is accessible via ArrayExpress (http://www.ebi.ac.uk/microarray-as/ae/) [54]. The workflow for analysis of microarray data is described in Scharl et al. [55], and the gcExplorer is available at the Comprehensive R Archive Network (CRAN, http://cran.R-project.org/package=gcExplorer).

2.4.3 GFP Online Stress Marker

To obtain real-time information on the cellular stress level during production of recombinant proteins, an approach based on GFP fused to a stress-responsive promoter, introduced into the production host either on a plasmid or integrated into the genome, has been established. In a first step, the GFP signal and its capability for online stress monitoring of recombinant fermentation processes were evaluated [44]. The fluorescence signal of expressed GFP was measured by 2D multiwavelength fluorescence spectroscopy, thereby allowing noninvasive in vivo stress monitoring online (Fig. 3). Results of these stress monitoring approaches are detailed by Nemecek et al. [56].

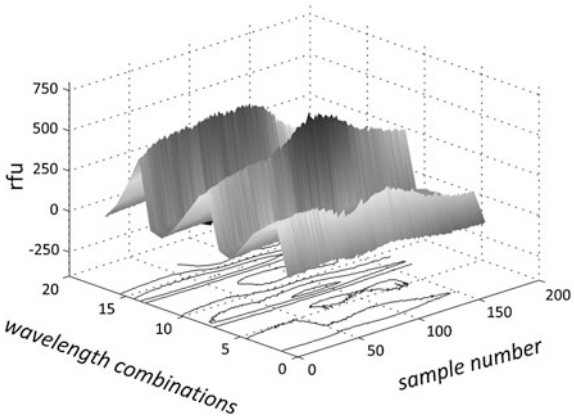

Fig. 3 Difference in online fluorescence signals of two fed-batch cultivations of *E. coli* HMS174(DE3) pET30ahSOD with and without the stress-monitoring plasmid pMMB67HE:dnaKp:GFPmut3.1. Selected wavelength combinations: ex450/em490/510/530/550/570/590; ex470/em510/530/550/570/590; ex490/em530/550/570/590. Sample number indicates measurement intervals of 5 min; sample 0 represents the first measurement after feed start

3 Chemometry and Prediction

The process monitoring platform described above delivers comprehensive online and offline datasets, but although advanced sensor systems are employed, direct measurement of key biological process variables (cell dry mass, product titer, and stress markers) is not possible. The problem is that physiology-relevant information delivered by offline analytics is not accessible in real time and most of the online sensors reflect changes in the process not directly linked to biological process variables per se. Statistical and mathematical modeling is indispensable to bridge this gap to key physiology-relevant variables to improve understanding of the process [57]. The application of mathematical methods, including multivariate data analysis, chemometrics, and statistical methods, enables the extraction of meaningful information from a variety of signals from online sensors and their assignment to complex offline variables.

The applicability of this approach has been demonstrated for the prediction of biomass, TCN, recombinant protein content, and plasmid copy number in *E. coli* cultivation processes. PLS regression and a radial basis function neural network (RBF-NN) model were tested for their predictive power. The best results were obtained with the RBF network model with a selected set of online data highly correlated to offline variables (Fig. 4).

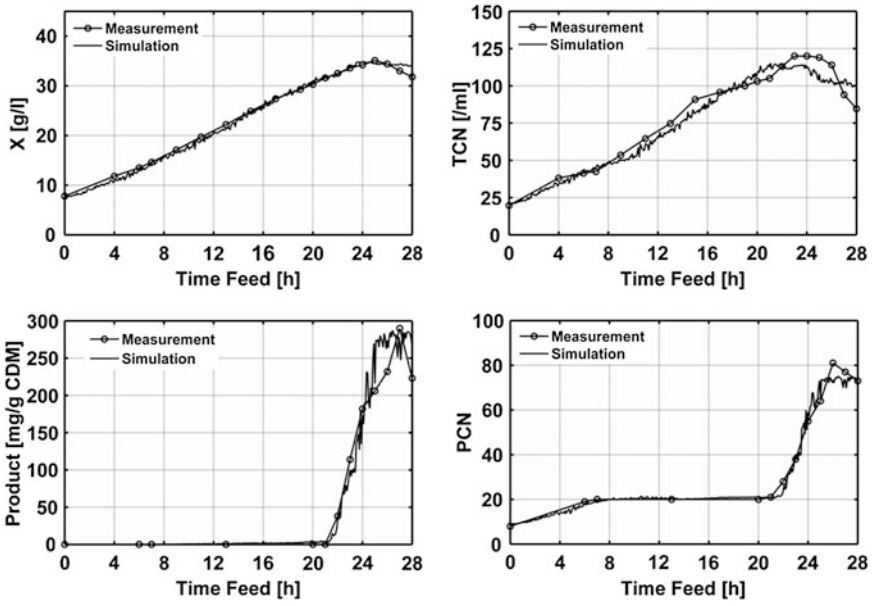

Fig. 4 Prediction of target process variables (CDM, TCN, DC, product, and PCN) by a RBF model generated from selected input signals

As the precision of PLS improves with the number of input signals, PLS cannot benefit from selected input signals.

The lowest root-mean-square errors of prediction (RMSEPs) for the selected set of target offline variables were obtained when applying a nonlinear RBF model that used selected online signals of dielectric and optical spectroscopy as inputs (Fig. 4). The availability of versatile datasets from the different analytical devices enabled the individual assessment of each dataset with regard to the quality of prediction of the particular variable. In addition, benchmarking of different statistical approaches could be carried out. To apply the previously created model for online prediction during a cultivation process, a MATLAB^{TM} function was programmed to perform data input, chemometric modeling, and display of estimated results online during a cultivation process [38].

Recently, an additional analytical device, the proton transfer mass spectrometry (PTR-MS) for analysis of VOCs, became available. This instrument fits perfectly into the monitoring platform due to its continuous and noninvasive measurement, which allows more accurate assignment of the acquired signals to individual compounds than the signal derived from 2D multiwavelength fluorescence and dielectric spectroscopy [16]. Datasets from such analyses will further contribute to prediction quality.

4 Process and Systems Design

Optimization of recombinant protein production in *E. coli* can be obtained by either engineering- or genetic-based solutions. In retrospect, it is obvious that concepts for cultivation of cells (e.g., high-cell-density cultivation, fed-batch cultivation with diverse feed profiles, or chemostat cultivation), including process monitoring and control strategies, were successfully developed in the first period of recombinant protein production. After this period, progress in process engineering slowed down because of limited monitoring capabilities and fragmentary understanding of the biological systems. In this situation, genetic-based solutions became dominant drivers for higher yields in recombinant protein production. Today, a multitude of very powerful expression systems are available, but in many cases the capacity of the cell factory is not only exploited but overstrained in these systems. As a consequence, cultivation of such overbred strains is far more demanding, process stability and reproducibility are reduced, and in many cases, product quality negatively affected. The logical conclusion is that further optimization calls for harmonization of measures in process engineering and host cell engineering.

In this section, such an integrated process optimization approach is presented. The frequently used T7-based *E. coli* expression system serves as a model system. A typical example of the output of a standard process is shown in Fig. 5.

Induction of recombinant gene expression initiates a very high recombinant gene expression level. In parallel, a drastic increase in plasmid copy number from

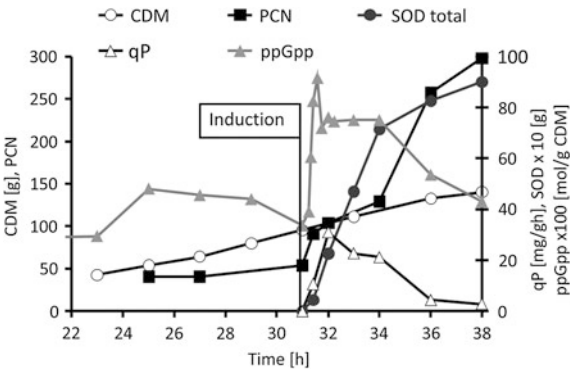

Fig. 5 Response of *E. coli* HMS174(DE3)(pET11aSOD) to induction of recombinant gene expression in a standard fed-batch cultivation regime (exponential carbon-limited feed profile; defined medium; growth rate of 0.1 h^{-1}; single pulse induction with 0.4 g L^{-1} IPTG)

40 to 300 takes place. These two phenomena are interlinked in a kind of self-amplifying stress-generating cascade, resulting in a high stress level, metabolic overload, and a significantly reduced period of product formation. Cellular growth can no longer be maintained, and simultaneously process control by limiting the carbon source no longer works. The challenging question is which measures should be followed, as this cannot be determined from the obtained data if the induction level or the increase in plasmid copy number and gene dosage is the main source of cellular stress. Therefore, these two phenomena are treated in separate approaches.

4.1 Transcription Tuning

Expression of recombinant protein at high rates is an energy-demanding process that drains a significant part of the host cells' synthesis capacity. If this exceeds a certain level, cell functionality becomes impaired because the required cellular infrastructure can no longer be maintained. The solution to this problem is to reduce the recombinant gene expression to a physiologically tolerable level. This can be accomplished by limiting the amount of inducer continuously supplied to the process [58]. The outcome of this engineering-based transcription tuning strategy is presented in Table 1.

A physiologically tolerable induction level allows for a prolonged product formation period, and thereby a higher specific product concentration was obtained. The volumetric yield in experiments with IPTG as inducer was increased by a factor of more than three. As the inducer was continuously supplied to the culture, use of lactose as the native inducer is also possible, and again higher specific and volumetric yields can be obtained. The data on growth behavior, PCN, and ppGpp indicate that there remains potential for further process optimization. Even though cells are able to maintain growth under such conditions, the glucose yield is reduced after induction of recombinant protein production. The transcription tuning concept is based on the assumption of a constant glucose yield

Table 1 Comparison of production processes with *E. coli* HMS174(DE3)(pET11aSOD): conventional full induction versus limited induction with IPTG or lactose

	Standard process	Limited induction with IPTG	Limited induction with lactose
Spec. SOD [mg/g CDM]	64	179	88.1
Volumetric yield [g/L]	0.92	2.87	2.02
Increase of volumetric yield	1	3.12	2.20

coefficient ($Y_{X/S}$), and consequently reduction of $Y_{X/S}$ results in an increasing inducer/CDM ratio.

The ppGpp level increases to even higher values than in the standard process, indicating high stress levels, and there is still an increase in PCN after induction. This effect is attenuated compared with the standard process, but as the *lac*I repressor gene is encoded on the plasmid, a variation in the number of repressor molecules must be expected, which interferes with the concept of transcription tuning.

4.2 Stabilized Plasmid Copy Number

Increasing the plasmid copy number imposes additional stress on the host cell. The plasmid used in our experiments, pET11a, is a ColE1-derived plasmid (with a ColE1 origin of replication). Replication control of this plasmid type based on two RNA molecules (RNAII, the primer for replication, and RNAI, an antisense RNA) is well investigated and described in detail [59–62]. Under starvation conditions, uncharged transfer RNAs (tRNAs) can interact with these regulatory molecules, thereby interfering with plasmid replication control [63, 64]. The shortage of metabolic resources and the depletion of amino acids after induction lead to increased levels of uncharged tRNAs and generate starvation-like conditions. Therefore, we conclude that the drastic increase in plasmid copy number is driven by interactions of uncharged tRNAs with RNAI and RNAII, respectively. To reduce interference of the tRNA with the replication regulatory system, homologies between tRNAs and RNAI were decreased by changing the RNAI sequence into its complementary sequence without inverting it [65]. The experiments with *E. coli* HMS174(DE3) harboring the newly designed pEARL plasmid clearly show that replication control can be maintained even under conditions of full induction (Fig. 6).

The product formation rate in this experiment is similar to that of the reference experiment with the wild-type plasmid (Fig. 5), and consequently the influence of the stabilized plasmid copy number on cell growth and stress level after induction is not significant. This means that a copy number of approximately 40 plasmids per cell is already too high and cellular capacities are exceeded even with the pEARL plasmid. Consequently, reduction of the gene dosage in T7-based systems should be the next step in process optimization.

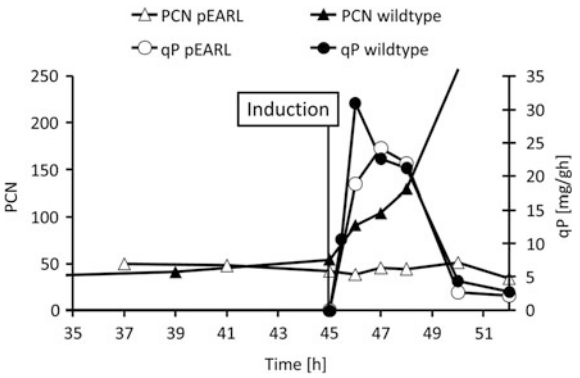

Fig. 6 Response of *E. coli* HMS174(DE3)(pEARL) to induction of recombinant gene expression in a standard fed-batch cultivation regime compared with the reference system *E. coli* HMS174(DE3)(pET11aSOD) with a conventional plasmid (exponential carbon-limited feed profile; defined medium; growth rate of 0.1 h^{-1}; single pulse induction with 0.4 g L^{-1} IPTG)

5 Plasmid-Free Systems

To protect host cells from too high a gene dosage and plasmid-mediated increases in metabolic load, we developed a plasmid-free, T7-based *E. coli* expression system in which the target gene is site-specifically integrated in the genome of the host. As shown in Fig. 7, the performance of plasmid-free genome-encoding expression systems is superior to plasmid-based reference systems. Depending on the host and protein, up to threefold increases in yield are obtained without any significant effects on cell growth. In combination with the transcription tuning concept, genome-encoding systems allow for unlimited control of the expression level, and in chemostat culture the period of product formation can be maintained for more than 10 doublings. Relative to conventional pET systems, this system

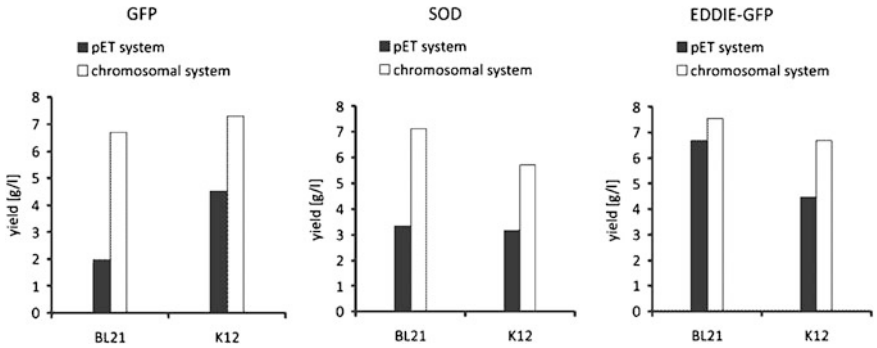

Fig. 7 Process performance of conventional pET and plasmid-free T7-based *E. coli* expression systems

permits improved process stability and increases the host cell's capacity for recombinant gene expression, resulting in higher product yields [66].

6 Conclusions

The established integrated approach for process and systems optimization is mainly based on a process monitoring platform. This platform significantly contributes to improve process understanding and to implement PAT and QbD concepts in bioprocessing. Moreover, the platform approach complies with pharmaceutical quality guidelines, in particular with ICH Q8, aiming at increasing product and manufacturing knowledge, which in turn will decrease the time required for marketing authorization.

Using the platform in combination with modeling techniques and predictive soft sensors, new and better process control strategies and improved process performance can be accomplished. Variables otherwise only available offline can be acquired in real time, enabling novel control regimes; For instance, real-time access to CDM could allow implementation of a model-based inducer feeding regime, and thereby the unwanted effects of increasing inducer/CDM ratios can be eliminated by using the established transcription tuning concept. This strategy could also allow growth of cells under conditions that have not been practically possible to date, where detailed analysis of such cells could deliver new insights into their metabolism.

Iterative process and systems optimization generates knowledge on the process and the cellular system. Based on the improved understanding of the cellular response to recombinant gene expression, such as metabolic bottlenecks, imbalances in the supply of building blocks, and interaction of the recombinant protein with the host metabolism, rational design of the host cell and process operation is possible.

References

1. Food and Drug Administration HHS (2009) International conference on harmonisation; guidance on Q8(R1) pharmaceutical development; addition of annex; availability, vol 200974. pp 27325–27326
2. Kourti T (2006) The process analytical technology initiative and multivariate process analysis, monitoring and control. Anal Bioanal Chem 384:1043–1048
3. Sonnleitner B (1997) Bioprocess automation and bioprocess design. J Biotechnol 52:175–179
4. Sonnleitner B (2000) Instrumentation of biotechnological processes. Bioanalysis Biosensors Bioprocess Monit 66:1–64
5. Schügerl K (2001) Progress in monitoring, modeling and control of bioprocesses during the last 20 years. J Biotechnol 85:149–173
6. Ritzka A, Sosnitza P, Ulber R, Scheper T (1997) Fermentation monitoring and process control. Curr Opin Biotechnol 8:160–164

7. Jørgensen P, Pedersen JG, Jensen EP, Esbensen KH (2004) On-line batch fermentation process monitoring (NIR)—introducing 'biological process time'. J Chemom 18:81–91
8. Read EK, Shah RB, Riley BS, Park JT, Brorson KA, Rathore AS (2010) Process analytical technology (PAT) for biopharmaceutical products: part II. Concepts and applications. Biotechnol Bioeng 105:285–295
9. Roggo Y, Chalus P, Maurer L, Lema-Martinez C, Edmond A, Jent N (2007) A review of near infrared spectroscopy and chemometrics in pharmaceutical technologies. J Pharm Biomed Anal 44:683–700
10. Lindemann C, Marose S, Nielsen HO, Scheper T (1998) 2-Dimensional fluorescence spectroscopy for on-line bioprocess monitoring. Sens Actuators B Chem 51:273–277
11. Hisiger S, Jolicoeur M (2005) A multiwavelength fluorescence probe: is one probe capable for on-line monitoring of recombinant protein production and biomass activity? J Biotechnol 117:325–336
12. Hisiger S, Jolicoeur M (2005) Plant cell culture monitoring using an in situ multiwavelength fluorescence probe. Biotechnol Prog 21:580–589
13. Marose S, Lindemann C, Scheper T (1998) Two-dimensional fluorescence spectroscopy: a new tool for on-line bioprocess monitoring. Biotechnol Prog 14:63–74
14. Markx GH, ten Hoopen HJ, Meijer JJ, Vinke KL (1991) Dielectric spectroscopy as a novel and convenient tool for the study of the shear sensitivity of plant cells in suspension culture. J Biotechnol 19:145–157
15. Noll T, Biselli M (1998) Dielectric spectroscopy in the cultivation of suspended and immobilized hybridoma cells. J Biotechnol 63:187–198
16. Clementschitsch F, Bayer K (2006) Improvement of bioprocess monitoring: development of novel concepts. Microb Cell Fact 5:19
17. Warth B, Rajkai G, Mandenius CF (2010) Evaluation of software sensors for on-line estimation of culture conditions in an Escherichia coli cultivation expressing a recombinant protein. J Biotechnol 147:37–45
18. Schügerl K, Seidel G (1998) Monitoring of the concentration of [beta]-lactam antibiotics and their precursors in complex cultivation media by high-performance liquid chromatography. J Chromatogr A 812:179–189
19. Pons MN, Engasser JM (1998) Monitoring of alcoholic fed-batch cultures by gas chromatography via a gas-permeable membrane. Anal Chim Acta 213:231–236
20. Oeggerli A, Heinzle E (1994) On-line exhaust gas analysis of volatiles in fermentation using mass spectrometry. Biotechnol Prog 10:284–290
21. Broger T, Odermatt RP, Huber P, Sonnleitner B (2011) Real-time on-line flow cytometry for bioprocess monitoring. J Biotechnol 154:240–247
22. Sitton G, Srienc F (2008) Growth dynamics of mammalian cells monitored with automated cell cycle staining and flow cytometry. Cytometry A 73:538–545
23. Harms P, Kostov Y, Rao G (2002) Bioprocess monitoring. Curr Opin Biotechnol 13:124–127
24. Mashego MR, van Gulik WM, Vinke JL, Visser D, Heijnen JJ (2006) In vivo kinetics with rapid perturbation experiments in Saccharomyces cerevisiae using a second-generation BioScope. Metab Eng 8:370–383
25. Barman TE, Bellamy SR, Gutfreund H, Halford SE, Lionne C (2006) The identification of chemical intermediates in enzyme catalysis by the rapid quench-flow technique. Cell Mol Life Sci 63:2571–2583
26. Luttmann R, Bracewell DG, Cornelissen G, Gernaey KV, Glassey J, Hass VC, Kaiser C, Preusse C, Striedner G, Mandenius CF (2012) Soft sensors in bioprocessing: a status report and recommendations. Biotechnol J 7(8):1040–1048
27. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW (1990) Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol 185:60–89
28. Bentley WE, Mirjalili N, Andersen DC, Davis RH, Kompala DS (1990) Plasmid-encoded protein: the principal factor in the "metabolic burden" associated with recombinant bacteria. Biotechnol Bioeng 35:668–681

29. Diaz Ricci JC, Hernández ME (2000) Plasmid effects on Escherichia coli metabolism. Crit Rev Biotechnol 20:79–108
30. Hoffmann F, Rinas U (2000) Kinetics of heat-shock response and inclusion body formation during temperature-induced production of basic fibroblast growth factor in high-cell-density cultures of recombinant *Escherichia coli*. Biotechnol Prog 16:1000–1007
31. Hoffmann FRU (2004) Stress induced by recombinant protein production in *Escherichia coli*. Adv Biochem Eng/Biotechnol 89:73–92
32. Wick LM, Egli T (2004) Molecular components of physiological stress responses in Escherichia coli. Adv Biochem Eng Biotechnol 89:1–45
33. Kiviharju K, Salonen K, Moilanen U, Eerikäinen T (2008) Biomass measurement online: the performance of in situ measurements and software sensors. J Ind Microbiol Biotechnol 35:657–665
34. Kiviharju K, Salonen K, Moilanen U, Meskanen E, Leisola M, Eerikäinen T (2007) On-line biomass measurements in bioreactor cultivations: comparison study of two on-line probes. J Ind Microbiol Biotechnol 34:561–566
35. Sonnleitner B, Locher G, Fiechter A (1992) Biomass determination. J Biotechnol 25:5–22
36. Yardley JE, Todd R, Nicholson DJ, Barrett J, Kell DB, Davey CL (2000) Correction of the influence of baseline artefacts and electrode polarisation on dielectric spectra. Bioelectrochemistry 51:53–65
37. Yardley JE, Kell DB, Barrett J, Davey CL (2000) On-line, real-time measurements of cellular biomass using dielectric spectroscopy. Biotechnol Genet Eng Rev 17:3–35
38. Clementschitsch F, Kern J, Pötschacher F, Bayer K (2005) Sensor combination and chemometric modelling for improved process monitoring in recombinant E. coli fed-batch cultivations. J Biotechnol 120:183–196
39. Weigang F (1987) Off-line Fermentationskontrolle mit Hilfe der High Performance Liquid chromatography. Dissertation, University of Natural Resources and Life Sciences, Vienna
40. Luchner M, Gutmann R, Bayer K, Dunkl J, Hansel A, Herbig J, Singer W, Strobl F, Winkler K, Striedner G (2012) Implementation of proton transfer reaction-mass spectrometry (PTR-MS) for advanced bioprocess monitoring. Biotechnol Bioeng 224:384–393
41. Hansel A, Jordan A, Holzinger R, Prazeller P, Vogel W, Lindinger W (1995) Proton transfer reaction mass spectrometry: on-line trace gas analysis at the ppb level. Int J Mass Spectrom 149–150:609–619
42. Lindinger W, Hansel A, Jordan A (1998) Proton-transfer-reaction mass spectrometry (PTR-MS): on-line monitoring of volatile organic compounds at pptv levels. Chem Soc Rev 27:347–354
43. Achmüller C, Kaar W, Ahrer K, Wechner P, Hahn R, Werther F, Schmidinger H, Cserjan-Puschmann M, Clementschitsch F, Striedner G, Bayer K, Jungbauer A, Auer B (2007) N(pro) fusion technology to produce proteins with authentic N termini in E. coli. Nat Methods 4:1037–1043
44. Reischer H, Schotola I, Striedner G, Pötschacher F, Bayer K (2004) Evaluation of the GFP signal and its aptitude for novel on-line monitoring strategies of recombinant fermentation processes. J Biotechnol 108:115–125
45. Porstmann T, Wietschke R, Schmechta H, Grunow R, Porstmann B, Bleiber R, Pergande M, Stachat S, von Baehr R (1988) A rapid and sensitive enzyme immunoassay for Cu/Zn superoxide dismutase with polyclonal and monoclonal antibodies. Clin Chim Acta 171:1–10
46. Kramer W, Elmecker G, Weik R, Mattanovich D, Bayer K (1996) Kinetics studies for the optimization of recombinant protein formation. Ann N Y Acad Sci 782:323–333
47. Dürrschmid K, Marzban G, Dürrschmid E, Striedner G, Clementschitsch F, Cserjan-Puschmann M, Bayer K (2003) Monitoring of protein profiles for the optimization of recombinant fermentation processes using public domain databases. Electrophoresis 24:303–310
48. Dürrschmid K (2006) Application of transcriptome and proteome profiling for accelerated process optimization of E.coli host/vector systems. University of natural resources and life sciences, Vienna

49. Breuer S, Marzban G, Cserjan-Puschman M, Dürrschmid E, Bayer K (1998) Off-line quantitative monitoring of plasmid copy number in bacterial fermentation by capillary electrophoresis. Electrophoresis 19:2474–2478
50. Dürrschmid K, Reischer H, Schmidt-Heck W, Hrebicek T, Guthke R, Rizzi A, Bayer K (2008) Monitoring of transcriptome and proteome profiles to investigate the cellular response of E. coli towards recombinant protein expression under defined chemostat conditions. J Biotechnol 135:34–44
51. Cserjan-Puschmann M, Kramer W, Duerrschmid E, Striedner G, Bayer K (1999) Metabolic approaches for the optimisation of recombinant fermentation processes. Appl Microbiol Biotechnol 53:43–50
52. Mairhofer J, Cserjan-Puschmann M, Striedner G, Nöbauer K, Razzazi-Fazeli E, Grabherr R (2010) Marker-free plasmids for gene therapeutic applications—lack of antibiotic resistance gene substantially improves the manufacturing process. J Biotechnol 146:130–137
53. Leparc GG, Tüchler T, Striedner G, Bayer K, Sykacek P, Hofacker IL, Kreil DP (2009) Model-based probe set optimization for high-performance microarrays. Nucleic Acids Res 37:e18
54. Rustici G, Kapushesky M, Kolesnikov N, Parkinson H, Sarkans U, Brazma A (2008) Data storage and analysis in ArrayExpress and Expression Profiler. Curr Protoc Bioinformatics Chapter 7:Unit 7.13
55. Scharl T, Striedner G, Pötschacher F, Leisch F, Bayer K (2009) Interactive visualization of clusters in microarray data: an efficient tool for improved metabolic analysis of E. coli. Microb Cell Fact 8:37
56. Nemecek S, Marisch K, Juric R, Bayer K (2008) Design of transcriptional fusions of stress sensitive promoters and GFP to monitor the overburden of Escherichia coli hosts during recombinant protein production. Bioprocess Biosyst Eng 31:47–53
57. Mandenius C-F (2004) Recent developments in the monitoring, modeling and control of biological production systems. Bioprocess Biosyst Eng 26:347–351
58. Striedner G, Cserjan-Puschmann M, Pötschacher F, Bayer K (2003) Tuning the transcription rate of recombinant protein in strong Escherichia coli expression systems through repressor titration. Biotechnol Prog 19:1427–1432
59. Lin-Chao S, Wong TT, McDowall KJ, Cohen SN (1994) Effects of nucleotide sequence on the specificity of rne-dependent and RNase E-mediated cleavages of RNA I encoded by the pBR322 plasmid. J Biol Chem 269:10797–10803
60. Merlin S, Polisky B (1995) Assessment of quantitative models for plasmid ColE1 copy number control. J Mol Biol 248:211–219
61. Tomizawa J (1990) Control of ColE1 plasmid replication. Interaction of Rom protein with an unstable complex formed by RNA I and RNA II. J Mol Biol 212:695–708
62. Tomizawa J (1990) Control of ColE1 plasmid replication. Intermediates in the binding of RNA I and RNA II. J Mol Biol 212:683–694
63. Wróbel B, Wegrzyn G (1998) Replication regulation of ColE1-like plasmids in amino acid-starved Escherichia coli. Plasmid 39:48–62
64. Yavachev L, Ivanov I (1988) What does the homology between E. coli tRNAs and RNAs controlling ColE1 plasmid replication mean? J Theor Biol 131:235–241
65. Grabherr R, Nilsson E, Striedner G, Bayer K (2002) Stabilizing plasmid copy number to improve recombinant protein production. Biotechnol Bioeng 77:142–147
66. Striedner G, Pfaffenzeller I, Markus L, Nemecek S, Grabherr R, Bayer K (2010) Plasmid-free T7-based Escherichia coli expression systems. Biotechnol Bioeng 105:786–794

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Modelling Approaches for Bio-Manufacturing Operations

Sunil Chhatre

Abstract Fast and cost-effective methods are needed to reduce the time and money needed for drug commercialisation and to determine the risks involved in adopting specific manufacturing strategies. Simulations offer one such approach for exploring design spaces before significant process development is carried out and can be used from the very earliest development stages through to scale-up and optimisation of operating conditions and resource deployment patterns both before and after plant start-up. The advantages this brings in terms of financial savings can be considerable, but to achieve these requires a full appreciation of the complexities of processes and how best to represent them mathematically within the context of in silico software. This chapter provides a summary of some of the work that has been carried out in the areas of mathematical modelling and discrete event simulations for production, recovery and purification operations when designing bio-pharmaceutical processes, looking at both financial and technical modelling.

Keywords CFD · Modelling · Sensitivity analysis · Simulation · Window of operation

Contents

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1 Introduction

1.1 Overview of Bioprocess Modelling

Simulation as a strategy for assisting with bioprocess design is a field that has developed within the last 25 years, with many publications on the subject focussing upon commercial packages such as BioProcess Simulator (AspenTech) and SuperPro Designer (Intelligen). For example, Varadaraju et al. [40] described the creation of a process and economic model in SuperPro Designer to evaluate a membrane-based process as an alternative to the packed bed capture and purification of monoclonal antibodies. A number of papers using these packages have also been presented by the creators of the software, e.g. Petrides et al. [33], who looked at the simulation of fill-finish facilities using the Intelligen software. Nevertheless, historically the use of software to drive bioprocess design and optimisation has been relatively uncommon compared with sectors such as chemical engineering, in part due to the absence of adequately predictive mathematical models and a lack of trained personnel available for model development [30]. More recently, however, growing manufacturing costs and the shorter timescales available for development have resulted in simulations attracting greater interest for process design and the evaluation of flowsheets on technical, resource, scheduling and economic grounds. The need to change existing processes to satisfy unexpectedly high market demands or to reduce the impact of process inefficiencies has created additional cost drivers for process modelling and simulation. In the simplest cases such as for a straightforward material balance calculation, modelling can be conducted on a custom basis using spreadsheet software. Such an approach becomes difficult or impossible, however, if one needs to represent dynamic properties such as resource allocation and its impact upon plant throughput [25]. In these circumstances, using dynamic computer simulations instead can help to improve the efficiency of design activities. These methods can be used early to evaluate multiple process flowsheets and operating conditions rapidly while potentially reducing the need for expensive pilot studies and focussing later work on the most feasible manufacturing strategies. Decisions can

thus be made earlier and with due attention paid to aspects such as facility or corporate constraints that may affect the choice of process strategy.

1.2 Advantages in Developing Bioprocess Models

In respect of some of the challenges outlined above, modelling can be a very useful way to combat the resulting cost and time pressures for a wide variety of scenarios that require process- or strategic-related decision making (Table 1). Mathematical simulations are a potentially useful method for designing and developing bioprocesses [48], reducing the costs and times required to conduct process development and manufacturing activities while maximising productivity [21, 22, 29, 39]. Simulations can be used to identify input parameters or parts of a process which have an especially pronounced effect upon technical or cost performance, hence giving an estimation of process robustness [29]. Models can help to compare different flowsheet choices in a virtual setting [25], enabling selection of robust manufacturing protocols and optimising plant capacity utilisation in order to maximise throughput at minimised production costs. Manufacturing routes can also be evaluated in terms of likely capital expenditure when deciding whether to commit resources to a project or not [8, 25]. If implemented across process development groups and other functions, simulations can provide a common language to facilitate communication between different groups such as fermentation, primary recovery and purification [26, 31]. Models can be technical in nature for determining material balances for individual unit operations or whole processes, or as is increasingly the case, they can also address business concerns. This allows an engineer to answer both technical and financial questions simultaneously [9], enabling a more holistic optimal process synthesis to be completed more rapidly than if done by experimentation alone. Models help to provide focus to experimental studies, thus reducing the total amount of time spent in the laboratory or pilot plant [32]. Models can also allow the investigation of an experimental design in order to shed light on the validity of model assumptions [19]. Another area in which models are of use is that of determining resource utilisation. Simulations provide a useful way to synthesise many manufacturing activities into a single portal from which it becomes possible to see where resources are in high demand and where bottlenecks may exist [32, 46]. The availability of resources such as labour, equipment and ancillary supplies is critical in allowing a plant to run smoothly. At times of high demand, simulations can indicate where a process is at greatest risk if resources become unavailable e.g. due to the maintenance or emergency shutdown of equipment or because operators are unavailable.

Evaluating process changes is another task to which models are well suited, and they can be applied for quantifying the impacts to determine whether the additional expenditure is worth the effort and to determine whether a process or facility can accommodate changes; For example, if titre improvements are made in a fermentation stage, then models can be used to check whether the downstream

Table 1 Some examples of typical scenarios examined by modelling approaches

Modelling scenario	Example
Process design	Determining which process flowsheet out of several alternatives is the best one
Investment decisions	Determining whether to build a new facility or contract out the manufacturing process
Economic evaluation	Determination of financial feasibility (NPV) of a project
Facility fit	Determining whether a process as designed and at a specific scale can be accommodated within the capabilities and footprint of the facility and whether the available resources are sufficient to support manufacture without causing bottlenecks. Simulation results could be used to determine whether plant retrofitting is required
Scheduling	Organising batches or campaigns to maximise throughput
Process improvement	Determining how changing an existing process might affect its operating capabilities e.g. the impact of increasing upstream titre upon downstream scheduling and process duties, or moving to new or unconventional technologies e.g. disposable systems versus stainless steel

process can cope with the potentially higher recovery and purification duty placed upon it or whether the plant can supply adequate additional quantities of ancillary services such as steam or water. Such insights can help to determine whether an upstream improvement will really deliver whole process benefits in terms of yields, costs, times and throughputs [16, 35]. This is especially useful where the impacts of process change are unexpected or counter-intuitive [43]. A classic example considers a pair of steps in which a homogeniser used for cell rupture is followed by a clarifying centrifugation step [16]. Although increasing the homogeniser pressure or the number of passes can increase product release, it will also micronise the debris, making it harder for the clarifying centrifuge to remove debris from the supernatant. Hence there is a trade-off between the operation of these two steps, and this would need to be determined on a case-by-case basis. Evaluating the impact of process interactions and identifying conditions for every individual unit operation that deliver the overall required process performance is a task that is well suited to a computer simulation that can evaluate many combinations of conditions to determine the best trade-off.

1.3 Challenges in Developing Bioprocess Models

Driven by the needs outlined in the previous section, modelling software has gained prominence rapidly within the manufacturing sector as a whole, driven also by a growth in the size, cost and intricacy of projects and the need to achieve success the first time around [15]. Despite the aggressive deployment of simulators for process design in sectors such as polymers or chemicals [25], however, as

noted above, their acceptance in the bioprocessing industry has been notably slower [30, 37] and the technique remains underdeveloped for the description of industrial-scale bio-manufacturing processes due to a number of factors. One key challenge lies in performing process optimisation in the face of complex and often multi-variable design problems [45]. Models aimed at assisting with these must cope with the use of mixed operating modes (e.g. batch and semi-batch), strong process interactions and run-to-run process variability with complex feeds [47]. Although the basic theory and fundamental equations share at least some commonality with the chemical engineering literature for a range of unit operations, naturally one must account for the specific nature of bioprocess problems. Complex and uncertain biophysical phenomena can mean additionally that unit operations may be only poorly characterised, and the use of different modes i.e. both discrete pass and continuous processing [26] can complicate modelling efforts. Often, the complex nature of bioprocess feedstocks can mean that physical property data of adequate quantity and quality may be absent and default assumptions need to be made. This can reduce the predictive power of a model. In such situations, empirical or semi-empirical models may be needed, with experimental data used to calibrate the equations. Even with such approaches, in many cases, the properties of such material can vary within and between studies, complicating attempts to quantify process behaviour. Process materials can sometimes be highly sensitivity to even small changes in the design of a manufacturing unit or facility. Added to this complexity is the wide variety of product types that are now considered for bio-manufacturing processes, including proteins, nucleic acids and whole cells, for which properties may be unknown or be highly variable from one batch to the next. Where default values are used for selected mass transport properties, this can make it difficult to construct valid, robust simulations. This lack of available process data is one of the key stumbling blocks when starting to create a model, and in such cases, it is necessary to acquire information from as many sources as possible, including known expertise in the literature, accepted industrial best practices, pre-existing development studies and full-scale manufacturing experience. In the latter case, a key problem is that data are often highly correlated or co-linear, meaning that it can fail to cover a wide search space and will instead focus upon selected combinations of operating conditions alone. In such cases, it may be necessary to develop qualified scale-down process mimics in order to acquire the additional information (Chap. 7).

1.4 Technical Versus Business Modelling

Historically, the task of modelling the technical performance of manufacturing processes has been treated as separate from examining the business aspects. This has resulted in a gap between models that use sometimes quite sophisticated

engineering equations to quantify technical performance on one side and to explore issues of expenditure, facility fit, scheduling and risk on the other. Process modelling has focussed on relating input variables to outputs through explicit mathematical relationships in order to complete material balances or to analyse process sensitivities. In some cases, such models have been conceptually simple enough to require only spreadsheets or non-specialist simulation engines to solve the necessary equations [9]. In other cases, more complex modelling frameworks have been used, such as computational fluid dynamics, in which powerful software is used to simulate momentum or mass transport properties. A key challenge lies in linking different model types together to provide whole process/facility level understanding and not just evaluating the technical outcomes of a specific unit operation. Traditional software approaches for bioprocess development have tended to make limited provision for incorporating business information. Growing cost pressures mean that it is now becoming increasingly important to make manufacturing decisions from both financial and process-related perspectives [29]. Business-process modelling has therefore gained significant ground in recent years as a way of managing process development activities from both technical and corporate perspectives.

2 Setting Up a Model

2.1 Establishing the Mathematical Basis for Modelling

When creating a model, it is important to realise that not every feature of a bioprocess will be critical, and where possible, simplifying assumptions should be made. This should apply both for making the equations themselves more manageable as well as for minimising the data requirement. Of clear importance then is availability in the literature of a sufficient, accurate set of values and default choices for bioprocess properties. With the types of highly complex biological material processed industrially, however, the assumptions underlying these data may not be valid and experimental studies will then be needed to acquire the appropriate values. Depending upon the depth of information needed, various types of modelling equations can be formulated for a range of unit operations, and thus the type and quantity of data will bear a close relation to the nature of the models. These range from the simple to the complex and are specified according to end-user needs; For example, a straightforward capture chromatography model may assume a given dynamic binding capacity and then use that to evaluate outputs such as processing times or consumable costs. On the other hand, more complicated models will calculate dynamic binding capacity by quantifying uptake and adsorption/desorption events from first principles using mass transport equations, calibrated using experimental studies.

2.2 User Interfaces

When developing a model, one important feature is to provide a user-friendly front end for data entry or retrieval of the simulated outcomes. A simulation may be used by those involved directly in bench-scale process development activities or those in more supervisory or managerial positions. Hence where applicable and to cater for this potentially wide range of users, it is important that the software is as intuitive as possible. Appropriate user interfaces are needed to make it easy for people to enter data, and a simple, clean interface with clear labels for exactly which data values are needed is essential, ideally with the capability to validate the inputs so as to avoid basic mistakes.

2.3 Modelling Inputs

Depending upon the type of model, various classes of input parameters (or choices for modelling) may be needed. Some of these may be categorised as follows under the appropriate property heading, although depending upon the nature of the model there may be additional factors to consider such as those relating to other parts of the supply chain or production that sit 'outside' the scope of the principal manufacturing process steps:

- Feed *E.g. selection of volumes or titres*
  These properties have a direct impact upon processing schedules, since larger quantities of product will require either a larger downstream process capacity or may necessitate the process to be cycled more often to cope with the higher overall throughput. The use of higher feed titres may also have other effects that may need to be modelled e.g. to adjust column loading times to prevent excessive breakthrough and product loss.
- Equipment *E.g. the number, type and sequence of unit operations*
  The quantity and sizing of unit operations are critical in ensuring that the production, recovery and purification duty can be satisfied e.g. making sure that a centrifuge bowl or a filter membrane provides an adequate separation area to carry out its task. Models can be used to determine whether the individual unit operations in a process are the most suitable e.g. whether a pre-clarification membrane step before a column is indeed adequate or whether additional steps are needed to remove foulants and thus maximise column lifetime.
- Operational *E.g. flow rates, agitator driveshaft speeds and bowl RCF values*
  These key parameters have a direct influence upon the performance of a process step. Appropriate values should be chosen so as to satisfy the objectives of the entire process sequence. Such terms

form the basis of mathematically 'rigorous' equations used to calculate yield, purity, throughput etc. and can thus enable material or volumetric balancing.

- Facility *E.g. related to provision of ancillary utilities*
  General plant-related items will have an impact upon overall process costs as well as the capability to run a process on time and within schedule e.g. reliable access to services such as steam, glycol, chilled water etc.
- Times *E.g. process durations and hold times*
  Inputs such as processing times can be set either directly or calculated based upon other values e.g. flow rates, process volumes etc. Models can be used to determine how best to arrange batches within a campaign in order to ensure that all can be processed on time. Hold times may also be critical if a product is sensitive or if excessive downtime causes undesirable events such as degradation, and such aspects should be included within a model. Using a model, the impact of process changes upon manufacturing times can be determined to see whether bottlenecks will be formed and how to avoid them. Such scheduling considerations are related to resource availability aspects, as discussed below.
- Resources *E.g. disposable bags, chromatography resins, membranes, buffers or labour*
  Typically, resource pools will exist within a facility and a model will need to represent the quantity of these and their availability (e.g. for labour, the impact of shift patterns will need to be reflected). Resource availability will also impinge upon the likelihood of a process running into bottlenecks and may prompt the acquisition of additional capital or consumable resources to prevent these from impeding processing. Costs will also be associated with the acquisition and usage of these resources, and so this needs to be factored into a process simulation.
- Costs *E.g. for capital, development or running costs, including overheads*
  In addition to those costs mentioned already (e.g. for resources or overheads), there are other expenses which need to be included e.g. capital expenditure, maintenance, taxes and also the cost of failed batches (see below).
- Risk factors *E.g. probabilities of batch failure*
  Risk factors and their impact upon annual product throughput and process costs should also be incorporated, as this can give an indication of not only the likely outcome on process or economic grounds but also the probability that a process will succeed or fail to meet its annual targets.

Related to the last point, although it is possible to model solely using fixed values for the input parameters identified above (i.e. a deterministic analysis), it is

also possible that, in reality, values will vary between batches or over time; For example, the initial product concentration in a bioprocess feedstock may vary, as indeed may market demand from 1 year to the next. Calculating production requirements and process costs in the face of uncertainty can be achieved by assigning probability distributions to ranges of the input factors and then running the model repeatedly i.e. using Monte Carlo simulation to obtain probability ranges for output values [10]. This can enable identification of both the most likely outcome as well as the range of other potential outcomes and hence the probability of satisfying a threshold performance. This can enable one to plot the risk associated with a given development or manufacturing option against the reward that may be possible if it can be made to work. The types of risks include technical (i.e. process-related), economic, scale-up or technology transfer, facility-level and strategic. Assimilating uncertainties into the analysis can enable a more realistic comparison of production scenarios when selecting the most favourable option. Examples of these are discussed later.

2.4 Modelling Outputs

For technical process modelling, normally some sort of material and volumetric balancing data set will be generated as an output, but depending upon the type of model and the depth or detail of the input parameters and equations, other outputs may also be created; For example, as one moves closer towards incorporating business-related issues, other aspects become significant:

- In-process and final product specifications (yield, quality, purity, concentration and volume)
- Product/batch throughput and hence manufacturing times/schedules
- Resource utilisation profiles
- Capital and operating costs (e.g. net present value)
- Probabilities of achieving specified results

In respect of these outputs, it becomes possible to make many types of decisions, such as those at the facility level (e.g. whether to use stainless steel or disposables), at the process level (e.g. using expanded bed chromatography or conventional recovery steps) and also from the capacity planning perspective (e.g. selecting a downstream process capacity to cope with current demands and expected requirements resulting from future upstream improvements). Hence one can determine whether a process within a facility as designed and operated is likely to meet its targets or whether alterations are needed. Although some outputs may be strategic or corporate in nature and others are more process related, in reality they are all connected to one another and separating outputs into different groups is somewhat artificial. In such cases, the different output values must be judged together when deciding whether a manufacturing strategy is worthwhile or not. It is possible that multiple outputs may conflict with one another,

improvements in one being obtained at the expense of others deteriorating. To ensure that a holistically balanced result is achieved, it may be necessary for several output metrics to be integrated together into a single objective value. One way to do this is to use multi-attribute decision making techniques, which combine several output values—possibly with the inclusion of additional weighting values to provide greater prominence to the more critical outputs—to generate a single assessment value to judge the overall feasibility of a process strategy [10]. This can apply to both quantitative metrics and qualitative measures including intangible items such as process reliability and flexibility. By assigning arbitrary, user-defined numerical values to these and then using multi-attribute decision making to obtain a single output metric, this can simplify the evaluation of a strategy. Such an approach can enable multiple conflicting outputs to be traded off to find the most appropriate final balancing point.

3 Types of Bioprocess Models

3.1 Process Models of Bio-Manufacturing Operations

A convenient way to set up a model is to apply simple percentage step efficiencies to individual unit operations to determine overall process recoveries. Such an approach is clearly straightforward and may be valid during the preliminary stages of assessing project feasibility, but more accurate models that use engineering understanding are more helpful when specifying operating conditions. Thus models which rely upon more mathematically 'rigorous' equations (i.e. those which provide a systematic connection between design/operating parameters and process outputs) can offer valuable insight. Some of these may be derived from standard engineering theory, while others may be developed on an empirical or semi-empirical basis using laboratory data. As indicated above, such models may be established using spreadsheets if the mathematical basis permits, or alternatively one may need a more specialised numerical solver. The technical modelling of biopharmaceutical operations draws at least in part upon standard process engineering concepts for describing mass or momentum transport phenomena. These may include mixing conditions, aeration rates or impeller specifications (for fermenters), pressures and the number of passes (for homogenisers), flow rates and separation surface areas (for centrifuges and membranes), uptake kinetics, equilibrium or diffusive properties (for chromatography) etc. An exhaustive list of the equations used to model all the main bioprocess unit operations is beyond the scope of this chapter, and the exact equations used for specific steps can be found in numerous texts and journal articles. Specific unit operation models vary in terms of complexity, with some being relatively straightforward to solve e.g. the first-order cell rupture expression [18] or the sigma concept for equating the flow rate-to-sedimentation area ratio between centrifuges at different scales [1]. Other

expressions become more complicated, e.g. in chromatography, the mass transport phenomena are often represented by a complex series of non-linear partial differential equations that may require access to powerful PCs equipped with appropriate numerical solving software. Hence simplifying assumptions can often be useful in reducing model complexity. This becomes especially true if one seeks to link models of different unit operations in a process flowsheet, since the computational time can otherwise become significant.

The current generation of bioprocess simulations produced by academic research groups or end-users themselves have focussed predominantly on individual unit operations alone [16, 34], often involving the solution of complex equations to evaluate material balances such as for fermentation [7, 13, 20], homogenisation [38, 44], primary recovery such as filtration or centrifugation [6, 28, 36, 37] and chromatography [12, 24]. By definition, models of individual unit operations fail to consider the likely interactions between process steps, and although they constitute a useful first step toward the production of robust and predictive modelling packages, it is the simultaneous consideration of all operations and replication of the interactions between them that is needed for the assessment of whole process feasibility. Unit operations do not operate in isolation, and even small changes at any given stage can affect operation there and further downstream dramatically. Optimising individual operations separately with respect to objectives defined for one stage alone runs the risk that performance of the overall process may well be suboptimal [16]. Thus linkage is vital for quantifying trade-offs between successive process steps, and this can be done most easily by simulations. Examples of integrated process modelling are provided by Groep et al. [16] and Varga et al. [41], who used models to investigate how, for example, varying the number of homogeniser cycles affected technical and economic outcomes during the recovery of an intracellular protein (alcohol dehydrogenase) produced by yeast fermentations.

3.2 Computational Fluid Dynamics

Computational fluid dynamics (CFD) modelling provides the capability to produce an in silico imitation of the hydrodynamic environment within large-scale bioprocessing equipment. This can enable one to quantify phenomena such as shear stresses at solid–liquid or gas–liquid interfaces. Such information may be necessary in the context of design space mapping because small-scale devices on their own may be inadequate for mimicking the hydrodynamic large-scale environment, and hence any predictions made may need to be adjusted. A classic example of this is in centrifuge bowls operated in a non-flooded manner. Small-scale rotors tend to invoke far less powerful shear than at commercial scale, where forces exerted as fluids impinge upon solid surfaces such as in the feed zone can damage delicate materials including cells or shear-sensitive precipitates. Thus some form of regime analysis is necessary to quantify large-scale shear, e.g. by using CFD to

characterise the energy dissipation profile. The damaging effects of this upon the material can be replicated at bench scale e.g. by using shear devices to induce an equivalent level of solids damage observed upon scale-up. Such strategies have been employed in several studies, e.g. by Boychyn et al. [4]; Boulding et al. [2] and Boychyn et al. [5], to improve the accuracy of scale-up predictions.

Other unit operations can also benefit from CFD, such as chromatography. Determining the rate of mass transport in either the bulk fluid or the stationary phases can be facilitated by CFD to solve equations such as the general rate model in order to predict breakthrough and elution profiles [14]. Modelling uptake profiles can require access to data such as mass transfer equilibrium parameters or rate coefficients; these can be used to calibrate the terms within the CFD models, and normally such information will come from small-scale (μL or mL) experiments (see Chap. 7). Computational fluid dynamics requires considerable experience with the available commercial software packages to perform such types of analysis and also far more processing power than is found in a conventional PC. This may owe much to the need to perform repeated iterations of the same equations; e.g. the solution to the general rate model [17] involves discretising the column dimensions into a series of coordinate points and then numerically solving a large system of ordinary differential equations. Hence as indicated above, it is important to introduce simplifying assumptions wherever possible to ensure that the numerical solution does not become intractable. Often, the nature of the operation (and similar prior examples) can provide much valuable data about which mass transport phenomena are the most significant and thus how the equations can be simplified; For example, in cases where mass transfer inside the macropores of the resin beads is known to be the dominating mass transport step, the pore diffusion model of chromatography can be solved using CFD software. If, however, the isotherm is sufficiently rectangular, then the shrinking core assumptions [42] may be used to reduce the pore diffusion model into a simpler set of equations that may be evaluated using a spreadsheet.

3.3 Factorial Design Models

One way to model a process is to use purely empirical data and to fit regression models to the data values, and in principle this could be done in a custom manner by an end-user in a spreadsheet or similar piece of software. In practice, it is more common to use commercial factorial design software which assists with selection of an experimental design, statistical data analysis and the generation of a regression model accompanied by statistical parameters that indicate the quality of fit between data and model. Choosing the most suitable regression model from the many which may be suggested by the software requires some skill and in particular may rely upon the understanding possessed by an end-user when interpreting the fitness statistics. Before conducting a factorial design, it is necessary to have some idea of which parameters are likely to be important, since this can be used to limit

the size of the experimental study. For a given unit operation, there are normally a set of operating parameters which are conventionally known to be potentially significant, and this list can be supplemented using information gained during process development, e.g. by using Ishikawa diagrams or failure modes and effects analysis (FMEA). Subsequently, one would conduct a series of factorial designs, at each stage with increasing density of test points and/or fewer variables, as one seeks to characterise the process design space with respect to its controlling parameters. From a quality-by-design perspective, such an approach would be carried out using qualified small-scale models to generate the empirical data; For example in one study, Looby et al. [27] employed a combination of FMEA, small-scale experiments, analysis of variance (ANOVA) modelling and Monte Carlo analysis to define a process design space to identify operating conditions that minimised the risk of manufacturing out-of-specification material. Thus design of experiments (DOE) can help to achieve thorough process characterisation and gain valuable understanding of which parameters are important to control, along with their acceptable ranges. This information can be particularly useful in uncovering interactions between steps and ultimately can help to achieve the most robust manufacturing strategy that tolerates process variations without compromising on product quality.

3.4 Business-Process Models

The types of models discussed above have focussed predominantly upon creating mathematical relationships to connect process parameters with technical manufacturing outcomes such as recovery, clarification, purity and quality. Another class of models considers financial aspects instead to evaluate process economics in terms of both capital and running expenditure. Ideally, such models must examine not only the main manufacturing tasks but also ancillary activities such as equipment, intermediate or buffer preparation, cleaning-in-place, steaming-in-place and validation. Such facility- or process-related issues must be looked at in context with strategic or corporate priorities such as clinical supply schedules. Although business and process concerns have often been seen and modelled as separate aspects, in reality they must be connected quantitatively to deliver the most efficient and cohesive process development programme. Thus recently, there has been an attempt to combine these model types together to perform process flowsheet evaluations from both perspectives. Such models evaluate both direct and indirect process expenditure by assigning costs to objects such as resources and then accumulating a cost within the simulation environment every time a resource item is either used or purchased (e.g. for consumable items such as resins or membranes). Other costs may be calculated as a fraction of capital investment or running costs (e.g. for estimation of overheads, depreciation, maintenance or taxes). Visualising these costs on either a unit operation basis (i.e. for each process step) or on a cost category basis (e.g. buffers, labour, ancillary items, overheads,

energy etc.) can enable a process development group to target optimisation efforts at the most significant cost centres. Such methods have been used to explore a number of issues in recent years, including:

- Deciding how to structure multiple campaigns within a multi-use facility
- Determining how increasing the number of manufactured batches per annum affects throughput and scheduling
- Exploring how to manage process changes within an existing manufacturing facility e.g. how upstream changes may affect downstream purification or scheduling capabilities
- Evaluating whether to use stainless steel or disposable bioprocessing equipment
- Comparing the outcomes of different fermentation strategies e.g. batch, fed-batch or perfusion cultures

The following sections outline some of these examples.

Example 1

Farid et al. [9] discuss the creation of a prototype in silico method for biopharmaceutical manufacturing, driven by a hierarchical framework to maximise user flexibility when constructing the simulation. The hierarchical framework is modular and enables a simulation to be built to varying levels of detail depending upon available information. Thus, additional details can be added whenever needed, enabling a production batch or campaign to be represented at different levels of complexity; For example, simulating tasks at a very high level can deliver an overview of the process to obtain a summary of key technical and financial outcomes, whereas drilling down to lower levels enables the higher-level activities to be split into individual tasks of increasing definition, with a concomitant increase in the amount and accuracy of data that are required. In the hierarchical decomposition, a facility can be said to house one or more manufacturing campaigns, which can be broken down into individual feed/in-process batches, which comprise an ordered series of unit operations and ancillary supplies. These can be split into a set of individual unit operations and associated specific tasks, all requiring their own resources and with associated costs, resource utilisation profiles and material balancing properties. Hence, using a hierarchical approach, one can obtain many different pieces of knowledge such as:

- Facility level, e.g. capital expenditure, ancillary equipment requirements, ancillary costs and resource pools
- Campaign level, e.g. the number of campaigns scheduled in a year (especially important for cases such as contract manufacturers where batches from different customers need to fit in around one another), operating expenditure (direct and indirect) and the final manufactured product mass
- Batch level, e.g. the number of batches, batch time and cost, productivity and failure rates
- Unit operation level, e.g. the time taken to complete a task/sub-task, the expenditure associated with those specific tasks and resource requirements for those tasks

The approach was exemplified by using it to evaluate the impact of manufacturing decisions upon technical and business outcomes in a case study focussing upon a mammalian cell culture process delivering clinical trial material. The model was used to track resource utilisation profiles and running costs incurred by different manufacturing options, thus enabling the provision of adequate resources, material balancing and cost calculation. Cost models were based upon conventional chemical engineering-style calculations, but they also accounted for the additional expenditure incurred by implementing stringent cGMP rules. The manufacturing process was represented as a set of unit operation and ancillary tasks in a dynamic, discrete event simulation environment, for which resource pools were available for entities such as capital equipment, membranes, chromatography columns, bags, buffers and labour. Values for these were initialised at the start of the simulation and updated as appropriate during the run whenever new stocks were purchased or prepared. Hence the financial and time-related impacts of operating in a resource-constrained environment could be evaluated. This meant that batches were processed only if adequate resources were available for that to occur. Otherwise, a batch was forced to wait until resources become available in sufficient quantities. This represents the same situation that would occur in a real plant and can thus enable identification of bottlenecks. The simulation contained pre-programmed blocks that represented specific tasks such as a culture or purification operation. The blocks were cloned to a workspace and then connected together to simulate the sequence of steps in an entire process flowsheet, with the generic blocks customised for specific requirements of the relevant step for its location in the process. A batch of process material is represented by an 'item' i.e. a computer-generated entity that is 'loaded' with stream properties and which is modified as it passes from one simulated unit operation to the next. Items were passed through the model, updating cost, resource and material balance property data such as volume, titre, impurity level etc. over the course of the simulation.

Example 2

Models are also useful when deciding whether or not to conduct a plant retrofit, where typical commercial questions might focus upon the timing of the retrofit and also for determining whether the altered final process merits the production downtime and retrofit cost. Mustafa et al. [30] describe a model which evaluated the economic impacts of an expanded bed retrofit in place of a centrifuge and packed bed-based protein separation. The study sought to simulate and trade off a higher product yield on one hand with the costs of plant shutdown (loss of production), capital investment and process re-validation on the other. The method enabled strategic, process and economic evaluation of the two options. In this specific case, the results indicated that the re-validation cost and the timing of the retrofit were important, as was the effective downtime cost associated with lost production. In a related study, Mustafa et al. [29] developed a software method to evaluate the business and process aspects of two different manufacturing flowsheets employing packed bed chromatography in one case and expanded bed adsorption in the other. The method assessed the advantages of expanded bed in terms of capital cost reduction and higher product yield compared with the higher

cost of the more expensive expanded bed matrices with lower resin lifetimes. The effects upon cost of goods by parameters such as the yield emerging from the expanded bed process as well as matrix cost, capacity and lifetime were examined. Both direct product handling activities and ancillary tasks were modelled explicitly to ensure that the costs included categories that might otherwise be overlooked, and in this case, the expanded bed option was preferential owing to a lower cost of goods and higher robustness.

Example 3

In a similar dynamic discrete-event example, Lim et al. [25] proposed another hierarchically structured simulation which, alongside material balancing, cost calculations etc., additionally included regulatory compliance activities such as QA/QC, batch documentation and post-batch lot review. Animation features enabled visualisation of batch (item) flow through the simulated process, thus helping to identify and eliminate mistakes. Model outputs included annual throughput, the total number of manufactured batches and resource utilisation curves over time (thus identifying both resource under-utilisation and bottlenecks hindering product throughput). The software was used to simulate the hypothetical full-scale manufacture of monoclonal antibodies using perfusion culture to determine the throughput, cost and resource impact of varying the culture broth pooling frequency, assuming an unchanged downstream process. Determining the optimal pooling interval is important to maximise resource utilisation but also to minimise expenditure and contamination risks. One could either employ only a small DSP capacity and then pool more frequently for purification or conversely choose a plant with a larger DSP capacity with less frequent pooling of the supernatant. Pooling more often makes greater use of DSP capital equipment since there are larger numbers of downstream batches that need to be purified, although with a smaller capacity the capital expenditure is lower in the first place. Using equipment more often, however, increases the need for equipment preparation and draws more heavily upon ancillary services, in addition to higher QC/QA costs since these activities are completed more frequently. Conversely, less frequent pooling makes lower demands for ancillary services or preparation of equipment or regulatory requirements, but will lead to a higher capital investment. Product stability is also an important factor since this affects the optimal choice of pooling frequency. The authors used modelling approaches to examine the trade-offs between such strategies.

3.5 Deterministic Versus Stochastic Modelling

As discussed earlier, modelling approaches should provide ideally not just a deterministic analysis of feasible operating conditions but also an indication of the impact of uncertainty within routine bio-manufacturing. Deterministic models will assign just one value to every parameter, giving a ‘single-point’ output. In reality, there are many possible risks that affect outcomes such as annual production levels

or operating costs, and modelling can help to determine the effects of these risks. Stochastic modelling involves assigning probabilities to ranges for specified inputs to enable a user to determine the range of possible outcomes and hence the confidence one can have in a given outcome or the risk of failing to meet specific process performance criteria. Modelling the random nature of a bioprocess or market-related forces can help to predict the most likely outcome, thus forming the basis of risk assessments and mitigation strategies (e.g. to ensure that market demand is satisfied or to avoid going over budget). Following on from the Lim et al. paper discussed in the preceding section, another study was used to assess perfusion pooling strategies for a mammalian cell culture expressing mAbs. Monte Carlo analysis was used to simulate random run-to-run changes [26] by averaging the outputs achieved over repeated model runs. Hence frequency distributions were created for the amount of mAb produced annually and the cost of goods. Risk factors that were modelled included the chances of contamination caused by having larger numbers of interventions over extended culture durations as well as the impact of uncertainties in mAb titre and the cost of the broth media. As part of this, the study also tried to identify a manufacturing strategy which used resources most efficiently at commercial scale. Other variations which could be modelled include resource costs, dosage level and market demand. A similar Monte Carlo business-process modelling approach was used by Farid et al. [11] in a case study designed to determine whether a start-up company should invest in either stainless facilities or disposable equipment for making material for early clinical trials. The impact of variable product demand and product concentration in the culture step were analysed in order to quantify the attractiveness of different manufacturing options.

Aside from Monte Carlo techniques, another way of determining the impact of variability is to use sensitivity analysis techniques which determine the effect of changing chosen input parameters upon the required outputs. This is discussed in more detail below.

4 Sensitivity Analysis

The task of choosing which parameters to test in a model is complicated by the plethora of choices. Flow rates, feed concentrations, residence times etc. are just a few of the parameters that can influence the performance of many unit operations. Although Ishikawa diagrams and FMEA-style analysis coupled to heuristics and prior experience can help to narrow down the parameters to the most fruitful ones, a more ostensibly quantitative assessment can also be invaluable. Quantitative understanding about which input variables are the most significant can help to focus modelling and thus ultimately experimental development efforts upon the key attributes that control manufacturing performance. This can be particularly relevant when considering interactions between inputs that can complicate the selection of bioprocess operating conditions. In a simple model with a clear

mathematical structure, it may be relatively intuitive to work out the most critical inputs by visual inspection. On the other hand, where there are many terms and when numerous or complicated mathematical equations are involved (e.g. a series of highly non-linear or differential equations), inspection may be impossible and more sophisticated approaches such as sensitivity analysis techniques are required. These enable the identification of critical variables, thus reducing the dimensionality of the search space and decreasing the scope of both modelling and subsequently experimental work.

Sensitivity analysis methods involve determining how variations in model outputs are affected by input changes. Local sensitivity analysis (LSA), for example, involves determining the importance of a given input by computing the partial derivative at a specific point on an output function. Although this is useful in providing sensitivity information about that location and its immediate proximity, it is not satisfactory for describing model characteristics elsewhere. In such instances, partial derivatives would need to be evaluated at every point of interest, which depending upon the type of model and the number of points could be computationally intensive. Additionally, local sensitivity analysis is restricted to analysing only one input at a time, meaning that complete characterisation would necessitate separate calculations for every variable. Alternatively, global sensitivity analysis (GSA) is a more powerful method that assesses all input variables simultaneously over the whole model and ranks them to determine the average contribution made by every parameter over a stipulated set of input ranges. Variable rankings quantify both the impact of individual variables as well as their interactions. King et al. [23] showed how GSA could be applied with beneficial effects in bioprocess development when determining sensitivities during the disk-stack centrifugation of mammalian and yeast cell culture broths. The impact of feed flow rate, particle size, solid–liquid density difference and viscosity on the achievable clarification was quantified using a validated centrifuge model. Variation in the values of process sensitivities as a consequence of making significant changes to the manufacturing set-point conditions could also be investigated. This can be important when dealing with post-approval process changes; For example, if upstream improvements result in higher cell densities, this may change the product–impurity profile that passes into the recovery and purification process, necessitating downstream changes to maintain product quality at its validated level. As these changes occur, it can be useful to determine whether previously important variables remain critical or whether other ones become more significant. This can prompt changes in either the design or the control mechanisms downstream to avoid ill effects caused by variations in specific parameters, thus maintaining satisfactory process robustness.

Multivariable analysis such as GSA is consistent with other approaches such as factorial design where one seeks to explore the influence of all variables over their full ranges to determine whether any synergistic effects exist between parameters. Where one has potentially several variables in a factorial design and depending upon the number of levels for each factor, this can result in fairly large experimental matrices. If a model of the experimental system exists already, the

application of GSA has the potential to reduce the number of design variables, thus limiting laboratory effort and cost.

5 Visualising Search Spaces

Once the critical parameters have been identified and evaluated through modelling approaches, one needs comprehensible ways to understand the resulting data in order to make decisions. Models can generate large amounts of information, and although in certain cases simple graphs may suffice to understand the location of an operating optimum, in other situations this may not be the case. The issue of process interactions is a case in point; to develop operating policies that select values of process variables that lead to optimal whole process performance, they must account for the interplay between process steps and thus trade off the potential beneficial effects of an upstream improvement against additional complexities that this may induce further downstream. As indicated earlier, a classical example of this considers a high-pressure homogeniser in which elevated pressures or numbers of passes cause greater product release but at the expense of a more heavily micronised debris that is more difficult to remove from the supernatant in a following centrifugation step. Working out the best trade-off in conditions between the two steps for yield and clarification requires use of suitable methods for graphically identifying the shape and size of the search space. Hence there is a need for approaches that can visualise outputs intuitively to identify combinations of operating parameters that satisfy required performance levels. Methods such as windows of operation can assist with this activity, in the homogeniser–centrifuge case, for example, by selecting conditions which meet levels for both minimum product recovery and debris removal. Thus, windows are a powerful way to extract design information from quite complex mathematical models and so inform an engineer about which conditions are process-relevant.

Windows of operation are formed by plotting critically important input parameters on the axes and then applying chemical, physical, biological, process or financial constraints to the search space to identify a bounded region that simultaneously satisfies all threshold values for process outputs and product specifications. Windows may be used to define feasible regions of either parts of processes or sequences of steps. The method can be used during both initial process design as well as for post-approval changes to identify operating boundaries and to judge process robustness in response to changes in constraints or parameter ranges. Sensitivity analysis as discussed above can also be useful for determining how the ranges of operating variables and their interactions may affect the shape and size of the windows. Woodley and Titchener-Hooker [45] describe how bioprocess design windows can be used to find acceptable operating regions in both qualitative and quantitative forms. In the former case, theoretical information can be used to get a rough idea of the characteristics of a search space, while in the latter case and with the right models, input data and understanding of

process constraints, these can provide numerical understanding of where to operate. Windows are shown conventionally as a series of two-dimensional maps; three-dimensional windows can also be used and are defined by an operating volume by plotting three variables on each of three axes [22]. Ultimately, such information can enable the definition of suitable operating protocols. In one example for chromatography, windows of operation were used by Boushaba et al. [3], in which the general rate model was calibrated using experimental breakthrough curves from feedstocks generated at variable levels of centrifugal preclarification. The calibrated model was then used to plot windows of operation that related the input load volume and flow rate to the yield and throughput. The results showed how the size and position of the feasible load volume–flow rate operating window changed as the clarification efficiency of the preceding centrifuge was altered.

6 Conclusions

This chapter has sought to evaluate some of the modelling approaches used in the bioprocessing sector to evaluate the technical and economic performance of both individual unit operations and also process flowsheets. Modelling methods have the potential to augment development approaches by enabling the rapid, cost-effective evaluation of process options. To achieve this however requires access to suitable modelling equations and adequate amounts of data or use of sensible assumptions to deliver relevant, accurate predictions of the technical and economic performance of a manufacturing process or facility. Aside from issues of yield, purity or cost of goods, other important aspects addressed by models include scheduling, facility fit and wider strategic issues which determine how best to enhance the development and manufacturing platforms of a company. Modelling frameworks need to be seen in the context of other approaches such as microscale methods (Chap. 7) and rapid analytical techniques that form a portfolio of technologies developed in the last decade for accelerating the design, development and optimisation of biopharmaceutical production operations.

References

1. Ambler CM (1959) The theory of scaling up laboratory data for the sedimentation type centrifuge. J Biochem Microbiol Technol Eng 1:185–205
2. Boulding N, Yim SSS, Keshavarz-Moore E, Ayazi Shamlou P, Berry M (2002) Ultra scaledown to predict filtering centrifugation of secreted antibody fragments from fungal broth. Biotechnol Bioeng 79:381–388
3. Boushaba R, Baldascini H, Gerontas S, Titchener-Hooker NJ, Bracewell DG (2011) Demonstration of the use of windows of operation to visualize the effects of fouling on the performance of a chromatographic step. Biotechnol Progr 27:1009–1017

4. Boychyn M, Yim SSS, Ayazi Shamlou P, Bulmer M, More J, Hoare M (2001) Characterization of flow intensity in continuous centrifuges for the development of laboratory mimics. Chem Eng Sci 56:4759–4770
5. Boychyn M, Yim SSS, Bulmer M, More J, Bracewell DG, Hoare M (2004) Performance prediction of industrial centrifuges using scale-down models. Bioproc Biosyst Eng 26:385–391
6. Chan SH, Kiang S, Brown MA (2003) One-dimensional centrifugation model. AIChE J 49:925–938
7. Chowdhury BR, Chakraborty R, Chaudhuri UR (2003) Modelling and simulation of diffusional mass transfer of glucose during fermentative production of pediocin AcH from Pediococcus acidilactici H. Biochem Eng J 16:237–243
8. Ernst S, Garro OA, Winkler S, Venkataraman G, Langer R, Cooney CL, Sasisekharan R (1997) Process simulation for recombinant protein production: cost estimation and sensitivity analysis for heparinase I expressed in Escherichia coli. Biotechnol Bioeng 53:575–582
9. Farid S, Novais JL, Karri S, Washbrook J, Titchener-Hooker NJ (2000) A tool for modelling strategic decisions in cell culture manufacturing. Biotechnol Progr 16:829–836
10. Farid SS, Washbrook J, Titchener-Hooker NJ (2005a) Combining multiple quantitative and qualitative goals when assessing biomanufacturing strategies under uncertainty. Biotechnol Progr 21:1183–1191
11. Farid SS, Washbrook J, Titchener-Hooker NJ (2005b) Decision-support tool for assessing biomanufacturing strategies under uncertainty: stainless steel versus disposable equipment for clinical trial material preparation. Biotechnol Progr 21:486–497
12. Fee CJ (2001) Economics of wash strategies for expanded bed adsorption of proteins from milk with buoyancy-induced mixing. Chem Eng Proc 40:329–334
13. Gebicke KW, Johl H-J, Sternad W, Trösch W, Chmiel H (1993) Application of modelling and simulation for optimisation of a continuous fermentation process. Comp Chem Eng 17(supplement 1):177–182
14. Gerontas S, Asplund M, Hjorth R, Bracewell DG (2010) Integration of scale-down experimentation and general rate modelling to predict manufacturing scale chromatographic separations. J Chromatogr A 1217:6917–6926
15. Giannasi F, Lovett P, Godwin AN (2001) Enhancing confidence in discrete event simulations. Comput Ind 44:141–157
16. Groep ME, Gregory ME, Kershenbaum LS, Bogle IDL (2000) Performance modelling and simulation of biochemical process sequences with interacting unit operations. Biotechnol Bioeng 67:300–311
17. Gu T, Hsu K-H, Syu M-J (2003) Scale-up of affinity chromatography for purification of enzymes and other proteins. Enzyme Microb Tech 33:430–437
18. Hetherington PJ, Follows M, Dunnill P, Lilly MD (1971) Release of protein from bakers' yeast (Saccharomyces cerevisiae) by disruption in an industrial homogeniser. Trans IChemE 49:142–148
19. Holford NHG, Kimko HC, Monteleone JPR, Peck CC (2000) Simulation of clinical trials. Annu Rev Pharmacol 40:209–234
20. Jang JD, Barford JP (2000) An unstructured kinetic model of macromolecular metabolism in batch and fed-batch cultures of hybridoma cells producing monoclonal antibodies. Biochem Eng J 4:153–168
21. Karri S, Davies E, Titchener-Hooker N, Washbrook J (2001) Biopharmaceutical process development: part III, a framework to assist decision making, Biopharm Europe, September, pp 76–82
22. King JMP, Griffiths P, Zhou Y, Titchener-Hooker NJ (2004) Visualising bioprocesses using, 3D-windows of operation. J Chem Technol Biotechnol 79:518–525
23. King JMP, Titchener-Hooker NJ, Zhou Y (2007) Ranking bioprocess variables using global sensitivity analysis: a case study in centrifugation. Bioproc Biosyst Eng 30:123–134
24. Li Z, Gu Y, Gu T (1998) Mathematical modelling and scale-up of size exclusion chromatography. Biochem Eng J 2:145–155

25. Lim AC, Zhou Y, Washbrook J, Titchener-Hooker NJ, Farid S (2004) A decisional-support tool to model the impact of regulatory compliance activities in the biomanufacturing industry. Comput Chem Eng 28:727–735
26. Lim AC, Zhou Y, Washbrook J, Sinclair A, Fish B, Francis R, Titchener-Hooker NJ, Farid SS (2005) Application of a decision-support tool to assess pooling strategies in perfusion culture processes under uncertainty. Biotechnol Prog 21:1231–1242
27. Looby M, Ibarra N, Pierce JL, Buckley K, O'Donovan E, Heenan M, Moran E, Farid SS, Baganz F (2011) Application of quality by design principles to the development and technology transfer of a major process improvement for the manufacture of a recombinant protein. Biotechnol Prog, (in press; http://dx.doi.org/10.1002/btpr.672)
28. Maybury JP, Mannweiler K, Titchener-Hooker NJ, Hoare M, Dunnill P (1998) The performance of a scaled down industrial disk stack centrifuge with a reduced feed material requirement. Bioprocess Eng 18:191–199
29. Mustafa MA, Washbrook J, Lim AC, Zhou Y, Titchener–Hooker NJ, Morton P, Berezenko S, Farid, SS (2004) A software tool to assist business–process decision–making in the biopharmaceutical industry. Biotechnol Prog 20:1096–1102 [Correction: Biotechnol Prog (2005) 21:320]
30. Mustafa MA, Washbrook J, Titchener-Hooker NJ, Farid SS (2006) Retrofit decisions within the biopharmaceutical industry: an EBA case study. Food Bioprod Process 84 C1:84–89
31. Petrides D (1994) Biopro designer: an advanced computing environment for modelling and design of integrated biochemical processes. Comput Chem Eng 18(supplement):S621–S625
32. Petrides D, Koulouris A, Siletti C (2002) Throughput analysis and debottlenecking of biomanufacturing facilities: a job for process simulators, BioPharm, August pp 2–7
33. Petrides D, Siletti C, Jiménez J, Psathas P, Mannion Y (2011) Optimizing the design and operation of fill-finish facilities using process simulation and scheduling tools. Pharmaceut Eng 31:1–10. http://www.intelligen.com/literature.shtml)
34. Pinto JM, Montagna JM, Vecchietti AR, Iribarren OA, Asenjo JA (2001) Process performance models in the optimization of multiproduct protein production plants. Biotechnol Bioeng 74:451–465
35. Pisano G (1996) Learning-before-doing in the development of new process technology. Res Policy 25:1097–1119
36. Reynolds T, Boychyn M, Sanderson T, Bulmer M, More J, Hoare M (2003) Scale-down of continuous filtration for rapid bioprocess design: recovery and dewatering of protein precipitate suspensions. Biotechnol Bioeng 83:454–464
37. Shanklin T, Roper K, Yegneswaran PK, Marten MR (2001) Selection of bioprocess simulation software for industrial applications. Biotechnol Bioeng 72:483–489
38. Siddiqi SF, Titchener-Hooker NJ, Ayazi Shamlou P (1996) Simulation of particle size distribution changes occurring during high-pressure disruption of bakers' yeast. Biotechnol Bioeng 50:145–150
39. Titchener-Hooker NJ, Zhou Y, Hoare M, Dunnill P (2001) Biopharmaceutical process development: part II methods of reducing development time, Biopharm Europe September, pp 68–74
40. Varadaraju H, Schneiderman S, Zhang L, Fong H, Menkhaus TJ (2011) Process and economic evaluation for monoclonal antibody purification using a membrane-only process. Biotechnol Progr 27:1297–1305
41. Varga EG, Titchener–Hooker NJ, Dunnill P (2001) Prediction of the pilot-scale recovery of a recombinant yeast enzyme using integrated models. Biotechnol Bioeng 74:96–107
42. Weaver LE, Carta G (1996) Protein adsorption on cation exchangers: comparison of macroporous and gel-composite media. Biotechnol Prog 12:342–355
43. Williams HP (1999) Model building in mathematical programming, 4th edn. Wiley, Chichester
44. Wong HH, O'Neill BK, Middelberg APJ (1997) A mathematical model for Escherichia coli debris size reduction during high pressure homogenisation based on grinding theory. Chem Eng Sci 52:2883–2890

45. Woodley J, Titchener-Hooker NJ (1996) The use of windows of operation as a bioprocess design tool. Bioprocess Eng 14:263–268
46. Workman RW (2000) Simulation of the drug development process: a case study from the pharmaceutical industry, proceedings of the winter simulation conference
47. Zhou YH, Titchener-Hooker NJ (1999) Visualising integrated bioprocess designs through, "windows of operation". Biotechnol Bioeng 65:550–557
48. Zhou YH, Holwill ILJ, Titchener-Hooker NJ (1997) A study of the use of computer simulations for the design of integrated downstream processes. Bioprocess Eng 16:367–374

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Extreme Scale-Down Approaches for Rapid Chromatography Column Design and Scale-Up During Bioprocess Development

Sunil Chhatre

Abstract Chromatography is a ubiquitous protein purification step owing to its unparalleled ability to recover and purify molecules from highly complex industrial feedstocks. Traditionally, column development has been driven by a combination of prior experience and empirical studies in order to make the best choices for design variables. Economic constraints now demand that companies engage with a more systematic exploration of a chromatographic design space. To deliver this capability using purely conventional laboratory columns, however, would require considerable resources to identify practical and economical operating protocols. Hence, recently there has been increased use of extremely small-scale devices that gather data quickly and with minimal feed requirements. Such information can be obtained either during early development for screening and trend-finding purposes or later for more accurate scale-up prediction. This chapter describes some of the key drivers for these small-scale studies and the different types of extreme scale-down chromatography formats that exist and illustrates their use through published case studies. Since extreme scale-down experimentation is linked to fundamental mechanistic engineering approaches as well, the utility of these in delivering process understanding is also highlighted.

Keywords Batch incubation · Extreme scale-down · High-throughput screening · Miniature columns · Pipette tips

Contents

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1 Introduction

Designing a chromatographic separation for a protein product is one of the most important steps in biopharmaceutical development for achieving satisfactory levels of purification [22], and achieving this capability may require up to a few dozen column runs during development studies [7]. Initial studies may employ small columns which are both significantly shorter and narrower than at final manufacturing scale; For example, pre-packed columns of a few millilitres in size may be used to determine whether a putative separation space exists and, if so, to identify a smaller subset of conditions to explore in larger laboratory columns of up to around 20 cm length (40–50 mL volume). At this stage, the bed height is fixed along with the linear operating velocity and the feed load expected for larger columns, and the bed diameter is changed whenever required to accommodate the larger feed challenges that are representative of later development. Cumulatively, the feed material needed to supply such work may be considerable (tens to hundreds of millilitres) and may become a limiting factor if many resins need to be evaluated before finding one that offers the required capacity and selectivity. Additionally, development timelines are often compressed, and this prevents lengthy experimentation across a wide parameter space using resource-intensive laboratory columns. Such studies are also limited by the availability of the expensive laboratory equipment required to operate these columns [4, 34]. Although some degree of automated control is possible with these systems, e.g. using predefined method protocols, there can still be many occasions where manual intervention is required; e.g. if pre-packed columns of the required matrix type, volumes or dimensions are unavailable, then column set-up, packing and HETP/asymmetry testing are needed. As a result of these factors, sample throughput at laboratory scale tends to be fairly low, and since columns tend to be operated in series, only one combination of operating conditions can be tested at a time, making the sequential examination of many parameters time-consuming [27, 31].

Consequently, very few manufacturing options may be studied in practice [19], and instead column design may be driven more by prior experience or heuristics when selecting a resin or buffer composition. This can result in sub-optimal processes that overlook superior operating conditions in order to accelerate commercialisation [30]. Techniques developed recently in the extreme scale-down field can alleviate some of these problems by evaluating feed, resin and mobile phase properties more systematically and at lower resource cost to try and identify operating strategies for economic and robust column separations. Extreme scale-down enables the collection of large amounts of empirical knowledge that can improve process understanding and manufacturing flexibility. Although such studies do not eliminate larger laboratory or pilot column studies, they focus efforts upon what is most feasible upon scale-up. The scale-down data restrict larger column experiments to verifying, refining or fine-tuning scale-down predictions (Fig. 1), and if operated in a way that can represent column hydrodynamics, the data can assist ultimately in specifying an optimal column operating protocol [37]. There are a number of extreme scale-down devices, including small columns of around 1 mL in volume, batch incubation methods, miniature columns and chromatography pipette tips which use up to a few hundred microlitres of resin, and microfluidic devices (with a few microlitres of resin). Such methods are consistent with the limited feed quantities available in early development and thus can cover a larger number of conditions in parallel and thus in a shorter period of time than might otherwise be the case in a conventional laboratory column. This makes development faster and cheaper and provides better focus for larger trials [15, 16, 33]. By mapping chromatographic design spaces, extreme scale-down can help ultimately in the design and control of processes that deliver the required product specification consistently. Nevertheless, extreme scale-down requires consideration of many factors, including which device is the most suitable and how easily it can be operated to deliver the required information. This chapter includes some of the general considerations for extreme scale-down process development and presents a summary of the different types of devices. It also provides an overview of selected case studies involving these devices. Allied issues of analysis and experimental design are also discussed.

2 General Considerations for Extreme Scale-Down

In extreme scale-down devices, the physical layout of matrix may be quite different from that of a normal packed column owing to the difference in geometries of the methods at the two scales. Thus, if extreme scale-down data are gathered early in development in the form of high-throughput screening (HTS), these differences may allow the acquisition of only qualitative trends in the search space, rather than quantitatively predictive information about scale-up outcomes. Although these qualitative data in themselves do not constitute a design space, they provide an approximate idea about the location of an operating area and can

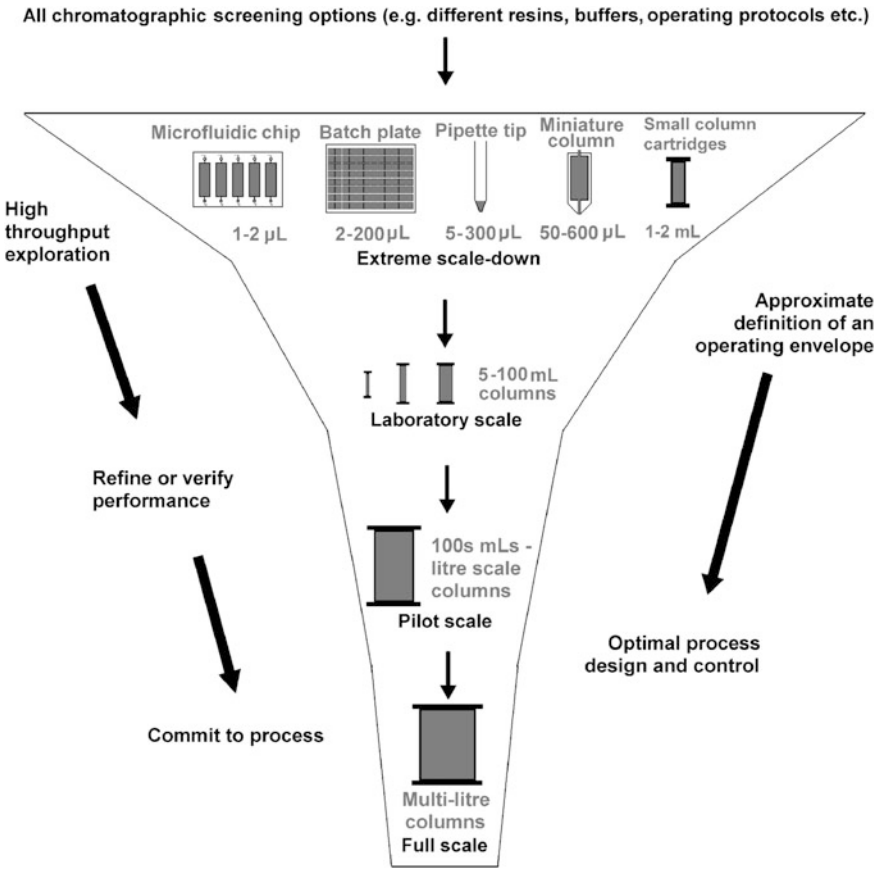

Fig. 1 Data derived from extreme scale-down operation focusses subsequent experimentation upon key manufacturing choices and ultimately enables the development of an optimal column design

thus act as a precursor to more detailed operating space characterisation by conventional column runs. Alternatively, it may be possible to use engineering understanding, empirical equations or mathematical models to adjust the scale-down outputs and thereby create quantitative column predictions. On this basis, the necessary transient and equilibrium properties may need to be measured at scale-down and then used in conjunction with appropriate correlations to predict the rate and extent of adsorption or desorption at scale-up. In either case, there are four principles that govern extreme scale-down chromatography, which are applicable to varying degrees depending upon exactly which type of device is used and its mode of operation [3]:

- *Miniaturisation*: This can lower the volume of reagents required and results in faster experimental processing, which reduces costs and times. Miniaturisation

during process development has evolved from test-tubes using millilitre-scale quantities through to micro-well-plate-based development and now into chip-based applications.

- *Automation*: This encourages fast, accurate and reliable process development and requires liquid handling systems capable of transferring microlitre- to millilitre-scale sample volumes. At these scales it is important to control phenomena such as hold-up loss, evaporation and surface tension effects to enable fast, accurate and precise dispensing. Automation reduces operator burden, enabling that person to conduct other activities while routine operations are carried out.
- *Parallelisation*: Running multiple conditions simultaneously brings clear throughput benefits and also enables more replicates to be run, increasing confidence in the data. Additionally, this reduces down-time when samples would otherwise have to be stored to await processing. Storage can be problematic with conventional serial column operations if one is dealing with crude feed material and unstable products, owing to the deleterious effects of phenomena such as enzymatic breakdown, aggregation or precipitation.
- *Data transformation*: For quantitative scale-up, empirical correlations may be needed to account for scale differences where these cannot be compensated fully by experimental means. Alternatively, mathematical models can use the scale-down data to simulate performance at larger scales. Regime analysis and computational fluid dynamics can be useful in providing the mechanistic framework for scale-up. The output results can then be displayed through diagrams such as windows of operation.

Implementing these extreme scale-down principles permits the generation of information-rich response surfaces [34] when searching for robust operating regions. Some of these principles are more applicable to microlitre operations than millilitre scale-down. The following sections of this chapter summarise the devices available, discuss key considerations and look at published examples of their application.

3 Millilitre Scale-Down

3.1 General Considerations

3.1.1 Scale of Operation

Millilitre-scale approaches use columns containing only one or a few millilitres of resin. First-principles or experimental correlations may be applied to the output data to correct for differences from larger columns. In comparison with standard laboratory beds, millilitre-scale columns reduce feedstock requirements considerably by using very small bed heights and diameters.

3.1.2 Feed Material

Ideally, the feed material used to conduct any form of scale-down chromatography should be as representative of the process-scale product and impurity profile as possible. This implies that the scale of the upstream process should be consistent with the quantities needed to conduct the scale-down chromatographic step. Furthermore, the feed consumed per test condition should be minimised to cover as much of the search space as possible.

3.1.3 Mechanistic Modelling

Chromatography models can help to increase process understanding when used in conjunction with extreme scale-down devices, but require the use of mechanistic equations to represent mass transport, diffusion, dispersion and equilibrium properties accurately [29]. Models that account for every one of these effects can be quite complex and may require advanced mathematical understanding. Fortunately, however, not all mass transport effects are critical in a given separation, and this can enable model simplification. Some mass transport terms can be combined together while still reflecting the necessary chromatographic properties. After a model has been chosen, the remaining unknown parameters need to be determined experimentally in order to calibrate the model. These can be obtained more quickly and cheaply using extreme scale-down devices than at conventional column scale. An example of mechanistic scale-up modelling is given later.

3.1.4 Graphical Representations

Beyond providing mechanistic outputs, an added requirement of in silico approaches is their facile representation in order that the consequence of process choices can be visualised easily. User interfaces can be constructed to portray likely windows of operation and thus enable the determination of specific combinations of process parameters that satisfy performance targets. Fractionation diagrams may also be useful in this context [21].

3.1.5 Techniques for Visualising Adsorption

Techniques for labelling proteins and visualising their adsorption onto resin beads can be a useful way of gathering intra-particle diffusion data [12]. These can give both qualitative and quantitative information about uptake rates and the maximum extent of adsorption to support scale-down studies; For example, confocal scanning laser microscopy can be used to track product uptake onto resin beads [32] or for fouling studies [28], determining the effectiveness of cleaning procedures and so column lifetimes.

3.2 Empirical Scale-Up Example

This example illustrates the use of equations to adjust elution peaks generated in a millilitre-scale column separation to predict the height, width and retention times of larger-scale elution profiles. Two key differences between the scales are the dispersion and retention volumes; For example, band broadening occurs inside and outside a chromatography column, and as column sizes reduce, extra-column effects become increasingly important in determining overall peak spreading. Failing to account for this can lead to incorrect selection of cut points and/or inaccurate peak volume estimation during scale-up, thus complicating the operation of subsequent steps; e.g. an unexpectedly dilute eluate may require longer downstream processing times. Correcting for the impact of retentive and dispersive effects upon an elution profile during scale-up is crucial and is especially important for columns of around 1 mL in size, since this packed bed volume may be similar to or smaller than the extra-column volume. Hutchinson et al. [11] describe how experimental correction factors derived from conductivity changes can be used to correct small-scale dispersion and retention effects and thus enable accurate prediction of the shape, size and position of larger laboratory and pilot elution profiles from a Protein A column. The approach was exemplified by using data from a 1-mL Protein A column challenged with a chimeric monoclonal antibody to predict the elution peaks from 3-mL laboratory and 18.3-L pilot columns. Transition analysis was conducted in 5-mm-diameter columns with bed heights ranging between 20 and 205 mm. The transitions were brought about as a high-conductivity equilibration buffer was replaced by a low-conductivity elutant, and thus the relationship between total dispersion and column height was determined. This was used to correct the small-scale elution profiles for dispersion when predicting the larger packed bed outcomes. A simple mathematical approach was also used to correct for retention effects. The corrected 1-mL data provided good predictions of elution profiles of both the 3-mL and 18.3-L columns. Such information could then be used to determine the most suitable adjustments to apply at extreme scale-down to achieve the same eluate volume and concentration as obtained at scale-up and which would thus be suitable for experimentation in small-scale mimics of subsequent unit operations.

3.3 Mechanistic Scale-Up Example

Mechanistic understanding has also been used for scale-up predictions; For example, Gerontas et al. [9] developed methods to derive process understanding from a highly minimal set of column runs to predict larger-scale elution profiles generated by varying the salt concentration. Computational fluid dynamics (CFD) and 1-mL column data were used to make the predictions. This involved using the general rate model (GRM) to account systematically for properties such as axial dispersion, film mass transfer, intra-particle diffusion and binding kinetics. GRM parameters were

calibrated from runs with three cation exchange matrices in 1-mL pre-packed columns to separate bovine serum albumin (BSA) and lactoferrin. The principles of the Hutchinson et al. [11] study described above were also taken into account in the prediction of scale-up profiles. The model was used to predict the laboratory- and manufacturing-scale performance at different liquid velocities (at 50–100 % larger than the 1-mL column), different bed heights (from 2.5 cm at 1-mL scale to 20-cm-tall columns with volumes ranging between 40 mL and 160 L) and different sample volumes (from 1 CV in the 1-mL column to 0.4 CV in larger columns). When the model was used to simulate the scale-up chromatograms, there was tailing in the experimental peaks which was not observed in the modelled peak, but in all cases the location of the peaks (i.e. their retention times) were predicted well.

The decision as to whether to use mechanistic approaches or not depends upon factors such as the understanding gained from using similar feedstocks and the complexity of the separation challenge. Furthermore, the increased instance of protein heterogeneity in 'real' bioprocess feedstocks increases the separation difficulty and makes it harder to predict the scale-up column performance. Thus, the empirical equations used to quantify axial dispersion and the film mass transfer coefficient in the Gerontas et al. study for BSA and lactoferrin may be insufficient to describe the adsorptive behaviour of more realistic feeds involving the separation of a product from highly crude materials at high protein loading levels. Furthermore, a great deal of computing power and user experience is needed to achieve successful CFD-based scale-up predictions, whereas purely experimental approaches offer greater simplicity and familiarity to bench-scale scientists.

3.4 Other Issues

Although millilitre-scale columns can provide highly valuable process development data, issues of parallelisation and automation remain. A typical liquid pumping system with a single set of ultraviolet (UV)/conductivity detectors can be used to control only one column at a time. Although some automation is possible, there are still many manual steps involved, such as resin packing (where pre-packed beds of the required volume are unavailable). Ideally, the principle of miniaturisation could also be taken further by reducing the resin volume. Automating multiple miniature devices is a task more characteristic of microlitre scale-down, as described below.

4 Microlitre Scale-Down

4.1 Overview

Microlitre-scale chromatography takes the form of three different types of device: batch incubation , pipette tips and miniaturised columns, and all three formats can be used with many different off-the-shelf and custom resins. The formats differ in

operation, sample handling and how the matrix is arranged physically. These formats have become widely recognised as useful techniques in early process development (some of the variables examined by HTS are shown in Fig. 2). Compared with larger-scale operation, feed and time savings using high-throughput automated systems can be up to a couple of orders of magnitude. The resulting data can then be used to home in on feasible operating ranges. Microscale data can be used to elucidate capacity, selectivity, kinetic and thermodynamic data, but conversion of outputs into column predictions is complicated by scale and operating differences. Thus, the scale-down data can be used in their raw form for approximate screening, or through theoretical or empirical models for more accurate scale-up predictions.

4.2 General Considerations

4.2.1 Robotic Versus Manual Operation

It is possible to operate all three microlitre scale-down formats manually, although doing so with tips or miniature columns while maintaining a consistent fluid flow rate/contact time is prone to error. Robotic platforms can simplify sample handling, accuracy and reproducibility. Such systems have multiple pumping channels and can be specified with manipulating arms to grip microtitre plates and move them to other equipment e.g. for agitation, solid–liquid separation or spectrophotometry. The ultimate form of this is 'walkaway' automation, which minimises the requirement for user intervention for routine activities.

4.2.2 Scale and Convenience Issues

The selection of matrix volume depends in part upon the type of study being conducted; e.g. for an overloading study used to determine maximum binding capacities, using as small a resin volume as possible will minimise the sample volume needed for saturation. Nevertheless, there is a practical limit to how low the feed volume can be, and it may be limited by robotic precision and accuracy. Batch incubation filter plates are available for selected commercial matrices, containing small pre-dispensed resin aliquots (e.g. 2-μL GE plates). Atoll GmbH sells packed miniature columns with between 50 and 600 μL and PhyNexus sells chromatography pipette tips with between 5 and 320 μL of matrix. In all cases, resin volumes are available at discrete values within the specified ranges. Self-dispensed plates can be used for any combination of resin type/volume from pre-existing stocks, leaving the filter plate as the main consumable cost and making this format cheaper than self-dispensed plates. Conversely, pre-dispensed plates remove the need to aliquot matrix and offer consistent resin volumes.

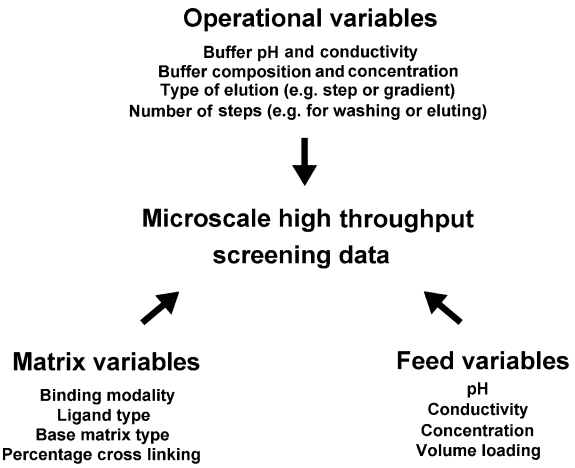

Fig. 2 Types of variables which can be examined using microlitre-scale chromatography devices

4.3 Microlitre Batch Incubation

4.3.1 Summary of the Technique

Millilitre-scale batch studies in stirred vessels or agitated tubes are used commonly in early development to examine many variables simultaneously and thus provide thermodynamic, selectivity and kinetic data [7, 37]. Normally, agitated 96-well dead-end or filter plates are used, where each well is dosed manually or robotically with matrix slurries or plaques of known volume. Feed and resin are mixed together and maintained in suspension for a specified contact time, and the resulting data can be used to extract information about dynamic column behaviour [2]. Incubation with feed or buffers requires a defined volume per wash and may be conducted several times if needed to ensure resin saturation or thorough immersion. After incubation, the supernatant is removed, and in dead-end plates, this can be achieved by gravity or centrifugal sedimentation, followed by careful manual liquid removal. This method may be time-consuming and risks accidental resin particle removal; the alternative involves drawing liquid quickly through a filter plate into a collection plate by centrifugation or under vacuum. Batch plates may be self- or pre-dispensed, and there are several key factors for successful operation, including resin and liquid handling, mixing and separation conditions, liquid hold-up and phase ratios [7], as discussed below.

4.3.2 Robotic Slurry Dispensing

Resin is mixed in a reservoir to ensure a homogeneous particle distribution prior to aliquoting. Resin can be dispensed manually [20, 23], but consistently accurate resin volumes are more easily achieved robotically, provided that one regulates the many controlling factors [7]; For example, the full resin aliquot must be dispensed

such that little remains occluded inside the tip or on the lumen/outer tip surface. One key point is to minimise the distance that the tips descend into the slurry reservoir and to dip the tips into buffer after pipetting to eliminate anything adhering to the outside of the tips as well as to rehydrate dried resin at the base of the tip [20]. To avoid matrix retention inside the tip after dispensing, buffer should be aspirated prior to the slurry to wash the tip clean during dispensing.

4.3.3 Resin Aliquoting Devices

Another way of generating 96-well batch plates is to use devices that create resin plaques from loose slurries. Herrmann et al. [10] described a system in which resin cylinders were separated from liquid through a mesh by vacuum to form. A commercial version of this device is available (MediaScout ResiQuot, Atoll GmbH), creating cylinders of 8, 21 and 51 μL. The device comes with a 96-spike tool to push plaques into a microtitre plate. Such devices have been used to measure isotherms [8] and static capacities [17].

4.3.4 Mixing Conditions

Plates can be mixed magnetically, by an end-over-end stirrer or in an orbital plate shaker. Protein uptake depends upon agitation speed, and values of 1,000 rpm or above are useful as a starting point, using an orbital shaker with a throw radius of a few millimetres to ensure that resin remains suspended. For intra-particle diffusive cases where the mixing speed exceeds a threshold value, no significant change in uptake rate will be observed as speed increases, but if the speed is significantly below the threshold, uptake will be slow and bulk fluid mass transport up to the bead surface may be limiting. The threshold depends upon well dimensions, throw distance as well as matrix and feed properties.

4.3.5 Feed Incubation Times and Theoretical Plate Number Aspects

Choosing the incubation time is critical to acquire column-relevant kinetic and thermodynamic data. This time may be defined by uptake curves when an acceptably high percentage of equilibrium has been achieved. Also of relevance (if known) is the column loading time (contact time) i.e. the total duration for which matrix is exposed to feed. This is more relevant than column residence time (the average duration taken by a feed molecule to pass through the bed), because uptake depends more upon cumulative resin exposure to feed over the course of a loading step, which in a process column could be several hours. Such a time is incompatible with HTS, and during early studies, matrix exposure to feed should be greater than residence times but shorter than column loading times. As protein challenges increase (e.g. in later development), extended incubations may be needed to attain equilibrium.

If the resin in each well represents a defined number of theoretical plates found in a section of a column operated under the same mass transport regime, then choosing the correct incubation time enables the beads to be saturated to a similar level as a column. As a guide, a 20–60-min incubation may be relevant, although column beds contain thousands of theoretical plates per metre and reducing this number in a batch plate may necessitate use of mathematical models.

4.3.6 Other Mass Transport Aspects

Unlike a column, which uses constant fluid flow to achieve feed–resin contact, a plate involves agitation of the bulk liquid. Nevertheless, unless very high flow rates are used, adsorption rates of small proteins at moderate to high concentrations in porous media are governed by internal bead diffusion. The same may not apply for large macromolecules where bead exclusion reduces internal diffusion and where convective mass transfer or external film diffusion may be rate limiting. In such instances, using an incubation time that equals column load time may not imitate column mass transport [2].

4.3.7 Liquid Evacuation

Liquid evacuation can be accomplished in three ways. Direct pipetting for dead-end plates [31] is simple, but it is important to minimise resin entrainment, and manual pipetting is especially prone to variable liquid hold-up. Vacuum filtration is another option, but if foaming occurs, there may be carry-over between wells. Coffman et al. [7] found centrifugation to be robust in achieving consistent liquid recovery from each well with minimal carry-over, while vacuums failed to provide reproducible hold-up volumes. Hold-up after liquid removal can be measured using high and low salt buffer [7] or by a protein to account for dilution effects in the following stage [20].

4.3.8 Self-Dispensed Resin Plate Examples

There are several examples illustrating robotic HTS in self-dispensed plates e.g. for monoclonal antibodies by ion-exchange purification [13, 14]. Charlton et al. [4] used robotic self-dispensed plates to carry out HTS of different IEX ligands and buffers. In another study, Coffman et al. [7] used a 96-well plate to screen conditions using 50–100 μL of resin per well. A 20-min incubation was used as a fast way to approximate HIC and AEX column binding conditions for a monoclonal antibody and an Fc-fusion protein. The HTS generated hundreds of chromatograms in under an hour and compared well with millilitre- and litre-scale columns. Differences occurred due to hold-up and plate number issues, but agreement between batch and column data was sufficient to predict column yields/purities and guide subsequent activities.

4.3.9 Pre-Dispensed Resin Plate Examples

Bergander et al. [2] used pre-dispensed PreDictor filter plates (GE Healthcare) to predict column capacity measurements. This involved two different applications for binding human polyclonal IgG to Protein A and amyloglucosidase adsorption to a weak anion exchanger. To verify plate predictions, dynamic column binding capacities were generated by frontal analysis using loading flow rates calculated to achieve a desired residence time. The impact of this parameter upon dynamic binding capacity normally involves many tedious and feed-intensive column runs at different flow rates. Transient batch uptake data at varying feed–resin contact times offer a useful alternative to understand column dynamics. An uptake curve was developed in the filter plate, with the incubation data from every well being used to generate a single data point on the curve. Feed was added to one well at a time at suitable intervals, such that the first well exposed to protein represented the longest incubation time and the last one represented the shortest time (Fig. 3). After all wells were exposed to feed, liquid was recovered in UV-transparent plates for 280-nm quantification. Mass transport equations were applied to the data to calculate column binding capacities.

Batch uptake can be used either qualitatively with limited data to screen search space trends or with more comprehensive data for quantitative capacity predictions. Bergander et al. [2] used a qualitative approach to acquire trends of how varying the pH and ionic strength affected dynamic capacities of amyloglucosidase on an anion exchanger. By contrast, the quantitative method generated a more accurate relationship between binding capacity and column residence time by combining the larger data sets with a mathematical model. In this context, Bergander et al. [2] used filter plates to study human IgG adsorption to Protein A. In cases where macropore diffusion is the dominant rate-limiting step and when the isotherm is sufficiently rectangular, the shrinking core model can be used to predict binding capacities. Langmuir adsorption parameters were estimated from 60-min-long uptake curves, which were then sufficiently shallow to approximate equilibrium. Using these and fitted pore diffusion coefficients, 10 % dynamic binding capacities were calculated. Capacities found by the plate method correlated well with column data, and the authors estimated savings in time and sample of 10- and 50-fold, respectively, compared with packed columns.

4.4 Chromatography Pipette Tips

4.4.1 Summary of the Technique

There are important differences between packed pipette tips [26, 38] and a normal column. The height and diameter of the tip column are significantly smaller, and it is tapered in shape (wider at the top), meaning that the linear flow rate changes along the bed length. Bidirectional flow is used to bring feed molecules into contact with the

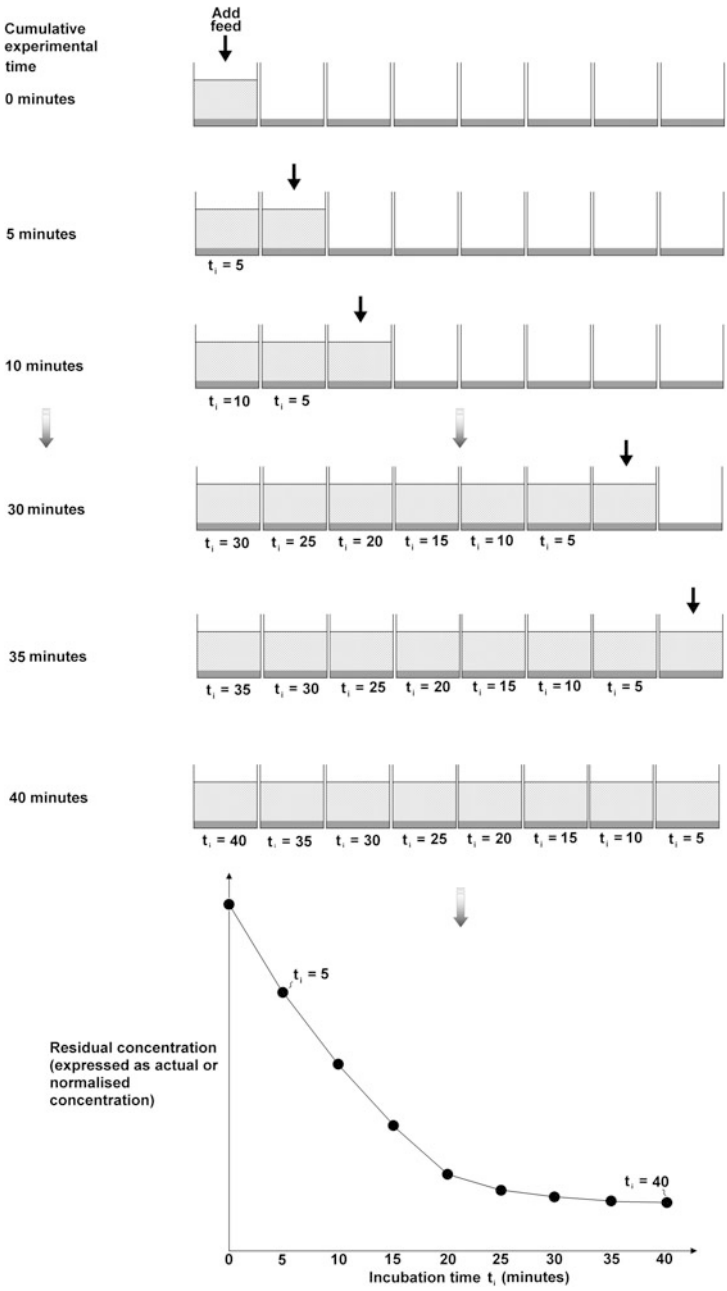

Fig. 3 A method for creating uptake curve data using batch filter plate experimentation

matrix. Automating batch devices may pose certain challenges [36], such as the need to move plates from the robot to plate shakers or solid–liquid separation equipment. Pre-prepared tips and miniature columns avoid some of these issues by passing liquid through a resin bed constrained between two frits/filters.

In general, tips are pre-packed (e.g. PhyNexus, CA, USA), with resin volumes varying between a few microlitres and a few hundred microlitres. Feed samples and buffers can be held in either a multi-well plate or troughs and are pipetted up and down through the resin at a specified flow rate to achieve a desired contact time. Selection of the number of up–down cycles and the flow rate needs to be determined on a case-by-case basis. The time for which matrix is exposed to feed influences uptake and affects the ability to predict scale-up performance. It is possible that mimicking a column contact time may be too time-consuming, and it may be necessary to reduce the contact time and then apply correction factors. Within this context, there are several considerations for pipette tips:

4.4.2 Contact Time

Since very short beds are used in pipette tips, the per-pass residence time is low and unlikely to reflect column uptake. Although increasing the number of cycles will increase adsorption, the chosen number needs to be practical to prevent excessive sample pipetting. The cut-off point represents a compromise between uptake and throughput, and it may be useful to create uptake curves to determine percentage adsorption after successive feed application cycles.

4.4.3 Volumetric Flow Rate

Volumetric flow rates should be lower than normal robotic pipetting speeds i.e. up to around 20 μL/s (compared with normal speeds of a few hundred μL/s). Depending upon the resin volume and the volumetric flow rate, it therefore becomes possible to realise linear velocities between a few hundred and a few thousand cm/h. Generally, the superficial flow rate is set to around 300–1,500 cm/h, resulting in an equivalent volumetric flow rate of around 5–20 μL/s.

4.4.4 Impact of Resin Bed Upon Aspirate–Dispense Operations

Compared with an empty tip, the packed bed at the base of the tip hinders liquid flow and means that, at a specified robotic flow rate, it may not be possible to pipette the full intended volume over the duration of the pipetting step. This can be overcome by adding a delay time after aspiration/dispensing to allow the liquid to ‘catch up’ or by adjusting the robotic flow rate or pipetted volume to compensate. The delay time is feed and matrix specific, and gravimetric analysis can be useful in this context [36].

4.4.5 Pipette Tip Example

Wenger et al. [36] used PhyNexus tips to mimic a chromatographic process used to purify recombinant VLP. In the downstream processing following fermentation, the cells were disrupted by high-pressure homogenisation, clarified by centrifugation and then purified by cation exchange followed by ceramic hydroxyapatite chromatography. Direct product titre analysis from the ruptured culture medium was found to be inadequate, as it did not correlate well with the amount of protein recovered after chromatographic purification. This necessitated an entire purification sequence to obtain accurate data about how changing the fermentation conditions affected downstream performance, using a two-step chromatographic purification at 30- and 80-mL laboratory column scales for the CEX and CHT, respectively. This scale meant that assessing the impact of changing fermentation conditions involved considerable feed volumes, time and labour. Purification throughput was increased by miniaturising the chromatography by three orders of magnitude using robotically controlled chromatography tips packed with either 40 or 80 μL of the relevant resin. The tips and the laboratory columns were compared by examining product recovery for a large number of fermentation conditions and for multiple product types for both the cation exchange and the hydroxyapatite steps. Despite the differences in scale and format, the tips were found to be predictive of laboratory VLP recovery and purity while increasing throughput tenfold. The comparison in recovery between the two scales showed close linear agreement.

4.5 Miniaturised Packed Columns

4.5.1 Summary of the Technique

Whereas the dimensions and operating protocols for tips and plates are significantly different from normal columns, the appearance, dimensions, matrix arrangement and principles of operation of packed miniature columns are closer to conventional columns. Very small columns of up to a few hundred microlitres are available (up to 600 μL) and can be controlled manually or robotically. Miniature columns have quite small heights (as low as a few millimetres) and are operated in a single direction. Especially with the shortest bed heights, this can result in very low residence times, and hence the potential effect upon breakthrough may be important. A supplier of miniature columns is Atoll GmbH (Weingarten, Germany), which provides pre-packed columns in a 96-array format that can be organised as a series of blocks, each containing eight columns arranged side-by-side. Different blocks can then be packed with different resins. Liquid that has passed through the columns can be collected in 96-well plates for analysis, and if required, individual liquid droplets can be pooled to create fractions for generation of pseudo-chromatograms.

4.5.2 Types of Miniature Columns

Centrifuge Columns

There are three types of miniature column supplied by Atoll. The first is operated centrifugally, with a reservoir located above the resin bed and through which buffers or feed solutions can be spun. The size of the reservoir varies according to the resin bed volume underneath; e.g. the 50-μL bed has a 315-μL reservoir, whereas the 200-μL bed has a 230-μL reservoir. Such columns are best employed where there are many samples available for processing but where robotic platforms are unavailable. Liquids are driven through at between a few hundred and a few thousand rpm (with recommended values of 1,000 rpm for 60 s, although the impact of this upon residence time needs to be borne in mind). Centrifuge columns enable very fast initial screening. It is important to check that the centrifuge bowl in question can accommodate the full height and footprint of the column stack.

Pipette Columns

The second column type relies upon manual or motorised handheld pipetting to pass liquid through a bed using a standard pipette tip and an adaptor at the top of the column which forms a seal around the tip. The recommended dispense rate is around 0.5 mL/min. This format is useful when one needs to regulate liquid flow through the bed but where automated workstations are unavailable and again are useful for early-stage resin screening. ‘Starter kits’ are also available, being designed for simultaneous screening of eight different resins in pipette-compliant miniature columns.

Robotic columns

The third column type is controlled robotically, with an inlet adaptor enabling a reversible seal to be formed with fixed tips to introduce buffers or feed. The column inlet possesses a conical duct which enables the fixed tip to be directed into the column inlet, where an O-ring ensures a tight seal. A frit is located at the top of the bed. The volume of liquid that can be delivered in one go is limited by the pipetting capacity of each liquid handling channel, and where the total volume exceeds the channel capacity, several cycles are used. Fractionation in microtitre plates can be achieved by automation, with devices used to position a plate underneath the column stack to collect liquid droplets.

4.5.3 Fraction Collection

When collecting fractions using the robotic shuttle set-up, the exact droplet volume can vary within and between studies depending upon the rate of liquid flow, geometry of the outlet piece on the column and buffer properties such as interfacial liquid tension [34]. Well-to-well variations will cause considerable noise, and so liquid sensing on the robot is useful to determine the real well volumes and so correct data values such as absorbance measurements for the actual liquid path length. Furthermore, if one seeks to quantify the droplet concentration by spectrophotometry and depending on the number of droplets collected per fraction, different types of collection plates may be needed to increase the height of the liquid column to obtain an adequate signal.

4.5.4 Hold-Up and Wall Effects

It is important to note that dead volumes upstream and downstream are particularly significant for very small beds such as miniature columns, since they can dominate peak retention and dispersion. Similarly, one other potentially important factor for an accurate miniature column (and pipette tip) separation that seeks to be potentially representative of a larger-scale column is the impact of the wall effect.

4.5.5 Miniature Column Examples

Wiendahl et al. [34] used a robot combined with 200-μL miniature columns to generate breakthrough and elution curves [35]. Breakthrough curves were generated and fractions were collected in a 96-well plate. Miniature columns were also operated on the ÄKTA chromatography pumping system, and scale-up runs were performed using a 5-mm-diameter, 10-cm-long column on the same system. The linear gradients on the ÄKTA used the same length, slope and initial concentrations as the step gradients on the robot. Generally, dynamic capacities reflect the quantity of a target molecule bound to a resin at a specific percentage on the breakthrough curve, and for those molecules which are highly valuable such as those found in the biopharmaceutical arena, a typical value quoted is at 10 % breakthrough or lower. Breakthrough curves were generated on miniature columns for bovine serum albumin when applied to a range of ion exchangers. The breakthrough curve method was shown to be acceptably reproducible

Elution studies involved separating three different protein mixtures. The first was BSA and Lipolase, where an identical sample load was used at scale-down and scale-up. After loading the protein solution, which contained 2 mg/mL BSA and 2 mg/mL Lipolase, a complex elution gradient was used consisting of three phases, each with different buffer concentrations and numbers of column volumes. The elution time of the BSA elution peak maxima in the miniature column matched closely with that of a larger column, and the use of successive incremental

elution steps at scale-down provided an adequate mimic of the linear gradient established over the larger column when operated on the ÄKTA. In the second study, separation of human growth hormone (hGH) and a precursor was attempted. This is challenging due to the structural similarity between the proteins as well as the use of only very small feed quantities, meaning that any spectrophotometric quantification may experience limit-of-detection problems. Elution was performed by an increasing salt gradient, and different resins and elution buffers were tested. The shape and position of the UV peaks using the HTS robot compared well with the curves produced by the miniature column and the laboratory column on the ÄKTA. Finally, an insulin precursor and a process-relevant contaminant were separated. Retention volumes and resolution were studied using an elution gradient, and this study showed general agreement in peak shape and position produced by the robotic separation compared with the ÄKTA.

In another case, Susanto et al. [29] used scale-down experiments to populate models that predicted scale-up column outcomes in which lysozyme adsorbs to a strong cation exchange matrix. This study looked at the suitability of HTS (batch uptake and miniature columns) to determine parameters for a lumped rate column model that accounted explicitly for axial dispersion effects while lumping other phenomena into a rate coefficient. These authors studied binding behaviour at different ionic strengths and determined isotherm parameters on a robot. Studies were conducted in a filter plate prepared with resin from the ResiQuot device. The model was validated by using it to predict the behaviour of a preparative column.

4.6 Microfluidic Chromatography

Especially when operated on robotic platforms, none of the microscale technologies discussed above provide a true representation of the continuous liquid flow obtained in a normal column. Microfluidic chip technology provides one possible route for achieving this. Shapiro et al. [24, 25] described the fabrication and flow characterisation of a microfluidic chromatography device for evaluating adsorption and resolution-based separations during early development. These authors developed a system for generating a packed IEX microfluidic column containing a 1-cm-high, 1.5-μL bed. This consisted of a glass chip with a 10-mm-long column into which compressible 6 % agarose beads were packed in a two-bead-thick layer. This was compared with a conventional-scale laboratory column with respect to the packing quality and similarity of breakthrough and elution profiles for egg proteins. Breakthrough curves were generated using a fluorescently labelled protein at linear velocities that fell within the range used in normal columns (between 60 and 270 cm/h). Binding capacities were found to be similar to those achieved in conventional laboratory beds, and microfluidic gradient elutions equated well with laboratory columns that were 1,000 times larger. Advantages of this method included a

reduction in the feed protein requirement by over 100-fold for breakthrough measurements and 3,000-fold for elution measurements between microfluidic and 30-mL laboratory columns.

5 Related Issues and Challenges

5.1 Analytics

Although scale-down work (especially that used robotically in a high-throughput format) reduces manual workload and increases throughput, this has the potential to shift the bottleneck away from process characterisation and over to the analysis [7]. To achieve the complete throughput benefits offered by high-throughput techniques requires one to address the potential impact of the analytical bottleneck. Depending upon the method used and the total number of samples, it is possible for the analysis to take longer than the primary high-throughput characterisation itself. In these circumstances, it is necessary to reduce analytical timescales as far as possible, and suitable approaches include:

- Using previously gathered knowledge to focus scale-down studies towards those regions of a search space that are most likely to be process-relevant, thus improving the intrinsic value of any analysis carried out in those areas;
- Stratifying the analysis by performing coarse screens such as simple absorbance measurements during initial studies and switching later to more rigorous, time-consuming techniques such as HPLC or ELISA for more detailed assessment of specific product or impurities. This minimises the upfront analytical burden and means that only the most feasible regions are subject to the most time-consuming assays;
- Managing data generated by the use of high-throughput analytics in an efficient way is important; For example, the 96-well batch format is compatible with a number of medium-throughput analytical devices such as plate readers for rapid spectrophotometric measurements. As a consequence, however, scale-down chromatography may generate very large, complex data sets that require efficient methods for their collection, storage and use. Failure to do so may overwhelm the capacities that a company has for analysing samples and archiving the data [1, 18];
- Employing high-throughput, parallel assays will become advantageous; commercial systems for doing this include microfluidic gel electrophoresis, chromatographic or ELISA-based assays. Since the ultimate aim of scale-down chromatography is to link such devices with scale-down mimics of other steps and since the smallest devices may use only minimal feed volumes themselves, significant loss to analysis or hold-up will mean that insufficient amounts remain to process in scale-down mimics of subsequent operations. This can force one to repeat a study to generate enough feed for the next step downstream. By

reducing analyte volumes, microfluidics may help to facilitate integrated micro-approaches for sequences of process steps;
- Automating assays wherever possible can improve data consistency and release operator time to perform other tasks such as data evaluation. Robots can also be used to perform preparative steps such as buffer make-up, potentially on the same system used to execute the main microscale experiment.

One example where assay automation and parallelisation can be beneficial lies in antibody titre determination by Protein G HPLC. HPLC systems work in a serial fashion, meaning that the timescales involved can become long. Although commercial equipment is available for processing many samples at the same time, this requires standalone and costly equipment. Especially if a high-throughput screen is conducted on a robotic system, then the use of a more integrated form of analysis would be preferential such that the analysis takes place on the same robot (reducing down-time by starting analysis directly after the main experiment and thus returning data to the end user more rapidly). A recent study developed a high-throughput antibody assay as a replacement for HPLC using an eight-channel liquid handling robot to manipulate chromatography pipette tips containing 40 μL of Protein G [5]. The study evaluated a number of analytical properties, including range and detection limits, linearity, accuracy, precision and specificity. After this, the method was tested by quantifying the titre in an ovine feedstock used commercially for making an approved therapeutic product. The average titre calculated using the chromatography tips was comparable to that determined by HPLC, but the eight-channel robotic tip approach delivered results in less than 40 % of the HPLC time. The potential for further time savings by using higher-capacity robots was also identified, with a 96-channel pipetting system offering the possibility of saving approximately 90 % of the time. Integrating the analytical set-up together with the main scale-down experiment such as robotic systems thus offers the potential for 'walkaway' automation.

5.2 Experimental Design Methods

5.2.1 Factorial and Iterative Designs

Appropriate experimental design techniques are needed for HTS to maximise search space coverage, although this must be balanced with the amount of laboratory effort required. Although it is possible to carry out 'brute force' experimentation by HTS, this would consume large quantities of materials, time and money and result in a considerable analytical workload. A better approach is to reduce the experimental requirements to the minimum needed to deliver the required process knowledge in a timely fashion. Thus test conditions can be chosen to provide approximately the same level of information as a brute force design but with a far lower experimental burden; For example, factorial designs

and response surface methods are used commonly to explore a search space. An initial design such as a fractional two-level factorial with replicate centre points might be sufficient to indicate where an optimal area might exist for closer examination by full factorial or response surface methods. There are many choices of design, and the selection of the number and location of test conditions must strike a balance between the depth of information on one hand and the time needed to gather the data on the other [19].

Ideally, experimental designs should minimise the total number of conditions that need to be evaluated, allowing a researcher to arrive at an optimum by selecting test conditions judiciously while still delivering sufficient process knowledge to enable efficient chromatographic operation [30]. One way to do this is to 'step' through a design space using the information gathered from previously tested conditions in order to determine the most suitable direction in which to continue searching for an upward gradient in the underlying response surface towards a good operating region. This can help to drive the experimental focus towards more desirable regions and so minimise the total number of experiments that are conducted before an optimum is found. One such technique is the simplex algorithm, which offers a rapid alternative to more conventional factorial designs during the very early stages of development. Design of experiments (DoE) involves regressing models to data values, but if the fitness of the regressed equation is found to be statistically insufficient, then additional laboratory work has to be carried out to supplement the existing data set and therefore provide more information to enable the identification of a more suitable regression model. The requirement for additional work may become difficult during very early development when feed material availability is highly limited. The use of such resources may be a particular wastage if a large number of the tested conditions transpire to be so highly sub-optimal that they are of no use whatsoever in defining a potential bioprocess design space for subsequent exploration at larger scales. The simplex method is different in that it uses accumulated information from conditions that have been tested already to direct its focus away from poor areas of the search space and instead towards better areas. A study conducted recently described the use of the simplex method in a chromatography case study [6]. When conventional two-level and face-centred central composite design models were created for the data sets, they were found to fit the data poorly, prompting further experiments to provide additional data in order to verify the location of the robust regions in the search space. By comparison, the simplex algorithm identified a good operating point using up to 70 % fewer conditions. Such methods therefore have some value during the very early stages of process development when resources are highly constrained and thus were it is necessary to extract as much value from the experimental studies as possible. An additional point to note is that the simplex method is sequential and therefore does not benefit directly from the parallel processing capabilities of a high-throughput screening device, but is relevant for systems where analytical timescales are long and so where it is advantageous that the majority of tested conditions should head successively towards a viable potential operating position. It should be noted, however, that the simplex method

is not necessarily suitable in every situation, and other design-of-experiments methodologies may be more suitable in those cases. The simplex method is essentially suited to early-stage process development when trying to identify a feasible operating point that merits further characterisation in later studies. Subsequent work (e.g. upon scale-up in later development) is perhaps best conducted using more conventional factorial design models such as two-level factorials or response surface methodologies to provide the type of robust design space characterisation that ends up supporting a regulatory filing. Table 1 summarises the circumstances in which one would use the simplex method versus an alternative.

5.2.2 Genetic Algorithm-Based Methods

There are other ways of structuring an experimental campaign, such as genetic algorithms, which are suitable for complex problems involving numerous variables and which are robust in the presence of experimental noise. Susanto et al. [30] proposed a way of performing chromatographic process optimisation at very small scales in a resource- and time-efficient manner by using closed-loop (i.e. no manual intervention) and semi-closed-loop strategies that integrated HTS robotics together with genetic algorithms. The procedure carries out process exploration, data analysis and optimization iteratively and was illustrated through two IEX case studies. The first was a closed-loop case looking at the capture of lysozyme from a solution also containing cytochrome and focussed upon how varying the equilibration buffer pH and NaCl concentration affected capacity and selectivity. The study was conducted in a robotically controlled batch plate to enable prediction of a 1-mL column. Resin slurries were aliquoted using the Atoll ResiQuot device to provide a 7.7 μL settled resin volume per well. Closed-loop optimisation was used to drive the search, and in spite of analytical noise, successive iterations enabled the search to converge to a global optimum within 4 h (96 experiments). After this, optimal and sub-optimal conditions identified by HTS were verified in a 1-mL bed.

The second study involved an interesting approach to predict the optimal elution gradient shape. The method involved deconvoluting the A280 ‘total’ protein absorbance signal into individual component peaks, alongside the use of mass balance and prior user knowledge, to distinguish the position and size of individual peaks for a three-protein mixture and so assess peak resolution. The study used robotically operated 200-μL miniature columns to look at the optimisation of the shape of a multilinear elution gradient consisting of two different linear slopes in order to maximise peak resolution for a mixture containing ribonuclease, cytochrome and lysozyme. Eight columns were run simultaneously using different elution conditions, and a 96-well UV-transparent plate was used to collect fractions by moving the plate underneath the outlet of the column row. The two successive linear slopes were converted into a succession of discrete step gradients that incremented the eluate salt concentration appropriately. The elution gradient was optimized by using a genetic algorithm, but instead of using a fully automatic closed loop, this second example involved a semi-closed case that demonstrated

Table 1 Situations in which to use the simplex method and where to employ an alternative

Issue	Use the simplex algorithm	Use an alternative
Stage of development	Deploy during early process development when the main aim is to head in the right direction within a design space	Use a factorial design later in process development, either as part of a design space submission to a regulator, or as the basis of a model for large-scale process control
Experimental cost versus value of process understanding	Use the simplex method when it is necessary to extract process knowledge from very small quantities of material, thus maximising the value of every experiment early in development. Alternatively, if a good scale-down model does not exist and hence when one is relying upon time- and resource-consuming operations e.g. at a large laboratory scale, then the simplex algorithm allows one to make best use of that experimental effort	If feed material availability is not an issue, or when achieving a good result from every data point is not as critical e.g. when one can afford the occasional misleading result or experimental failure, the simplex approach may not be as relevant; For example, if highly parallelised microscale operation on robotic platforms is being considered, typically this will generate large quantities of data and the general trends within the resulting response surfaces may be sufficient to overcome a small number of failures
Analytical burden	When the analytical burden is significant or time-consuming, it may be necessary to reduce the total number of test conditions to avoid bottlenecks. Thus again it becomes necessary to maximise the value of every data point in an experiment and the simplex technique can facilitate this aim	If analysis is fast enough that one does not need to resort to using only the bare minimum number of runs to prevent throughput bottlenecks, then the simplex technique may not be as pertinent

the utility of integrating user expertise into the optimization. A semi-closed loop enables data evaluation and optimisation during a GA iteration to be regulated by an operator to allow process understanding to be injected where appropriate. Thus, the initial experiments defined for the first GA iteration were chosen in part by the user to ensure good coverage of the entire variable range and to ensure that a large number of gradient shapes were evaluated during the study in the search for the optimal multilinear gradient shape.

6 Conclusions

This chapter has reviewed a range of resource-efficient methods for the extreme scale-down development of chromatographic separations along with selected case study examples. Such methods allow engineers to obtain information early in development about how a large-scale process could perform. The outcome of a scale-down device can be either information about general trends across a search space or the provision of more accurate information for predicting scale-up performance. Direct scale-up prediction will require knowledge of the large-scale engineering environment to be replicated satisfactorily in the small devices. This may be possible by direct calibration of the scale-down devices, or it may also need the use of mathematical modelling to adjust scale-down outputs. Of additional importance are the principles of parallelisation and automation, which increase throughput significantly and thus allow many more options to be considered than might otherwise be the case. This increases process confidence and understanding and allows a rational basis for choosing a specific process design, thus enabling efficient and cost-effective column development.

References

1. Bensch M, Wierling PS, von Lieres E, Hubbuch J (2005) High throughput screening of chromatographic phases for rapid process development. Chem Eng Technol 28:1274–1284
2. Bergander T, Nilsson-Välimaa K, Öberg K, Łącki KM (2008) High-throughput process development: determination of dynamic binding capacity using microtiter filter plates filled with chromatography resin. Biotechnol Progr 24:632–639
3. Bhambure R, Kumar K, Rathore AS (2011) High-throughput process development for biopharmaceutical drug substances. Trends Biotechnol 29:127–135
4. Charlton H, Galarza B, Leriche K, Jones R (2006) Chromatography process development using 96-well microplate formats. Biopharm Int. http://biopharminternational.findpharma.com
5. Chhatre S, Francis R, Bracewell DG, Titchener-Hooker NJ (2010) An automated packed Protein G micro-pipette tip assay for rapid quantification of polyclonal antibodies in ovine serum. J Chromatogr B 878:3067–3075
6. Chhatre S, Konstantinidis S, Ji Y, Edwards-Parton S, Zhou Y, Titchener-Hooker NJ (2011) The simplex algorithm for the rapid identification of operating conditions during early bioprocess development: case studies in FAb' precipitation and multimodal chromatography. Biotechnol Bioeng 108:2162–2170
7. Coffman JL, Kramarczyk JF, Kelley BD (2008a) High-throughput screening of chromatographic separations: I. Method development and column modeling. Biotechnol Bioeng 100:605–618
8. Dismer F, Petzold M, Hubbuch J (2008) Effects of ionic strength and mobile phase pH on the binding orientation of lysozyme on different ion-exchange adsorbents. J Chromatogr A 1194:11–21
9. Gerontas S, Asplund M, Hjorth R, Bracewell DG (2010) Integration of scale-down experimentation and general rate modelling to predict manufacturing scale chromatographic separations. J Chromatogr A 1217:6917–6926
10. Herrmann T, Schrőder M, Hubbuch J (2006) Generation of equally sized particle plaques using solid-liquid suspensions. Biotechnol Progr 22:914–918

11. Hutchinson N, Chhatre S, Baldascini H, Davies JL, Bracewell DG, Hoare M (2009) Ultra scale-down approach to correct dispersive and retentive effects in small-scale columns when predicting larger scale elution profiles. Biotechnol Progr 25:1103–1110
12. Jin J, Chhatre S, Titchener-Hooker NJ, Bracewell DG (2010) Evaluation of the impact of lipid fouling during the chromatographic purification of virus-like particles from *Saccharomyces cerevisiae*. J Chem Technol Biotechnol 85:209–215
13. Kelley BD, Switzer M, Bastek P, Kramarczyk JK, Molnar K, Yu T, Coffman J (2008a) High-throughput screening of chromatographic separations: IV ion-exchange. Biotechnol Bioeng 100:950–963
14. Kelley BD, Tobler SA, Brown P, Coffman J, Godavarti R, Iskra T, Switzer M, Vunnum S (2008b) Weak partitioning chromatography for anion exchange purification of monoclonal antibodies. Biotechnol Bioeng 101:553–566
15. Lye GJ, Ayazi Shamlou P, Baganz F, Dalby PA, Woodley JM (2003) Accelerated design of bioconversion processes using automated microscale processing techniques. Trends Biotechnol 21:29–37
16. Micheletti M, Lye G (2006) Microscale bioprocess optimisation. Curr Opin. Biotech 17: 611–618
17. Staby A, Jensen RH, Bensch M, Hubbuch J, Dünweber DL, Krarup J et al (2007) Comparison of chromatographic ion-exchange resins VI weak anion-exchange resins. J Chromatogr A 1164:82–94
18. Nfor BK, Ahamed T, van Dedem GWK, van der Wielen LAM, van de Sandt EJAX, Eppink MHM, Ottens M (2008) Review: design strategies for integrated protein purification processes: challenges, progress and outlook. J Chem Technol Biotechnol 83:124–132
19. Nfor BK, Verhaert PDEM, van der Wielen LAM, Hubbuch J, Ottens M (2009) Rational and systematic protein purification process development: the next generation. Trends Biotechnol 27:673–679
20. Nfor BK, Noverraz M, Chilamkurthi S, Verhaert PDEM, van der Wielen LAM, Ottens M (2010) High-throughput isotherm determination and thermodynamic modeling of protein adsorption on mixed mode adsorbents. J Chromatogr A 1217:6829–6850
21. Ngiam S-H, Zhou Y, Turner MK, Titchener-Hooker NJ (2001) Graphical method for the calculation of chromatographic performance in representing the trade-off between purity and recovery. J Chromatogr A 937:1–11
22. Przybycien TM, Pujar NS, Steele LM (2004) Alternative bioseparation operations: life beyond packed-bed chromatography. Curr Opin Biotechnol 15:469–478
23. Rege K, Pepsin M, Falcon B, Steele L, Heng M (2006) High-throughput process development for recombinant protein purification. Biotechnol Bioeng 93:618–630
24. Shapiro M, Haswell S, Lye G, Bracewell D (2009) Design and characterization of a microfluidic packed bed system for protein breakthrough and dynamic binding capacity determination. Biotechnol Progr 25:277–285
25. Shapiro M, Haswell S, Lye G, Bracewell D (2011) Microfluidic chromatography for early stage evaluation of biopharmaceutical binding and separation conditions. Separ Sci Technol 46:185–194
26. Shukla AK, Shukla MM and Shukla AM (2007) Incision-based filtration/separation pipette tip. US Patent 7276158
27. Stein A, Kiesewetter A (2007) Cation exchange chromatography in antibody purification: pH screening for optimised binding and HCP removal. J Chromatogr B 848:151–158
28. Siu SC, Boushaba R, Topoyassakul V, Graham A, Choudhury S, Moss G, Titchener-Hooker NJ (2006) Visualising fouling of a chromatographic matrix using confocal scanning laser microscopy. Biotechnol Bioeng 95:714–723
29. Susanto A, Knieps-Grünhagen E, von Lieres E, Hubbuch J (2008) High throughput screening for the design and optimization of chromatographic processes: assessment of model parameter determination from high throughput compatible data. Chem Eng Technol 31:1846–1855

30. Susanto A, Treier K, Knieps-Grünhagen E, von Lieres E, Hubbuch J (2009) High throughput screening for the design and optimization of chromatographic processes: automated optimization of chromatographic phase systems. Chem Eng Technol 32:140–154
31. Thiemann J, Jankowski J, Rykl J, Kurzawski S, Pohl T, Wittmann-Liebold B, Schlüter H (2004) Principle and applications of the protein-purification-parameter screening system. J Chromatogr A 1043:73–80
32. Thillaivinayagalingam P, O'Donovan K, Newcombe AR, Keshavarz-Moore E (2007) Characterisation of an industrial affinity process used in the manufacturing of digoxin specific polyclonal Fab fragments. J Chromatogr B 848:88–96
33. Titchener-Hooker NJ, Dunnill P, Hoare M (2008) Micro biochemical engineering to accelerate the design of industrial-scale downstream processes for biopharmaceutical proteins. Biotechnol Bioeng 100:473–487
34. Wiendahl M, Wierling PS, Nielsen J, Christensen DF, Krarup J, Staby A, Hubbuch J (2008) High throughput screening for the design and optimization of chromatographic processes – miniaturization, automation and parallelization of breakthrough and elution studies. Chem Eng Technol 31:893–903
35. Wierling PS, Bogumil R, Knieps-Grűnhagen E, Hubbuch J (2007) High throughput screening of packed-bed chromatography coupled with SELDI-TOFMS analysis: monoclonal antibodies versus host cell protein. Biotechnol Bioeng 98:440–450
36. Wenger MD, DePhillips P, Price CE, Bracewell DG (2007) An automated microscale chromatographic purification of virus-like particles as a strategy for process development. Biotechnol Appl Bioc 47:131–139
37. Wensel DL, Kelley BD, Coffman JL (2008) High-throughput screening of chromatographic separations: III. Monoclonal antibodies on ceramic hydroxyapatite. Biotechnol Bioeng 100:839–854
38. Williams JG, Tomer KB (2004) Disposable chromatography for a high throughput nano-ESI/MS and nano-ESI/MS–MS platform. J Am Soc Mass Spectr 15:1333–1340

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Applying Mechanistic Models in Bioprocess Development

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Abstract The available knowledge on the mechanisms of a bioprocess system is central to process analytical technology. In this respect, mechanistic modeling has gained renewed attention, since a mechanistic model can provide an excellent summary of available process knowledge. Such a model therefore incorporates process-relevant input (critical process variables)–output (product concentration and product quality attributes) relations. The model therefore has great value in planning experiments, or in determining which critical process variables need to be monitored and controlled tightly. Mechanistic models should be combined with proper model analysis tools, such as uncertainty and sensitivity analysis. When assuming distributed inputs, the resulting uncertainty in the model outputs can be decomposed using sensitivity analysis to determine which input parameters are responsible for the major part of the output uncertainty. Such information can be used as guidance for experimental work; i.e., only parameters with a significant influence on model outputs need to be determined experimentally. The use of mechanistic models and model analysis tools is demonstrated in this chapter. As a practical case study, experimental data from *Saccharomyces cerevisiae* fermentations are used. The data are described with the well-known model of Sonnleitner and Käppeli (Biotechnol Bioeng 28:927–937, 1986) and the model is analyzed further. The methods used are generic, and can be transferred easily to other, more complex case studies as well.

Keywords Fermentation · Identifiability · Modeling · Monte Carlo · PAT · *Saccharomyces cerevisiae* · Sensitivity · Uncertainty

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Abbreviations

API	Active pharmaceutical ingredient
EMEA	European Medicines Agency
FDA	Food and Drug Administration
MW	Molecular weight
NBE	New biological entity
NCE	New chemical entity
PAT	Process analytical technology
PSE	Process systems engineering
QbD	Quality by design
RTR	Real-time release
OD	Optical density
DW (Biomass)	Dry weight

Contents

1 Introduction

The pharmaceutical industry is changing rapidly nowadays. One important change, compared with the situation 10 or 20 years ago, is undoubtedly the increased focus on development of more efficient production processes. The introduction of process analytical technology (PAT) by the Food and Drug Administration [2] forms an important milestone here, since its publication ended a long period of regulatory uncertainty. The PAT guidance indeed makes it clear that regulatory bodies are in favor of more efficient production methods, as long as a safe product can be guaranteed. This opens up new and exciting possibilities for innovation in pharmaceutical production processes.

One of the central concepts in PAT is the *design space*, which is defined as "the multi-dimensional combination of critical input variables and critical process

parameters that lead to the right critical quality attributes" [2]. The term "critical" should be interpreted as "having a significant influence on final product quality." Changing the process within the design space is therefore not considered as a change. As a consequence, no regulatory postapproval of the process is required for a change within the design space. Almost naturally, this opens up the possibility of increased use of optimization methods for pharmaceutical processes in the future, methods that have been used for a long time in, for example, the chemical industry [3].

Small-molecule (MW $<$ 1,000) drug substances (APIs, NCEs) are typically produced via organic synthesis. In such a production system, the available process knowledge is often relatively large. Process systems engineering (PSE) methods and tools—especially those relying on mechanistic models to represent available process knowledge—are therefore increasingly applied in the frame of pharmaceutical process development and innovation of small-molecule drugs [4], with the aim of shortening time to market while yielding an efficient production process. In essence, mechanistic models rely on deterministic principles to represent available process knowledge on the basis of mass, energy, and momentum balances; given initial conditions, future system behavior can be predicted.

It is, however, not the intention here to provide a detailed review on mechanistic models for biobased production processes of pharmaceuticals. There are excellent textbooks and review articles on the general principles of mechanistic modeling of fermentation processes [5–8], biocatalysis [9, 10], and mammalian cell culture [11].

Biotechnology research has resulted in a new class of biomolecular drugs—typically larger molecules, also called biologics or NBEs—which includes monoclonal antibodies, cytokines, tissue growth factors, and therapeutic proteins. The production of biomolecular drugs is usually complicated and extremely expensive. The level of process understanding is therefore in many cases lower, compared with small-molecule drug substances, and as a consequence, PSE methods and tools relying on mechanistic models are usually not applied to the same extent in production of biomolecular drugs, despite the fact that quite a number of articles have been published throughout the years on the development of mechanistic models for such processes.

This chapter focuses on the potential use of mechanistic models within biobased production of drug products, as well as the use of good modeling practice (GMoP) when using such mechanistic models [12]. A case study with the yeast model by Sonnleitner and Käppeli [1] is used to illustrate how a mechanistic model can be formulated in a well-organized and easy-to-interpret matrix notation. This model is then analyzed using uncertainty and sensitivity analysis, an analysis that serves as a starting point for a discussion on the potential application of such methods. Strategies for mechanistic model-building are highlighted in the final discussion.

2 Case Study: Aerobic Cultivation of Budding Yeast

Saccharomyces cerevisiae is one of the most relevant and intensively studied microorganisms in biotechnology and bioprocess engineering; For example, out of 151 recombinant biopharmaceuticals that had been approved by the FDA and EMEA in January 2009, 28 (or 18.5 %) were produced in *S. cerevisiae* [13]. Sonnleitner and Käppeli [1] proposed a widely accepted mechanistic model describing the aerobic growth of budding yeast, and this model is used here to exemplify how a mechanistic model of a bioprocess can be applied to create more in-depth process knowledge. Optimally, the process knowledge should be translated into a mechanistic model, and the model should be updated whenever additional details of the process are unraveled. This model should capture the key phenomena taking place in the process, and be further employed in the development of process control strategies.

However, when developing and using mechanistic models, reliability of the model (hence the credibility of model-based applications) is an important issue, which needs to be assessed using appropriate methods and tools including identifiability, sensitivity, and uncertainty analysis techniques. Unfortunately, literature reporting on mechanistic model developments often lacks the results of such analysis—confidence intervals on estimated parameters, for example, are only sporadically reported—and as a consequence it is not possible to conclude about the quality of the model and its predictions. Seen from a PAT perspective, it is of utmost importance to document that one has constructed a reliable mechanistic model; For example, in case this model would be used later for simulations to help in determining where to put the borders of the design space, it would be difficult to defend the resulting design space—for example, towards the FDA—in case the reliability of the model cannot be documented sufficiently.

One of the challenges in modeling is the identifiability problem, defined as "given a set of data, how well can the unknown model parameters be estimated, hence identified." Typically, the number of parameters in a mechanistic model is relatively high, and therefore it is often not possible to uniquely estimate all the parameters by fitting the model predictions to experimental measurements. An indication of the parameters that can be estimated based on available data can be obtained by performing an identifiability analysis prior to the parameter estimation.

Furthermore, the model predictions will depend on the values of all parameters. Some of the parameters will, however, have a stronger influence than others. An uncertainty and sensitivity analysis can be performed to determine which are the parameters whose variability contributes most to the variance of the different model outputs.

In this case study, a systematic model analysis is performed following the workflow presented in Fig. 1. This workflow is rather generic, and could easily be transferred to another case study with a similar model.

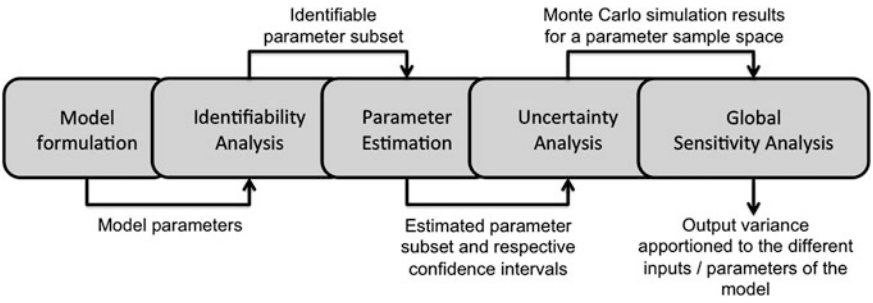

Fig. 1 Schematic workflow for the model analysis

2.1 Model Formulation

Under aerobic conditions, budding yeast may exclusively oxidize glucose (respiratory metabolism), or simultaneously oxidize and reduce glucose (fermentative metabolism) if the respiratory capacity of the cells is exceeded. The described overflow metabolism is commonly referred to as the Crabtree effect. Cells preferably oxidize glucose, as the energetic yield is more favorable for respiration than fermentation. In case the respiratory capacity is reached, the excess of glucose (i.e., overflow of glucose) is reduced using fermentative pathways that result in the production of ethanol. Moreover, in a second growth phase, yeast will then consume the produced ethanol, but only after depletion of glucose, as the latter inhibits the consumption of any other carbon source. Also acetate and glycerol are formed and consumed, although the corresponding concentrations are typically much lower than for ethanol.

The Sonnleitner and Käppeli [1] model describes the glucose-limited growth of *Saccharomyces cerevisiae*. This model is able to account for the overflow metabolism, and to predict the concentrations of biomass, glucose, ethanol, and oxygen throughout an aerobic cultivation in a stirred tank reactor. Acetate and glycerol are not included for simplification purposes. The model relies on three stoichiometric reactions describing the growth of biomass on glucose by respiration (Eq. 1) and by fermentation (Eq. 2), as well as the growth of biomass on ethanol by respiration (Eq. 3). The stoichiometry of the three different pathways can be summarized in a matrix form (Table 1) describing how the consumption of glucose, ethanol, and oxygen are correlated with the production of biomass and ethanol, i.e., the yields of the reactions. The mol-based stoichiometric coefficients can be converted into the corresponding mass-based yields, e.g., $Y_{XG}^{Oxid} = b \times$ MW(biomass)/MW(glucose).

$$C_6H_{12}O_6 + aO_2 + b\ 0.15\ [NH_3] \rightarrow bC_1H_{1.79}O_{0.57}N_{0.15} + cCO_2 + dH_2O \quad (1)$$

$$C_6H_{12}O_6 + g\ 0.15\ [NH_3] \rightarrow gC_1H_{1.79}O_{0.57}N_{0.15} + hCO_2 + iH_2O + jC_2H_6O \quad (2)$$

Table 1 Stoichiometric matrix describing aerobic growth of budding yeast

Component i →	C_1 Glucose		C_2 Ethanol		C_3 Oxygen		C_4 Biomass	
Symbols	G		E		O		X	
Units	mol l^{-1}	g l^{-1}	mol l^{-1}	g l^{-1}	mol l^{-1}	g l^{-1}	C-mol l^{-1}	g l^{-1}
Process j ↓								
Biomass growth by glucose oxidation (Eq. 1)	−1	−1	0	0	a	Y_{OG}	b	Y_{XG}^{Oxid}
Biomass growth by glucose reduction (Eq. 2)	−1	−1	j	Y_{EG}	0	0	g	Y_{XG}^{Red}
Biomass growth by ethanol oxidation (Eq. 3)	0	0	−1	−1	k	Y_{OE}	l	Y_{XE}

$$C_6H_6O + kO_2 + l\,0.15[NH_3] \rightarrow lC_1H_{1.79}O_{0.57}N_{0.15} + mCO_2 + nH_2O \quad (3)$$

For each pathway, a mass balance can be established for each atomic element (e.g. C or N). To solve such elemental balances for carbon, hydrogen, and oxygen, one stoichiometric coefficient for each pathway has to be assumed. Since the biomass yield coefficients are often easily estimated from experimental data, they are typically the ones that are assumed. Therefore, only the coefficients b, g and l, or the corresponding mass yields Y_{XG}^{Oxid}, Y_{XG}^{Red}, and Y_{XE} will be considered as model parameters; i.e., the other stoichiometric coefficients are fixed based on Eqs. 1–3.

Furthermore, a process matrix can be used to describe the rates of consumption and production of each of the model variables (glucose, ethanol, oxygen, and biomass), as well as the fluxes in each pathway. Details on the use of this matrix notation are provided by Sin and colleagues [14]. The interested reader can find additional details on elemental mass and energy balances applied to fermentation processes elsewhere [15, 16].

In the case of the model used as an example here, the total glucose consumption and ethanol consumption rates (when considered individually) are mathematically described using Monod-type kinetics (Eqs. 4–6). The maximum uptake rates for glucose, ethanol, and oxygen ($r_{i,max}$) are model parameters, and they are characteristic of the *S. cerevisiae* strain being used. The same goes for the substrate saturation constants: K_G, K_E, and K_O. The maximum oxygen uptake rate ($r_{O,max}$) corresponds to the respiratory capacity, as it reflects the maximum rate for oxidation of glucose or ethanol when any of these carbon sources is in excess. The ethanol uptake rate includes a term accounting for glucose repression; i.e., ethanol consumption is only observed for low concentrations of glucose. The strength of inhibition (i.e., how low the glucose concentration should be before ethanol consumption is allowed) is defined by the inhibition constant K_i. The specific growth rate of biomass is defined as the sum of the growth resulting from each pathway, and is estimated based on the yield of biomass on the substrate and the corresponding uptake rate (Eq. 7).

$$r_G^{Total} = r_{G,max}\frac{G}{G + K_G} = r_G^{Oxid} + r_G^{Red} \quad (4)$$

$$\mathrm{r_E} = \mathrm{r_{E,max}} \frac{\mathrm{E}}{\mathrm{E} + K_\mathrm{E}} \frac{K_i}{\mathrm{G} + K_i} \tag{5}$$

$$\mathrm{r_O} = \mathrm{r_{O,max}} \frac{\mathrm{O}}{\mathrm{O} + K_\mathrm{O}} \tag{6}$$

$$\mu_{\mathrm{Total}} = +Y_{\mathrm{XG}}^{\mathrm{Oxid}} \times \mathrm{r_G^{Oxid}} + Y_{\mathrm{XG}}^{\mathrm{Red}} \times \mathrm{r_G^{Red}} + Y_{\mathrm{XE}} \times \mathrm{r}_E^{Oxid} \tag{7}$$

The rate of oxidation and the rate of reduction of glucose are defined based on the maximum oxygen uptake rate: if the oxygen demand that is stoichiometrically required for oxidation of the total glucose flux ($Y_{\mathrm{OG}} \times r_\mathrm{G}^{\mathrm{Total}}$) exceeds the maximum oxygen uptake rate ($r_{\mathrm{O,max}}$), the difference between the two fluxes corresponds to the overflow reductive flux. With regard to the oxidation of ethanol, the observed rate of ethanol oxidation depends on the ethanol availability (Eq. 5) and it is further limited by the respiratory capacity: not only the maximum capacity of the cell, but also the capacity remaining after considering metabolism of glucose (Table 2).

In addition to the reactions taking place in the cells, oxygen is continuously supplied to the bioreactor. This supply is described based on the mass transfer coefficient ($k_\mathrm{L}a$) and the difference between the dissolved oxygen concentration (O) and the saturation concentration of oxygen in water (O^*) as a driving force. $k_\mathrm{L}a$ is dependent on the aeration intensity and the mixing conditions in a given fermentor. It is also dependent on the biomass concentration, although this dependence is often disregarded. The rates for each component can be obtained from the process model matrix (Table 2) by multiplying the transpose of the stoichiometric matrix ($\mathbf{Z}$') by the process rate vector ($\boldsymbol{\rho}$): $r_{m,1} = \mathbf{Z}\prime_{nxm} \times \boldsymbol{\rho}_{nx1}$, where m corresponds to the number of components (or model variables) and n is the number of processes. In Table 3, a nomenclature list of vectors and matrices is presented.

The model matrix in Table 2 provides a compact overview of the model equations. In the example here, it contains information about the biological reactions and the transfer of oxygen from the gas to the liquid phase. Of course, depending on the purpose of the model, the model matrix could be extended with additional equations, for instance, aiming at a more detailed description of the biological reactions, e.g., by including additional state variables, or aiming at the description of the mass transfer of additional components, e.g., CO_2 stripping from the fermentation broth. Sin and colleagues [14] provided an example of the extension of the model matrix with chemical processes for the kinetic description of mixed weak acid–base systems. The latter is important in case pH prediction is part of the purpose of the model. In the work of Sin and colleagues [14], the yield coefficients are all part of the stoichiometric matrix. In our case here, an alternative rate vector is presented, where all rates are normalized with regard to glucose.

Table 2 Process matrix describing the conversion rates and stoichiometry for each model variable: glucose, ethanol, oxygen, and biomass

Component $i \rightarrow$	C_1 Glucose	C_2 Ethanol	C_3 Oxygen	C_4 Biomass	Rate vector (ρ)
Symbols	G	E	O	X	
Units	g l^{-1}	g l^{-1}	g l^{-1}	g l^{-1}	g l^{-1} h^{-1}
Process $j \downarrow$					
Biomass growth by glucose oxidation	-1	0	$-Y_{OG}$	$-Y_{XG}^{Oxid}$	$\frac{1}{Y_{OG}}\left(\min\left(r_{O,max}\frac{O}{O+K_o}, Y_{OG}\times r_{G,max}\frac{G}{G+K_G}\right)\right)$
Biomass growth by glucose reduction	-1	Y_{EG}	0	Y_{XG}^{Red}	$r_{G,max}\frac{G}{G+K_G}-\frac{1}{Y_{OG}}\left(\min\left(r_{O,max}\frac{O}{O+K_o}, Y_{OG}\times r_{G,max}\frac{G}{G+K_G}\right)\right)$
Biomass growth by ethanol oxidation	0	-1	$-Y_{OE}$	Y_{XE}	$\frac{1}{Y_{OE}}\left(\min\left(r_{O,max}\frac{O}{O+K_o}-\min\left(r_{O,max}\frac{O}{O+K_o}, Y_{OG}\times r_{G,max}\frac{G}{G+K_G}\right), Y_{OE}\times r_{E,max}\frac{E}{E+K_E}\frac{K_i}{G+K_i}\right)\right)$
Oxygen supply	–	–	1	–	k_La (O*−O)

Table 3 Nomenclature list of matrices and vectors used in the model formulation and model analysis

Unit	Description
$\boldsymbol{Z}$	Stoichiometric matrix
$\boldsymbol{\rho}$	Process rate vector
$\boldsymbol{\theta}$	Vector of model parameters
$\mathbf{S}^{sc}$	Scaled sensitivity matrix
$\mathbf{s}_j$	Column vector of the sensitivity matrix: corresponding to sensitivity of the various model outputs to the parameter j
s_{ij}	Scaled sensitivity of the output i to the parameter j
δ_j	Importance index of parameter j
sc	Scaling factors

2.2 Parameter Identifiability Analysis

The model described in the previous sections has four variables—glucose (G), ethanol (E), oxygen (O), and biomass (X)—and 11 parameters. In addition, the oxygen saturation concentration in water (at growth temperature) is necessary for solving the model. A list of the parameters and their descriptions is provided in Table 4.

The maximum specific growth rate on ethanol $(\mu_{E,max})$ is defined as the product of the yield of biomass on ethanol (Y_{XE}) and the maximum specific ethanol uptake rate $(r_{E,max}^{Oxid})$. For consistency between parameters, the ethanol specific uptake rate is used as a parameter in this example.

The number of parameters is considerably larger than the number of model variables (or outputs), which is typical for this type of model. It is therefore questionable whether all parameters can be estimated based on experimental data, even if the four model variables were to be measured simultaneously. This is the subject of identifiability analysis, which seeks to identify which of the parameters

Table 4 Model parameters, corresponding units, and numerical values [12]

	Parameter	Value	Units
$r_{G,max}$	Maximal specific glucose uptake rate	3.5	g G g^{-1} X h^{-1}
$r_{O,max}$	Maximal specific oxygen uptake rate	8×10^{-3}	mol O g^{-1} X h^{-1}
Y_{XG}^{Oxid}	Yield of biomass on glucose (oxidation)	0.49	g X g^{-1} G
Y_{XG}^{red}	Yield of biomass on glucose (reduction)	0.05	g X g^{-1} G
Y_{XE}	Yield of biomass on ethanol	0.72	g X g^{-1} E
$\mu_{E,max}$	Maximal specific growth rate on ethanol	0.17	h^{-1}
K_G	Saturation parameter for glucose uptake	0.5	g l^{-1}
K_O	Saturation parameter for oxygen uptake	1×10^{-4}	g l^{-1}
K_E	Saturation parameter for ethanol uptake	0.1	g l^{-1}
K_i	Inhibition parameter: free glucose inhibits ethanol uptake	0.1	g l^{-1}
k_La	Mass transfer coefficient	1,000	h^{-1}

can be estimated with high degree of confidence based on the available experimental measurements.

The main purpose of such an identifiability analysis is in fact to increase the reliability of parameter estimation efforts from a given set of data [17]. One method available to perform such an analysis is the two-step procedure based on sensitivity and collinearity index analysis proposed by Brun and colleagues [18]. Accordingly, the method calculates two identifiability measures: (1) the parameter importance index (δ) that reflects the sensitivity of the model outputs to single parameters, and (2) the collinearity index (γ) which reflects the degree of near-linear dependence of the sensitivity functions of parameter subsets. A parameter subset (a combination of model parameters) is said to be identifiable if (1) the data are sufficiently sensitive to the parameter subset (above a cutoff value), and (2) the collinearity index is sufficiently low (below a cutoff value).

2.2.1 Local Sensitivity Analysis: Parameter Importance Indices δ

The local importance of an individual parameter to a model output for small changes ($\Delta\boldsymbol{\theta}$) in the parameter values ($\boldsymbol{\theta}$) at a specific location ($\boldsymbol{\theta}_0$) can be measured by the estimation of a dimension-free scaled sensitivity matrix $\mathbf{S}^{sc} = \{s_{ij}\}$, where the index i refers to a specific model variable (output) and j denotes the model parameter. For further details, the reader is referred to the original paper of Brun and colleagues [18]. The mean squared norm of column $\boldsymbol{s}_j$, denoted by δ_j, is a measure of the importance of parameter θ_j (see Eqs 8–10). A large norm indicates that the parameter is identifiable with the available data if all other parameters are fixed. A parameter importance ranking can be obtained by ranking the parameters according to their δ indices. The lower the value of δ, the lower the importance of that parameter.

For this first analysis, the parameter values (Table 4) provided in the original paper [1] are used as nominal values at which sensitivity functions are calculated. The scaled sensitivity matrix $\mathbf{S}$ and the resulting rank of δ importance indices were calculated using Eqs. 8–10, and are graphically compared in Fig. 2. It is noteworthy that the δ indices are very sensitive to: (1) the choice of variation range defined for each parameter ($\Delta\boldsymbol{\theta}$), (2) scaling factors ($sc$) used to calculate the sensitivity matrix, and (3) the original set of parameters ($\boldsymbol{\theta}_0$), naturally as this is a local analysis. In this example the sc were defined as the mean of the experimental observations for each variable.

$$v_{ij} = \left.\frac{\partial \eta_i(\theta_j)}{\partial \theta_j}\right|_{\theta-\theta_0} \tag{8}$$

$$S_{ij} = v_{ij}\frac{\Delta\theta_j}{SC_j} \tag{9}$$

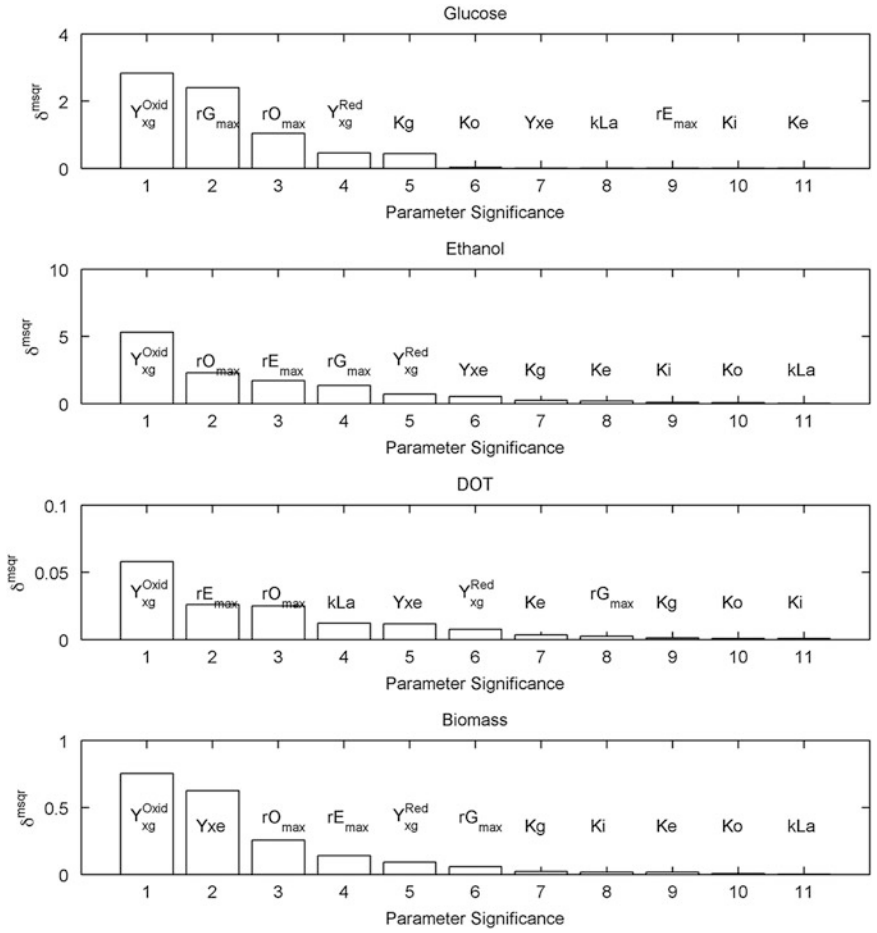

Fig. 2 Parameter importance indices (δ) for the four model variables: glucose, ethanol, dissolved oxygen and biomass

$$\delta_j = \sqrt{\frac{1}{n}\sum_{i=1}^{n} s_{ij}^2} \tag{10}$$

The results of the parameter significance ranking indicate that the yield coefficient $Y_{\mathrm{XG}}^{\mathrm{Oxid}}$ is the parameter that most affects all four model outputs. Variations in the maximum uptake rates will also have a significant effect on the model outputs. As may be expected, the glucose maximum uptake rate is most significant with regard to the model prediction for glucose, whereas the maximum uptake rate of ethanol is most important for the prediction of ethanol and dissolved oxygen.

The prediction of biomass is also greatly affected by the yield of biomass on ethanol, in addition to the yield on glucose (oxidative metabolism). The impact of the saturation constants is rather limited for any of the model variables.

2.2.2 Identifiability of Parameter Subsets: Collinearity Index γ_K

In addition to understanding the importance of individual parameters to the model output, it is necessary to take the joint influence of all parameters into account as well ($[\theta_1, \ldots, \theta_{j=J}]$). If columns $\boldsymbol{s}_j$ are nearly linearly dependent, the change of a parameter θ_j can be compensated by a change in the other parameter values. This means that the parameters $[\theta_1, \ldots, \theta_J]$ are not uniquely identifiable.

The collinearity index γ_K assesses the degree of near-linear dependence between a subset of K ($2 \leq K \leq J$) parameters, i.e., columns of the scaled sensitivity matrix.[1]

A high value of a collinearity index indicates that the parameter set is poorly identifiable. In practice, γ_K is calculated for all subsets of K parameters out of the 11 parameters and is plotted in Fig. 3. Also the subset size for each case is shown. In this case, a subset was considered identifiable if the corresponding collinearity index was smaller than 5. This threshold has to be defined a priori. Brun and colleagues [18] suggested as a rule of thumb that this threshold should lie in the range 5–20, where the lowest collinearity index corresponds to the strictest criterion. In practice, this decision on the threshold value is dependent on prior experience of the model user, and thus an iterative process.

All the model variables were considered in this analysis, implying as well that all could be measured experimentally. As illustrated in Fig. 3, a maximum of eight parameters can be identified, and the collinearity index increases with the number of parameters. The maximum collinearity index observed for combinations of eight parameters was 22.34, while the best identifiable sets of eight parameters correspond to a γ_K value of approximately 2.65. These parameter subsets are listed in Table 5.

It is indeed known that a change in the maximum uptake rate of glucose can be compensated with a change of biomass yield coefficients. Also, based on the model structure, it is clear that changes in yields for the oxidative and reductive consumptions of glucose can compensate each other. It is therefore not surprising that the parameter subsets that have higher collinearity index include these parameters. When comparing the subset of six parameters with the lowest collinearity index (last row in Table 5) with the "best" subset of eight parameters (shaded row in Table 5), the two parameters that have been removed in the subset of six parameters are the maximum uptake rates of ethanol and oxygen.

[1] Further discussion and equations are provided in the paper by Brun et al. (2002).

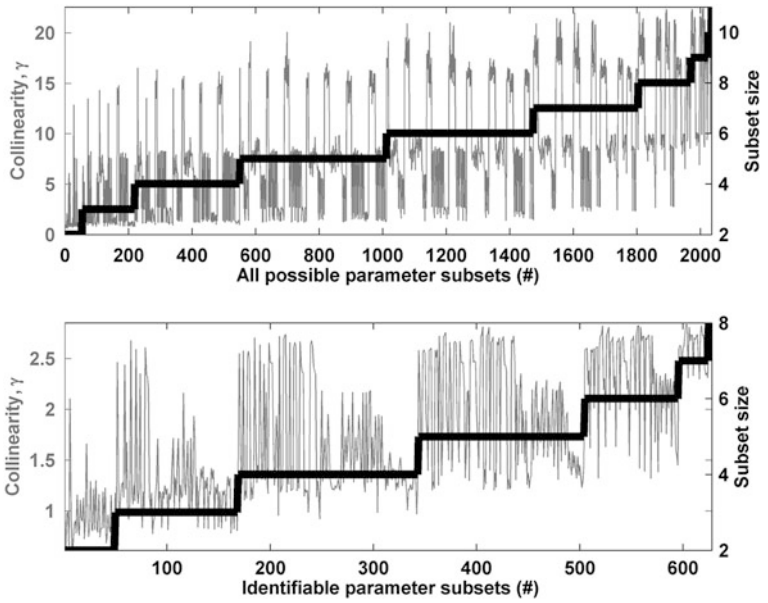

Fig. 3 Collinearity index and size corresponding to parameter subsets of increasing size. The top plot refers to all the parameter subsets evaluated in the analysis, whereas the bottom figure refers exclusively to the subsets that complied with the a priori defined collinearity threshold

The collinearity between the uptake rates and the yield coefficients explains why, even though they are the parameters with greatest importance for the model outputs (Fig. 2), they are not all included in the identifiable parameter subsets.

2.3 Parameter Estimation

Two datasets corresponding to two replicate batch fermentations of *S. cerevisiae* were available. For further details on the experimental data collection methods the reader is referred to the work of Carlquist et al. [19]. The dynamic profiles of glucose, ethanol, and biomass (as optical density, OD) were available for the two datasets, while oxygen data were only available for one of them. The OD measurements were converted into biomass dry weight (DW) values using a previously determined linear correlation (DW = 0.1815 × OD).

The parameters in the "best" identifiable subset were estimated by minimization of the weighted least-square errors. The weights for each variable i were defined by $w_i = 1 \Big/ (sc_i)^2$, and the scaled factors (also used in Eq. 9) were defined as the mean of the experimental observations for each given variable. The estimation was done simultaneously for the two datasets. The new estimates of the identifiable parameters

Table 5 Identifiable parameter subsets with maximum number of parameters and corresponding collinearity index

Parameter subset								Collinearity index	Identifiable parameter Set
$r_{G,max}$	$r_{E,max}$	Y_{XG}^{Oxid}	Y_{XG}^{Red}	Y_{XE}	K_G	K_E	k_La	22.34	No
$r_{G,max}$	$r_{O,max}$	$r_{E,max}$	Y_{XG}^{Oxid}	Y_{XG}^{Red}	Y_{XE}	K_G	k_La	22.10	No
$r_{G,max}$	$r_{E,max}$	Y_{XG}^{Oxid}	Y_{XG}^{Red}	Y_{XE}	K_G	K_i	k_La	22.10	No
$r_{G,max}$	$r_{O,max}$	$r_{E,max}$	Y_{XE}	K_G	K_E	K_i	k_La	2.65	Yes
$r_{G,max}$	$r_{E,max}$	Y_{XG}^{Red}	Y_{XE}	K_G	K_E	K_i	k_La	2.75	Yes
$r_{G,max}$	$r_{E,max}$	Y_{XE}	K_G	K_O	K_E	K_i	k_La	2.75	Yes
$r_{G,max}$	$r_{E,max}$	Y_{XG}^{Oxid}	Y_{XE}	K_G	K_E	K_i	k_La	2.85	Yes
$r_{G,max}$	Y_{XE}	K_G	K_E	K_i	k_La	–	–	1.75	Yes

Table 6 Estimated values for the identifiable subset of parameters

Parameter	Initial guess	Estimated value	Units
$r_{G,max}$	3.5	2.9	g G g^{-1} X h^{-1}
$r_{O,max}$	8×10^{-3}	5.5×10^{-3}	mol O g^{-1} X h^{-1}
$r_{E,max}$	0.24	0.32	g E g^{-1} X h^{-1}
Y_{XE}	0.72	0.47	g X g^{-1} E
K_G	0.5	0.17	g G l^{-1}
K_E	0.1	0.56	g E l^{-1}
K_i	0.1	0.31	g G l^{-1}
k_La	1,000	930	h^{-1}

are presented in Table 6. In Fig. 4, the model predictions obtained with the estimated set of parameters are compared with the experimental data.

Generally, the model predictions are in good agreement with the experimental data. An overprediction of the biomass concentration and a slight underestimation of the ethanol concentration are however observed. The oxygen profile describes the drop of the dissolved oxygen concentration during the growth, and a steep increase upon the depletion of ethanol and the resulting growth arrest. The dynamics of oxygen described by the model assumes a constant mass transfer coefficient (k_La) and equilibrium between the gas and liquid phases. It is worth mentioning that the formation of other metabolites (i.e., glycerol and acetate) that are not considered in the model may explain the discrepancies to some degree. In fact, the overestimation of biomass which can be observed in Fig. 4 may be caused by the fact that other carbon-containing metabolites have not been taken into account.

When assessing the goodness of fit of the mechanistic model, it is important to consider that the experimental measurements have an associated error as well. Model predictions may not give a "perfect" fit at first sight, but they may well be within the experimental error. While such error might be relatively low for the measurement of glucose and ethanol by high-performance liquid chromatography

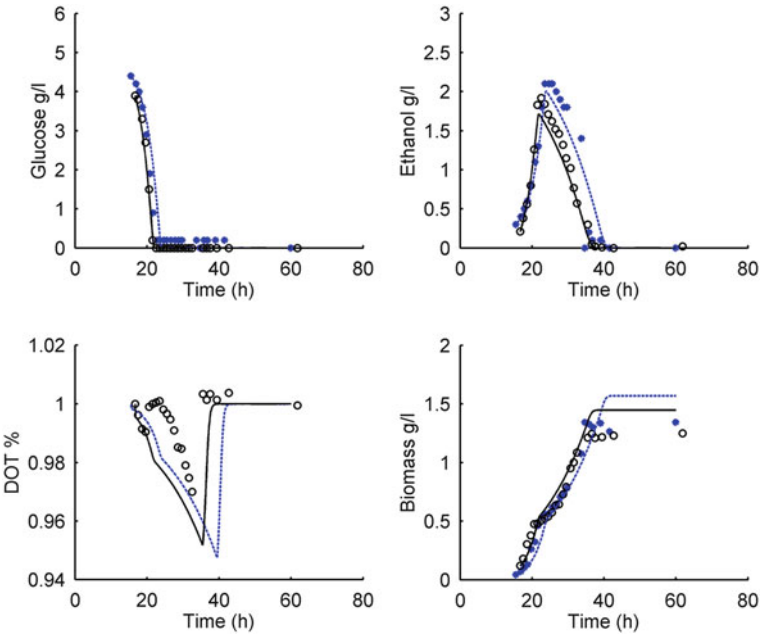

Fig. 4 Comparison of model predictions versus experimental data collected for cultivation 1 (*black line* model prediction, *black circles* experimental data) and cultivation 2 (*blue dashed line* model prediction, *blue stars*, experimental data)

(HPLC), it is significantly higher for dry weight measurements, which are less reliable, especially for low biomass concentrations (too large sample volumes would be required for increasing accuracy). Additionally, at the end of the fermentation, the biomass dry weight may include a fraction of nonviable and/or dormant cells.

2.3.1 Confidence Intervals for Estimated Parameters

The estimated parameter values as such only have limited value if they are not presented in combination with a measure of the degree of confidence that one can have in them. Therefore, the confidence intervals for each of the parameters are defined based on the covariance matrix and Student *t*-probability distribution. The covariance matrix is calculated using the residuals between model predictions and the standard deviations of the experimental measurements (further details are provided by Sin et al. [14]). An experimental error of 5 % was assumed for glucose and ethanol measurement by high-performance liquid chromatography (HPLC), as well as for the oxygen measurements using a gas analyzer for determining the composition of the exhaust gas, and a 20 % error for the determination of the cell dry weight. The confidence intervals at $(1 - \alpha)$ confidence level were

Table 7 Confidence intervals for the identifiable subset of parameters for 95 % confidence level

Parameter	Estimated value	Confidence interval	Units
$r_{G,max}$	2.9	$\pm 9.8 \times 10^{-2}$ (3.4 %)	g G g^{-1} X h^{-1}
$r_{O,max}$	5.5×10^{-3}	$\pm 6.3 \times 10^{-4}$ (11.6 %)	mol O g^{-1} X h^{-1}
$r_{E,max}$	0.32	±0.24 (75.7 %)	g E g^{-1} X h^{-1}
Y_{XE}	0.47	$\pm 3.1 \times 10^{-2}$ (6.6 %)	g X g^{-1} E
K_G	0.17	$\pm 8.4 \times 10^{-2}$ (50.2 %)	g G l^{-1}
K_E	0.56	±0.44 (78.9 %)	g E l^{-1}
K_i	0.31	±0.30 (97.5 %)	g G l^{-1}
k_La	930	±49 (5.2 %)	h^{-1}

calculated using Eq. 11, where COV is the covariance matrix of the parameter estimators, $t(N - M, \alpha/2)$ is the t-distribution value corresponding to the $\alpha/2$ percentile, N is the total number of experimental observations (45 samples for the two cultivations), and M is the total number of parameters. The confidence intervals for the estimated parameters are presented in Table 7.

$$\theta_{1-\alpha} = \theta \pm \sqrt{\text{diag}(\text{COV}(\theta))} \cdot t\left(N - M, \frac{\alpha}{2}\right). \tag{11}$$

None of the confidence intervals include zero, giving a first indication that all parameters are significant to a certain degree and the model does not seem to be overparameterized. In the case of the inhibition constant K_i, the confidence interval is rather large. This is most likely a consequence of the low sensitivity of model outputs to this variable (Fig. 2). Furthermore, the confidence intervals of the Monod half-saturation constants K_G and K_E are quite large as well, which might be related to the fact that their estimated values are rather low. The latter means that the collected data do not contain that many data points which can be used during the parameter estimation for extracting information on the exact values of K_G and K_E Indeed, only the data corresponding to relatively low glucose and ethanol concentrations can be used, since the specific rates will be relatively constant and close to maximum for higher substrate concentrations.

It is furthermore also a good idea to analyze the values of the parameter confidence intervals simultaneously with the correlation matrix (Table 8); For example, the correlation matrix shows that $r_{E,max}$ is correlated with K_E and that $r_{O,max}$ is correlated with K_O. Both correlations are inherent to the model structure; i.e., correlation between the parameters related to the maximum specific growth rate and the substrate affinity constant in Monod-like kinetics expressions are quite common, and point towards a structural identifiability issue.

Note also that the significant correlations found between some of the model parameters (Table 8) seem to conflict with the results of the collinearity index analysis which was reported earlier (Fig. 3; Table 5). That is one of the reasons also for the identifiability analysis to be an iterative process.

Table 8 Correlation matrix for all model parameters

	$r_{G,max}$	$r_{O,max}$	$r_{E,max}$	Y_{xg}^{Oxid}	Y_{xg}^{Red}	Y_{xe}	K_G	K_O	K_E	K_i	k_La
$r_{G,max}$	1	0.166	0.081	0.538	−0.546	−0.168	−0.356	−0.145	−0.047	−0.134	−0.076
$r_{O,max}$	0.166	1	0.546	−0.163	0.202	0.314	0.276	−0.904	−0.419	−0.548	0.114
$r_{E,max}$	0.081	0.546	1	−0.038	0.105	0.338	0.134	−0.648	−0.950	−0.652	0.434
Y_{xg}^{Oxid}	0.538	−0.163	−0.038	1	−0.987	−0.421	0.165	0.017	0.095	−0.387	−0.041
Y_{xg}^{Red}	−0.546	0.202	0.105	−0.987	1	0.493	−0.206	−0.108	−0.145	0.342	0.131
Y_{Xe}	−0.168	0.314	0.338	−0.421	0.493	1	0.042	−0.515	−0.352	0.003	0.490
K_G	−0.356	0.276	0.134	0.165	−0.206	0.042	1	−0.255	−0.091	−0.425	0.009
K_O	−0.145	−0.904	−0.648	0.017	−0.108	−0.515	−0.255	1	0.499	0.583	−0.398
K_E	−0.047	−0.419	−0.950	0.095	−0.145	−0.352	−0.091	0.499	1	0.552	−0.373
K_i	−0.134	−0.548	−0.652	−0.387	0.342	0.003	−0.425	0.583	0.552	1	−0.047
k_La	−0.076	0.114	0.434	−0.041	0.131	0.490	0.009	−0.398	−0.373	−0.047	1

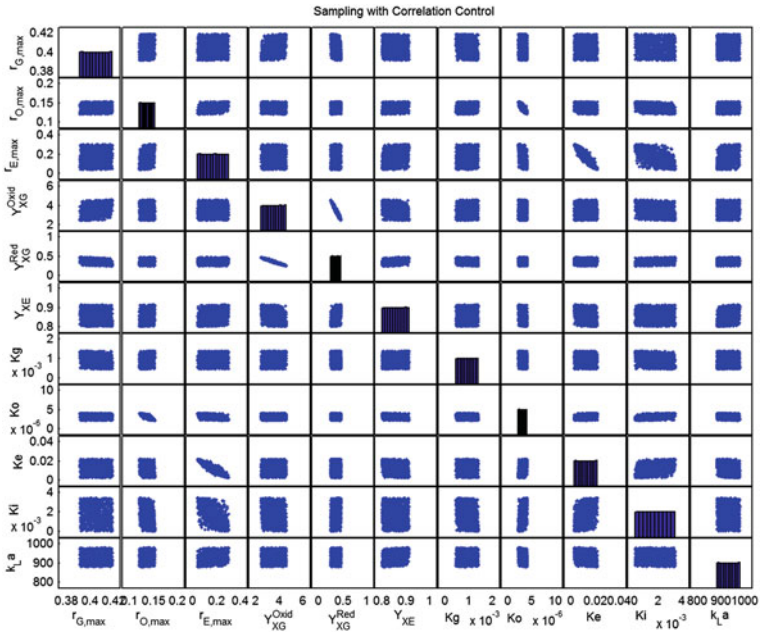

Fig. 5 Latin hypercube sampling for the model parameters, taking into account the correlation between them

2.4 Uncertainty Analysis

Uncertainty analysis allows for understanding the variance of the model outputs as a consequence of the variability in the input parameters. Such an analysis can be performed using the Monte Carlo procedure, which consists of three steps: (1) definition of the parameter space, (2) generation of samples of the parameter space, i.e., combinations of parameters, and (3) simulation of the model using the set of samples generated in the previous step. In this case study, a sample set of 1,000 combinations of parameter values was generated using the Latin hypercube sampling procedure [20]. This sampling technique can be set up such that it takes the correlations between parameters, i.e., information resulting from the parameter estimation, into account (as explained by Sin et al. [12]). The correlation matrix for all the parameters was estimated and is presented in Table 8. For each parameter, minimum and maximum values have to be defined: for the estimated parameters the limits of the 95 % confidence intervals were used, while a variability of 30 % around the default values was assumed for the remaining parameters.

The correlation between two parameters can take values between −1 and 1. A positive correlation indicates that an increase in the parameter value will result in an increase in the value of the other parameter as well. On the contrary, a negative value indicates an inverse proportionality. In Fig. 5, the sampling space is illustrated by scatter plots of combinations of two parameters. A high correlation

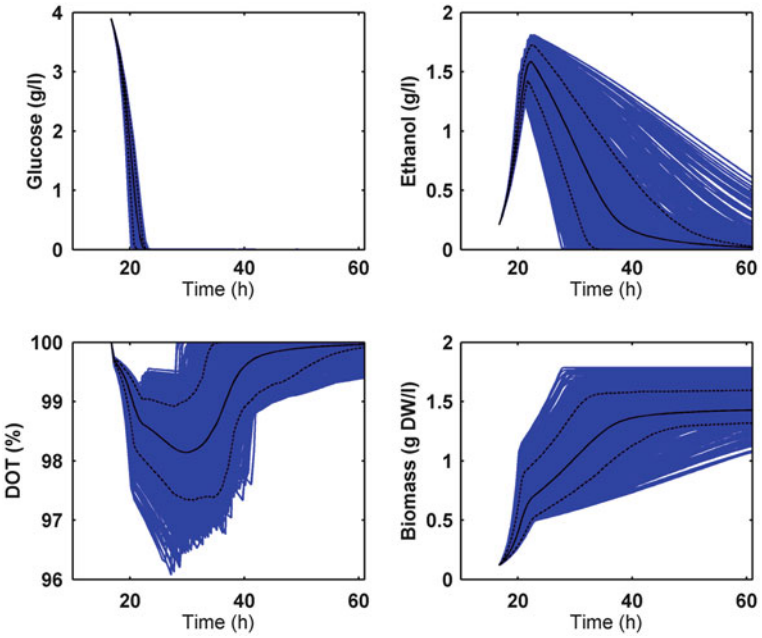

Fig. 6 Representation of uncertainty in the model predictions for glucose, ethanol, dissolved oxygen, and biomass: Monte Carlo simulations (*blue*), mean, and the 10th and 90th percentile of the predictions (*black*)

(in absolute value) will lead to an elliptical or linear cloud of sampling points, as, for example, for Y_{XG}^{Oxid} and Y_{XG}^{Red} [corr(Y_{XG}^{Oxid}, Y_{XG}^{Red}) = −0.98 in Table 8], as well as $r_{E,max}$ and K_E, and $r_{O,max}$ and K_O.

The number of samples and the assumed range of variability of each parameter (i.e., the parameter space) is defined by the expert performing the analysis. The higher the number of samples, the more effectively the parameter space will be covered, at the expense of increased computational time. The range of the parameter space should rely on previous knowledge of the process: (1) the initial guess of the parameter numerical values can be obtained from the literature or estimated in a first rough estimation where all parameters are included; (2) the variability (range) for each parameter can be determined by the confidence intervals, in case a parameter estimation has been done, or be defined based on expert knowledge as discussed by Sin et al. [12].

The estimations for the four model variables (outputs) and the corresponding mean and a prediction band defined by 10 and 90 % percentiles are presented in Fig. 6. The narrow prediction bands (including 80 % of the model predictions) for glucose reflect the robustness of the predictions for this model variable, while the wide bands observed, for example, for oxygen show the need for a more accurate estimate of the parameters in order to obtain a good model prediction.

2.5 Sensitivity Analysis: Linear Regression of Monte Carlo Simulations

Based on the Monte Carlo simulations, a global sensitivity analysis can be conducted. The aim of the sensitivity analysis is to break down the output uncertainty with respect to input (parameter) uncertainty. The linear regression method is a rather simple yet powerful analysis that assumes a linear relation between the parameter values and the model outputs. The sensitivity of the model outputs to the individual parameters, for a given time point, is summarized by a ranking of parameters according to the absolute value for the standardized regression coefficient (SRC). In a dimensionless form, the linear regression is described by Eq. 12, where sy_{ik} is the scalar value for the kth output, β_{jk} is the SRC of the jth input parameter, θ_j, for the kth model output, y_k, and its magnitude relates to how strongly the input parameter contributes to the output.

$$\frac{sy_{ik} - \mu_{sy_k}}{\sigma_{sy_k}} = \sum_{j=1}^{M} \beta_{jk} \times \frac{\theta_{ij} - \mu_{\theta j}}{\sigma_{\theta_j}} + \varepsilon_{ik} \tag{12}$$

In the case of nonlinear dependence of the model variable on a parameter, this method can still be used, although with caution. As a rule of thumb, if the model coefficient of determination (R^2) is lower than 0.7, this analysis is not conclusive. The SRC for each parameter has, by definition, a value between -1 and 1, where a negative sign indicates that the output value will decrease when there is an increase in the value of the parameter. Oppositely, a positive SRC indicates direct proportionality between the parameter value and the model output. Sin et al. [12] describe further details on how to perform the analysis.

In the model example, different growth phases are described, and therefore the importance of the parameters is expected to change with time. Therefore, the analysis was performed for a selection of time points up to 62 h.

The suitability of applying the linear regression method was in this case also assessed for each time point and each output. The R^2 values are presented in Fig. 7 as a function of time.

While the regression method seems to be suitable for all time points in the case of biomass, the same is not observed for glucose, ethanol, and oxygen.. With regard to glucose, the model uncertainty is very small (narrow spread of the model predictions plotted in Fig. 6). The depletion of glucose is estimated to occur at time of approximately 22 h for all cases. The sensitivity analysis when the glucose concentrations are virtually zero is not expected to be significant, and it is thus not surprising that the R^2 value decreases abruptly at approximately the same time point that glucose is depleted. Simultaneously, the uncertainty in ethanol concentration predictions increases substantially. This may explain the temporary drop in the R^2 value for ethanol at this time point. A similar drop in R^2 is observed for oxygen around the time that ethanol is depleted, and a sudden rise in the dissolved oxygen concentration is observed. Upon ethanol depletion, the R^2 value

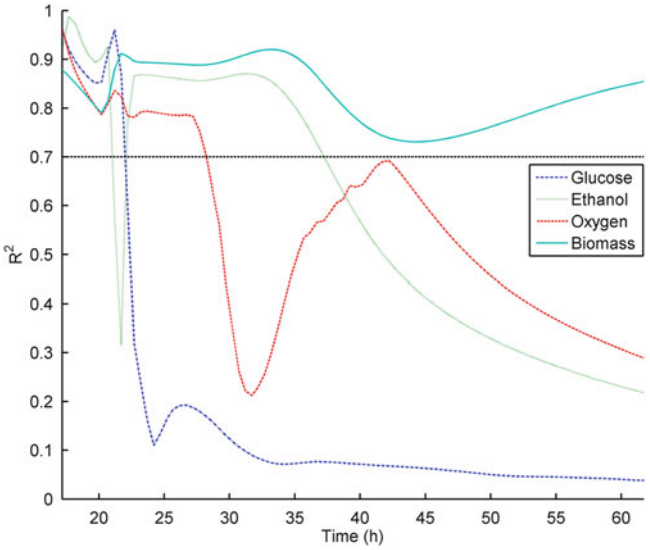

Fig. 7 Regression correlation coefficient (R^2) for each model output, indicating the goodness of the linear regression used for estimating the sensitivity of each model output to various parameters. For R^2 values lower than 0.7, the corresponding standardized regression coefficient (SRC) may yield erroneous information

for ethanol falls under the threshold, similarly to what was observed for glucose at its depletion.

In Fig. 8, an overview of the SRCs for each parameter and model output is presented. Interpretation of parameter ranking and SRC should be made cautiously. All model outputs seem to be sensitive to the yield coefficient of biomass on oxidized glucose, even during the growth phase on ethanol (after glucose depletion).

The ranking of each parameter according to the SRC for each model output is illustrated in Fig. 9. When analyzing this ranking, it is possible to see the decrease in sensitivity of the glucose prediction towards the maximum glucose uptake rate, as well as the simultaneous increase in sensitivity towards the maximum oxygen uptake rate, during the growth phase on glucose. This is in agreement with the fact that the consumption of glucose is initially only limited by the maximum uptake rate (excess of glucose in the media), and afterwards as the biomass concentration increases and glucose concentration decreases, the observed uptake rate is no longer maximal. Similar figures for the parameter ranking regarding ethanol, oxygen and biomass can be drawn.

With regard to the model predictions for ethanol, this model output is most sensitive to the maximum glucose uptake rate and biomass yield on glucose (reduction pathway) during the first growth phase, and later on the maximum ethanol uptake rate. This is in good agreement with the fact that the production of ethanol is a result of the reduction of glucose, and its consumption only takes place

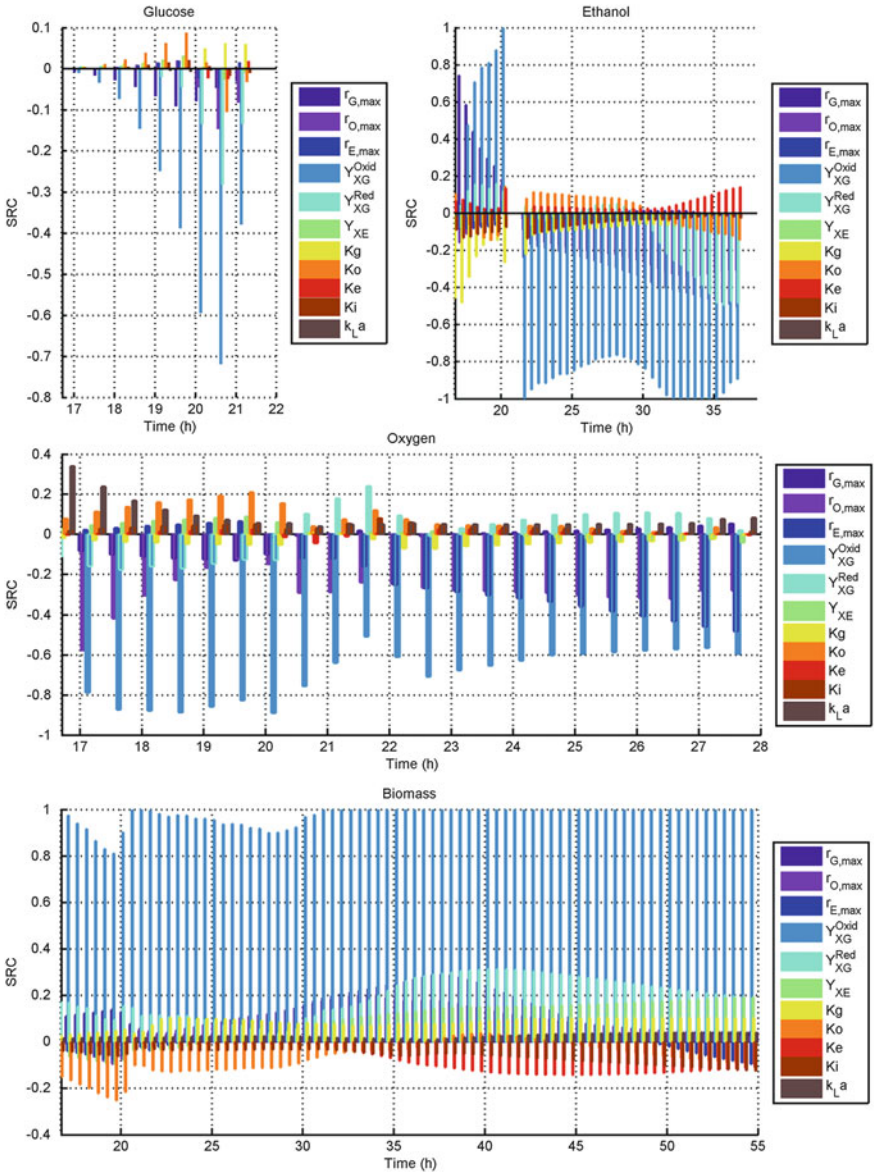

Fig. 8 Standardized regression coefficients (SRC) for the four model outputs as a function of time. Only the time points for which $R^2 > 0.7$ was observed are presented. Each color corresponds to a model parameter

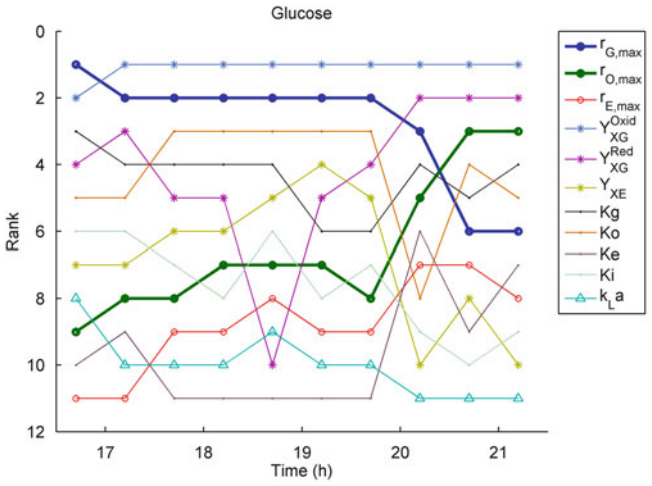

Fig. 9 Ranking of each model parameter according to the magnitude of the SRC for each model output: a rank of 1 indicates that the model output is most sensitive to that parameter, while a rank of 11 indicates that the parameter contributes the least to the variance of the model output

during the second growth phase following the depletion of glucose. A similar pattern was observed with regard to the model predictions for oxygen.

To analyze the sensitivity of the outputs to the parameters in more detail, two time points during the exponential growth phase on glucose ($t = 17$ h) and on ethanol ($t = 27$ h) were selected. The SRC and corresponding rank position for these time points are provided in Table 9a and b, respectively. As could be expected, during the growth on glucose, the parameters that most influence the prediction of glucose are the biomass yield parameters (for the two pathways) and the maximum uptake rate. The two yield coefficients have, however, a different effect on the glucose prediction: while an increase in the oxidative yield will lead to a lower predicted concentration, an increase in the reductive yield seems to imply an increase in the predicted concentration. This may reflect the fact that the oxidative pathway is the most effective way of transforming glucose into biomass.

The maximum glucose uptake rate is also the most influential parameter for the prediction of the ethanol concentration (produced by reduction of glucose), during this first growth phase. The glucose saturation rate plays an important role, however not as significant as the maximum uptake rate ($r_{G,max}$: SRC = 0.74; K_G: SRC = −0.48).

Obviously, the results of the global sensitivity analysis (SRC) should be compared with the results of local sensitivity analysis (Fig. 2). It can be seen that both methods rank the biomass yield on glucose (oxidation) as the most influential parameter. For the ranking of the other parameters, there are quite some differences between the results obtained by the two methods.

2.5.1 Morris Screening

As discussed by Sin et al. [11], an alternative to the linear regression method, especially when low R^2 values are observed, is Morris screening. Similarly to the linear regression method, a sampling-based approach is used. The method is based on Morris sampling, which is an efficient sampling strategy for performing randomized calculation of one-factor-at-a-time (OAT) sensitivity analysis. The parameters are assigned uniform distributions with lower and upper bounds defined by the confidence intervals for estimated parameters and by 30 % variability for the remaining ones (as done previously for the Latin hypercube sampling). The number of repetitions (r) was set to 90, corresponding to a sampling matrix with 1,080 [$90 \times (11 + 1)$] different parameter combinations. The model was simulated for all the parameter combinations, and the results are summarized in Fig. 10.

The elementary effects (EE) were estimated as described by Sin et al. [12]. These EEs are described as random observations of a certain distribution function F, and are defined by Eq. 13, where Δ is a predetermined perturbation factor of θ_j, $sy_k(\theta_1, \theta_2, \theta_j, \ldots, \theta_M)$ is the scalar model output evaluated at input parameters $(\theta_1, \theta_2, \theta_j, \ldots, \theta_M)$, whereas $sy_k(\theta_1, \theta_2, \theta_j + \Delta, \ldots, \theta_M)$ is the scalar model output corresponding to a Δ change in θ_j.

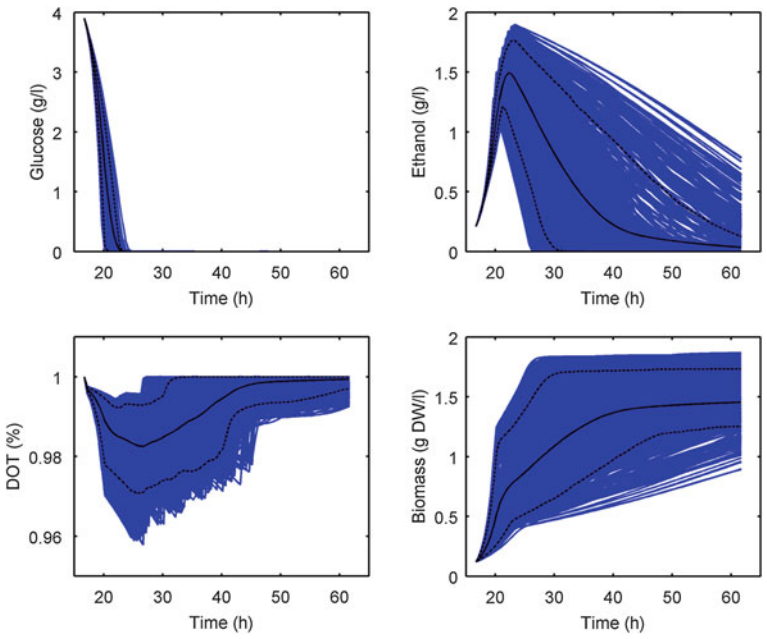

Fig. 10 Model simulation results using Morris sampling of parameter space: model simulations for glucose, ethanol, dissolved oxygen, and biomass showing simulations (*blue*), mean, and the 10th and 90th percentile of the simulations (*black*) (not to be confused with uncertainty analysis)

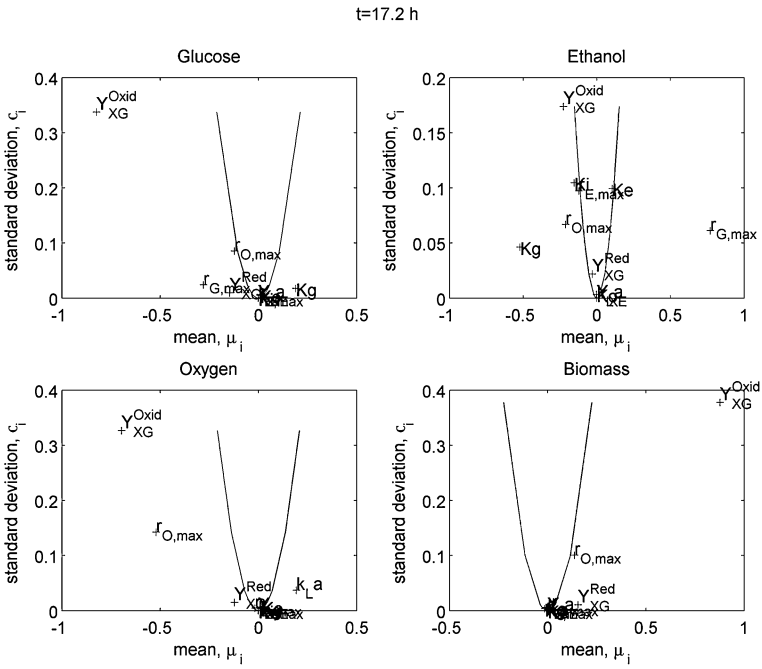

Fig. 11 Elementary effects during growth phase on glucose: estimated mean and standard deviation of the distributions of elementary effects of the 11 parameters on the model outputs. The two lines drawn in each subplot correspond to $\text{Mean}_i \pm 2\text{sem}_i$ (see text)

$$EE_{jk} = \frac{\partial sy_k}{\partial \theta_j} = \frac{sy_k(\theta_1, \theta_2, \theta_j + \Delta, \ldots, \theta_M) - sy_k(\theta_1, \theta_2, \theta_j, \ldots, \theta_M)}{\Delta} \tag{13}$$

The results obtained are compared with the mean and the standard deviation of this distribution. Often, the EEs obtained for each parameter are plotted together with two lines defined by $\text{Mean}_i \pm \text{sem}_i$, where Mean_i is the mean effect for output i and sem_i is the standard error of the mean ($\text{sem}_i = \text{std deviation}_i/\sqrt{r}$). The EEs are scaled, and thus a comparison across parameters is possible.

Also this analysis has to be performed for a selected time point, or using a time-series average. As the cultivation has distinct phases, several time points were selected. The results for the growth phase on glucose ($t = 17.2$ h) and the growth phase on ethanol ($t = 27.2$ h) are presented in Figs. 11 and 12, respectively.

Parameters that lie in the area in between the two curves (inside the wedge) are said to have an insignificant effect on the output, while parameters outside the wedge have a significant effect. Moreover, nonzero standard deviations indicate nonlinear effects, implying that parameters with zero standard deviation and nonzero mean have a linear effect on the outputs.

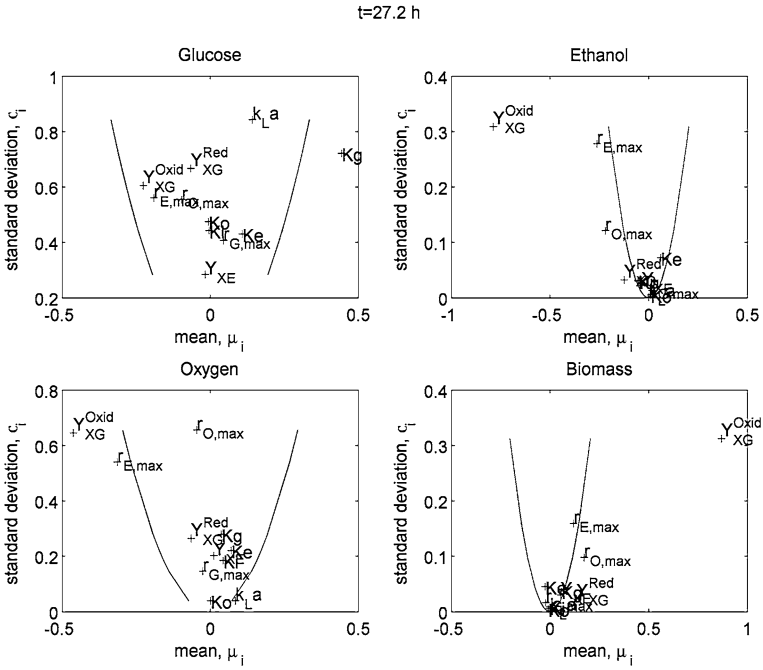

Fig. 12 Elementary effects during growth phase on ethanol: estimated mean and standard deviation of the distributions of elementary effects of the 11 parameters on the model outputs. The two lines drawn in each subplot correspond to $\text{Mean}_i \pm 2\text{sem}_i$ (see text)

During growth on glucose (Fig. 11) only a few parameters show a significant effect on the model outputs. While Y_{XG}^{Oxid} seems to have a nonlinear effect on the glucose prediction, $r_{G,max}$ has a linear one. The effects of other parameters are mostly nonlinear, as expected given the structure of the model used in the example. The former parameter has also a significant effect on oxygen and biomass, while the latter parameter has a significant effect on ethanol.

With regard to results for a time point during growth on ethanol, it is important to note that Y_{XG}^{Oxid} appears to have a significant effect on the ethanol, oxygen, and biomass predictions, although the glucose has been depleted. This may reflect the impact of the biomass concentration (originated during the prior growth on glucose) on the total amount of ethanol produced, as well as its consumption and the consumption of oxygen for the observed time point.

There is good agreement of the results of the Morris analysis with the previously presented SRC ranking obtained for the linear regression method. In Figs. 11 and 12, the parameters most distant from the wedge are the parameters ranked as the most influential on the model outputs (Table 9a, b).

Table 9a Ranking and SRC value of the model parameters for each model output, for a time point during the exponential growth phase on glucose

$t = 17.2$ h	Glucose		Ethanol		Oxygen		Biomass	
	SRC	Rank	SRC	Rank	SRC	Rank	SRC	Rank
$r_{G,\max}$	−0.0089	2	0.7423	1	−0.0858	6	0.1111	4
$r_{O,\max}$	0.0006	8	−0.1591	3	−0.5768	2	−0.0129	10
$r_{E,\max}$	0.0002	11	−0.0837	5	0.0210	10	−0.0400	7
Y_{xg}^{Oxid}	−0.0107	1	−0.0777	6	−0.7884	1	0.9746	1
Y_{xg}^{Red}	0.0060	3	0.0467	8	−0.1599	4	0.1697	2
Y_{Xe}	0.0008	7	0.0070	10	0.0452	7	−0.0643	5
K_G	0.0058	4	−0.4819	2	−0.0301	8	0.0328	8
K_O	0.0042	5	0.0279	9	0.1142	5	−0.1664	3
K_E	−0.0005	9	0.0756	7	0.0027	11	−0.0103	11
K_i	0.0013	6	−0.1324	4	0.0273	9	−0.0420	6
k_La	−0.0005	10	−0.0070	11	0.2380	3	0.0170	9

Table 9b Ranking and SRC value of the model parameters for each model output, for a time point during the exponential growth phase on ethanol

$t = 27.2$ h	Glucose		Ethanol		Oxygen		Biomass	
	SRC	Rank	SRC	Rank	SRC	Rank	SRC	Rank
$r_{G,\max}$			−0.0253	11	−0.0003	11	0.0356	8
$r_{O,\max}$			−0.1310	3	−0.2484	3	0.0409	5
$r_{E,\max}$			−0.1700	2	−0.2534	2	−0.0208	9
Y_{xg}^{Oxid}			−0.9504	1	−0.6101	1	0.9812	1
Y_{xg}^{Red}			−0.0653	7	0.0936	4	0.1157	3
Y_{xe}			0.0281	10	0.0105	9	−0.0357	6
K_G			−0.1195	4	−0.0710	6	0.0997	4
K_O			0.1150	5	0.0748	5	−0.1279	2
K_E			0.0394	8	−0.0010	10	−0.0120	11
K_i			−0.1110	6	0.0596	7	−0.0356	7
k_Lan			−0.0284	9	0.0560	8	0.0202	10

Values corresponding to the prediction of glucose are not shown, as the linear regression was found not to be suitable for this time point and model output ($R^2 < 0.7$)

3 Discussion

A mechanistic model of glucose oxidation by *Saccharomyces cerevisiae* has been taken as an example and has been analyzed rigorously with a number of methods. The chosen case study is purposely kept relatively simple in order to better illuminate how the different methods work and what kind of information is gained in each step. In practice, the presented analysis methods are generic and can be applied to a wide range of process models to assess their reliability. Each step of the analysis has been commented in detail already. However, one thing that cannot be emphasized enough is the importance of collecting proper datasets: biological

replicates (duplicate/triplicate fermentations) but also sample replicates are needed to know the error of the measurements. If the quality of the collected data is not sufficiently high, this might later raise severe questions about the reliability of the resulting model.

Assuming that a decision has been taken to develop a mechanistic model of a pharmaceutical production process, or one of its unit operations, one could, of course, wonder how such a model can be established, and how it can support PAT objectives. In general, construction of a mechanistic model is considered time-consuming, which may explain why data-driven models and chemometrics have been more popular than mechanistic approaches, despite the PAT guidance. However, during the past 5 years, this situation has already changed considerably for small-molecule drug substances [4]. According to us, the tools presented here can be helpful in setting up and structuring the model equations in an efficient way, for example, by making use of matrix notation, which can facilitate transfer of the model equations between different users. Such sharing of modeling knowledge is essential in multidisciplinary process development. As discussed by Sin et al [14], a significant part of such a model matrix can be transferred from one system to a second or a third, which undoubtedly makes the whole model-building exercise more efficient.

Finally, we would also like to emphasize that one should move ahead in small steps when constructing a mechanistic model of a process or unit operation. One should rather start with a smaller model with limited scope, for example, an unstructured model [21]. Such a model could then be gradually extended with more detail, while the development of the production process at laboratory and pilot scale is ongoing. The model analysis tools presented here can then be used in the different stages of the model-building as continuous quality checks of the model.

Once a model is considered ready for use, a first application that is relevant for such a model is to use simulations to propose more informative experiments leading to more accurate estimation of the model parameters, for example, by applying optimal experimental design (OED) [22]. Furthermore, the mechanistic model can be helpful in process design, optimization, and in development of suitable control strategies [23]. The latter applications of the model are essential for implementing PAT principles, and can potentially contribute to more efficient process development, replacing data collection and experiments by simulations whenever possible.

4 Conclusions

Mechanistic models form an attractive alternative for structuring and representing process knowledge, also for production processes in biotechnology. The reliability of such models can be confirmed by performing identifiability, uncertainty, and sensitivity analyses on the resulting model. Tools for performing such analyses can be considered as standard engineering tools and are increasingly available on

different software platforms. Once it can be documented that the model is reliable, it can be used for design of experiments, for process optimization and design, and for investigating the usefulness of novel control strategies.

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References

1. US Food and Drug Administration (FDA) (2004) PAT guidance
2. Bhatia T, Biegler LT (1996) Dynamic optimization in the design and scheduling of multiproduct batch plants. Ind Eng Chem Res 35:2234–2246
3. Gernaey KV, Cervera-Padrell AE, Woodley JM (2012) A perspective on PSE in pharmaceutical process development and innovation. Comput Chem Eng 42:15–29
4. Nielsen J, Villadsen J (1992) Modeling of microbial kinetics. Chem Eng Sci 47:4225–4270
5. Teusink B, Smid EJ (2006) Modelling strategies for the industrial exploitation of lactic acid bacteria. Nat Rev Microbiol 4:46–56
6. Gernaey KV, Eliasson Lantz A, Tufvesson P, Woodley JM, Sin G (2010) Application of mechanistic models to fermentation and biocatalysis for next generation processes. Trends Biotechnol 28:346–354
7. Villadsen J, Nielsen J, Lidén G (2011) Bioreaction engineering principles (3rd ed). Springer, New York, 561 p, ISBN 978-1-4419-9687-9
8. Sin G, Woodley JM, Gernaey KV (2009) Application of modeling and simulation tools for the evaluation of biocatalytic processes: a future perspective. Biotechnol Prog 25:1529–1538
9. Vasić-Rački D, Findrik Z, Vrsalović Presečki A (2011) Modelling as a tool of enzyme reaction engineering for enzyme reactor development. Appl Microbiol Biotechnol 91:845–856
10. Sidoli FR, Mantalaris A, Asprey SP (2004) Modelling of mammalian cells and cell culture processes. Cytotechnology 44:27–46
11. Sin G, Gernaey KV, Eliasson Lantz A (2009) Good modelling practice (GMoP) for PAT applications: propagation of input uncertainty and sensitivity analysis. Biotechnol Prog 25:1043–1053
12. Sonnleitner B, Käppeli O (1986) Growth of *Saccharomyces cerevisiae* is controlled by its limited respiratory capacity: formulation and verification of a hypothesis. Biotechnol Bioeng 28:927–937
13. Ferrer-Miralles N, Domingo-Espín J, Corchero JL, Vázquez E, Villaverde A (2009) Microbial factories for recombinant pharmaceuticals. Microb Cell Factories 8:17
14. Sin G, Ödman P, Petersen N, Eliasson Lantz A, Gernaey KV (2008) Matrix notation for efficient development of first-principles models within PAT applications: integrated modeling of antibiotic production with Streptomyces coelicolor. Biotechnol Bioeng 101:153–171
15. Roels JA (1980) Application of macroscopic principles to microbial metabolism. Biotechnol Bioeng 22:2457–2514
16. Esener AA, Roels J, Kossen NWF (1983) Theory and applications of unstructured growth models: kinetic and energetic aspects. Biotechnol Bioeng 25:2803–2841
17. Holmberg A (1982) On the practical identifiability of microbial growth models incorporating Michaelis–Menten type nonlinearities. Math Biosci 62:23–43
18. Brun R, Kuhni M, Siegrist H, Gujer W, Reichert P (2002) Practical identifiability of ASM2d parameters—systematic selection and tuning of parameter subsets. Water Res 36:4113–4127
19. Carlquist M, Lencastre Fernandes R, Helmark S, Heins A-L, Lundin L, Sørensen SJ, Gernaey KV, Eliasson Lantz A (2012) Physiological heterogeneities in microbial populations and implications for physical stress tolerance. Microb Cell Factories 11:94

20. McKay MD, Beckman RJ, Conover WJ (1979) A comparison of three methods for selecting values of input variables in the analysis of output from a computer code. Technometrics 21:239–245
21. Bailey JE (1998) Mathematical modeling and analysis in biochemical engineering: past accomplishments and future opportunities. Biotechnol Prog 14:8–20
22. Baltes M, Schneider R, Sturm C, Reuss M (1994) Optimal experimental design for parameter estimation in unstructured growth models. Biotechnol Prog 10:480–488
23. Singh R, Gernaey KV, Gani R (2009) Model-based computer aided framework for design of process monitoring and analysis systems. Comput Chem Eng 33:22–42

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Multivariate Data Analysis for Advancing the Interpretation of Bioprocess Measurement and Monitoring Data

Jarka Glassey

Abstract The advances in measurement techniques, the growing use of high-throughput screening and the exploitation of 'omics' measurements in bioprocess development and monitoring increase the need for effective data pre-processing and interpretation. The multi-dimensional character of the data requires the application of advanced multivariate data analysis (MVDA) tools. An overview of both linear and non-linear MVDA tools most frequently used in bioprocess data analysis is presented. These include principal component analysis (PCA), partial least squares (PLS) and their variants as well as various types of artificial neural networks (ANNs). A brief description of the basic principles of each of the techniques is given with emphasis on the possible application areas within bio-processing and relevant examples.

Keywords Data pre-processing · Feature extraction methods · Neural networks · Regression methods

Contents

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1 Introduction

Chapter 1 overviewed the state of routine and state of the art in bioprocess monitoring and provided an indication of the amount and the complex nature of data monitored routinely during bioprocess operation. Individual sensors measuring physical, chemical and biological variables at various stages of the production process typically yield extensive data sets with high sampling frequency. Sensor arrays, such as electronic noses and tongues referred to in Chapter 1, and multianalyte analysers, such as spectroscopic and fluorescence measurements, further increase the complexity of the data structures collected and stored for 'post mortem' analysis of process behaviour. A plethora of methods used to check the quality of the product during various stages of processing, such as various chromatographic techniques and ELISA assays, typically yield discrete data points with varying sampling frequencies as well as accuracy. In addition a rapid expansion and improvements in methods of genomic, trascriptomic, proteomic, metabolomic or environomic (Chap. 8) data collection open up the possibilities of much better process understanding during the development stage, as required by a quality by design (QbD) framework (Chaps. 4, 8 and 9). The latter methods result in data arrays with structures which are much more complex and extensive than the data routinely monitored during process operation; however these data also dramatically increase the potential benefits to be gained.

With such extensive data collection, the importance, and benefits of, effective and robust data pre-processing and analysis become critical from both the bioprocess development and monitoring and control points of view. This chapter concentrates on data pre-processing, conditioning, reconciliation and multivariate data analysis (MVDA) methods that enable advanced interpretation of process measurement data for effective monitoring and control, whilst also being applicable to process development. This chapter provides a basic overview of the fundamental principles of both linear and non-linear methods most frequently used for pattern recognition and regression building, with examples of application from various stages of bioprocess operation. However, it is not possible to provide a detailed description of each of the methods and all aspects of their applications. Readers are referred to appropriate literature sources for more details on individual techniques or relevant application examples.

It is important to stress that a number of data analysis methods described in this chapter are well established and applied routinely in other scientific disciplines (e.g. chemometric methods widely applied in chemistry) and industrial sectors, and thus extensive resources, in terms of both literature and computing packages

utilising these methods, are available. However, bioprocessing, and in particular the biopharmaceutical industry, can be said to lag significantly behind other industrial sectors, such as the chemical industry and manufacturing, in terms of routine application of these methods in large-scale processing. This is often explained by the strict regulatory nature of the industry, although there are also fundamental differences in the principles of application within each of these areas; For example in the chemical analysis area where chemometric methods were originally developed, there is usually a 'known' solution to the task, i.e. known concentrations of analytes in question, whereas in biological applications, MVDA techniques are often used as exploratory techniques to investigate what relationships exist (or are evident from the data) between measured variables.

However, the successful implementation of the QbD and PAT initiatives, introduced in this sector almost a decade ago, depends to a great extent on the effective integration of the MVDA methods within the monitoring and control framework. Thus, it is important that a wider understanding and application of these methods within this industry sector is championed and illustrated on successful, industrially relevant case studies. On the other hand, caution is advised in the use of chemometric methods, for example by Brereton [6], who expresses a concern at 'lots of people, often without a good mathematical or computational background, wanting to use' them quickly, without gaining much insight. The following material is presented very much with this caveat in mind.

2 Data Pre-Processing

Data pre-processing and reconciliation is by no means a novel concept, and it is widely applied in industrial facilities such as refineries or bulk chemical production plants. As early as 1961 Kuehn and Davidson [27] described data reconciliation, and alternative mathematical approaches have been explored since then with a multitude of applications of data reconciliation in the field of engineering being suggested.

The importance of data pre-processing is universally acknowledged, and most users of chemometric and MVDA methods would agree that the data preparation steps take up most of the analysis time and can either 'make or break' the success of any pattern recognition or model development efforts. One particular issue in MVDA analysis is the removal of outliers, as highlighted by MØller et al. [37]. They argue that, since MVDA methods are usually based on a least-squares or similar criterion, they are sensitive to outliers, which can lead to incorrect conclusions. They describe a range of robust methods for outlier removal.

Further data pre-processing is then usually required, depending on a particular application; For example in NIR spectra analysis, a range of specific data pre-processing approaches has been developed (for more detail on pre-processing of spectral data see Chap. 9). These include smoothing noisy data using a Savitzky-Golay filter, baseline shift correction, multiplicative scatter correction (MSC) and

standard normal variate (SNV) [38]. Frequently a range of data pre-processing methods are compared and their effectiveness for a particular application is assessed by the outcome of the classification or modelling task required.

2.1 Data Characteristics

The introduction section indicated how complex the data structures collected during bioprocess operation can be. Figure 1 illustrates this, albeit in a somewhat simplified manner, by including data from raw material quality assessment through to downstream process monitoring.

In order to gain maximum benefit from data analysis and modelling, the quality data on raw materials, monitored over time and often using several (multianalyte) sensors, will need to be linked with quality data monitored during the batch/fed-batch cultivation at various frequencies for various quality attributes and merged with online data available from both the cultivation and downstream processing unit operations.

The varying frequency of sampling and issues with missing, inaccurate and noisy data, often with significantly varying means and ranges of individual process variables, often require significant data pre-processing before any meaningful data interpretation and modelling can be carried out.

2.2 Data Scaling

Brereton [6] offers an extensive description of various data scaling approaches, ranging from single measurement transformations (which should not be required frequently) to scaling individual variables over all samples or individual samples over all variables. Various transformations, such as logarithmic or power transformations, are well established and used in a range of applications. Equally, there are various methods of scaling, from simple mean or weighted centring for applications with varying numbers of samples from different populations to standardisation (or normalisation or autoscaling, as this approach is often referred to). Whilst centring scaling simply aligns the means of all the variables without scaling their ranges, standardisation ensures, as a result of mean centring and dividing by the standard deviation, that each variable has a similar influence upon the resulting model. Alternative methods of adjusting the range of individual variables prior to the analysis and modelling stage include scaling within a particular range (usually between 0 and 1 by using the maximum and minimum values of each of the variables) or block scaling and weighting, which is particularly useful in applications where data from various analytical techniques, such as NIR spectra, are combined with a process data measurement matrix of much smaller dimensions.

In each application it is very important to apply process understanding at the data pre-processing stage and then to decide which method of scaling is appropriate for a particular application and will not result in the introduction of artificial features into the data set.

2.3 Data Reconciliation

Data scaling and pre-processing methods described in Sects. 2.1 and 2.2 represent only a small proportion of the extensive variety of data reconciliation methods applied in a range of industrial sectors. A plethora of methods for eliminating random and gross errors, such as calibration errors, malfunction in devices or post-calibration drifts, from process data employ model-based approaches to data reconciliation and treat this problem as a model identification and parameter estimation problem [3]. Various model representations have been used for this purpose, ranging from straightforward material and energy balances [58], extended Kalman filters [20], through to some of the MVDA approaches, such as support vector regression [35], described in more detail in Sect. 3.3.

The selection of an appropriate model structure for data reconciliation is not a straightforward issue, in particular in bioprocessing, where a range of models from black box to metabolic to synthetic mechanistic models can be used (see Chap. 6 and Sect. 3.3). The choice of the right level of model complexity is crucial, but might in reality also be influenced by the availability of measurements, although recent advances in real-time process measurement (Chaps. 1 and 9) have reduced this challenge significantly.

3 Feature Extraction Methods

Exploratory data analysis, feature extraction, pattern recognition, classification and clustering are very important in bioprocess data analysis for a number of reasons. The complexity and the amount of data obtained from the various measurements taken during bioprocess operation or an experiment preclude a comprehensive and robust analysis of trends ‘by eye’ even if carried out by the best trained and most experienced personnel. Traditional chemometric methods, such as principal component analysis (PCA), allow significant data reduction to eliminate correlation and noise in the data and thus provide a clearer depiction of the relationships captured by the data. However, the basic assumption of a linear correlation within the data structures is often highlighted as a major limitation of such techniques within bioprocess applications, as the clear non-linear and complex nature of the studied systems is assumed (and proven in a number of applications) to be difficult to capture by linear methods. Hence a range of non-linear feature extraction techniques have also been applied to bioprocess data analysis [39, 42].

Two major categories of methods can be distinguished regardless of the linear/non-linear character of the technique used. In the supervised mode, a 'known' solution is assumed to exist and examples of data representing various classes are used during model development to establish classification boundaries between these. On the other hand, when no underlining classification is assumed or known, a clustering of data features is identified on the basis of a defined similarity in data characteristics. In fact, the distinction between classification (supervised pattern recognition) and clustering (unsupervised) is often not fully recognised [12].

Only a selection of the most frequently used methods of clustering and classification with relevant bioprocessing examples can be discussed in this chapter, but an extensive range of literature is available covering various aspects of clustering and classification in the biosciences [12, 54].

3.1 Application Areas in Biosciences and Bioprocessing

Exploratory data analysis and clustering are widely applied in biosciences for a range of tasks. Probably the most widely described are the bioinformatics tools used for identifying patterns in gene expression data under various conditions, whether in medical applications for identifying biomarkers of particular diseases or for biosynthesis of particular products or taxonomic studies of biodiversity and evolution of microorganisms [25, 51]. Various clustering methods, ranging from hierarchical clustering and *k*-means clustering to soft clustering methods, fuzzy *c*-means and their variations [19], rely on the specification of a 'similarity' measure or distance metric that is used to assess whether two data points are sufficiently similar to be assigned to the same class of objects. These metrics significantly affect the resulting clustering, and given that the 'correct' clustering is not known in these applications, the biological plausibility of the resulting cluster structure is typically used to assess the effectiveness of the analysis. In bioprocessing, the situation is often simplified when analysing historical data, for which clusters can be user-defined. The cluster boundaries could be based on, for example, high/low final productivity or nominal behaviour/deviation, depending on the application [9].

The extension of the use of MVDA tools to the analysis of the remaining 'omics' data is an expected development in data analysis [17]. Although techniques and case studies currently more directly associated with bioprocess monitoring and control will be discussed in more detail below, it is important to point out that 'omics' data are increasingly used in combination with more traditional process monitoring [49], and thus MVDA methods used for the interpretation of such data are becoming more important from the bioprocessing point of view. The most frequently reported applications of MVDA will be highlighted with each technique in the following subsections.

3.2 Principal Component Analysis

Principal component analysis (PCA) [7] is probably the most established MVDA method of feature extraction. It works by generating a new group of uncorrelated variables (principal components, PCs) from a high-dimensional data set. The approach transforms a matrix [**X**], containing measurements from n measured variables, into a matrix of mutually uncorrelated PCs, $\mathbf{t}_k$ (where $k = 1$ to n), which are transforms of the original data into a new basis defined by a set of orthogonal *loading* vectors, $\mathbf{p}_k$. The individual values of the principal components are called *scores*. The transformation can be described by Eq. (1):

$$[\mathbf{X}] = \sum_{k=1}^{np<n} \mathbf{t}_k \mathbf{p}_k^T + \mathbf{E} \tag{1}$$

The loadings are the eigenvectors of the data covariance matrix, $\mathbf{X}^T\mathbf{X}$. The $\mathbf{t}_k$ and $\mathbf{p}_k$ pairs are ordered such that the first pair captures the largest amount of variation in the data and the last pair captures the least. This means that fewer PCs are required to describe the relationship than the original process variables. This data compression allows for easier visualisation of the data for the purposes of feature extraction, which can be used in a variety of applications within bioprocessing.

Literature sources on PCA applications within bioprocess data analysis are numerous and cover all aspects of bioprocessing from raw material, seed cultivation, production batch or downstream process quality monitoring [2, 9, 44]. Typically in these applications, high-dimensional process data are compressed into a set of principal components which are used either as inputs for further MVDA process models or as a monitoring tool within a multivariate statistical process control (MSPC) scheme.

3.2.1 Variants of PCA

There are a number of reported variants of PCA, introduced due to specific application requirements; for example, Alexandrakis [1] discusses the need to increase the robustness of PCA models in the analysis of NIR spectral data in order to deal with external factors influencing the spectra in the industrial setting. These factors include not only instrument effects not observed at laboratory scale during process development (e.g. temperature effects and stray light), but more significantly sample- (e.g. differences in particle sizes) and process-related effects (e.g. variability in unit operation conditions). Whilst a number of alternative methods have been discussed to address these issues, the use of orthogonal methods, such as external parameter orthogonalization (EPO), transfer by orthogonal projection (TOP) and dynamic orthogonal projection (DOP) [22, 46] are highlighted as useful tools for such an application.

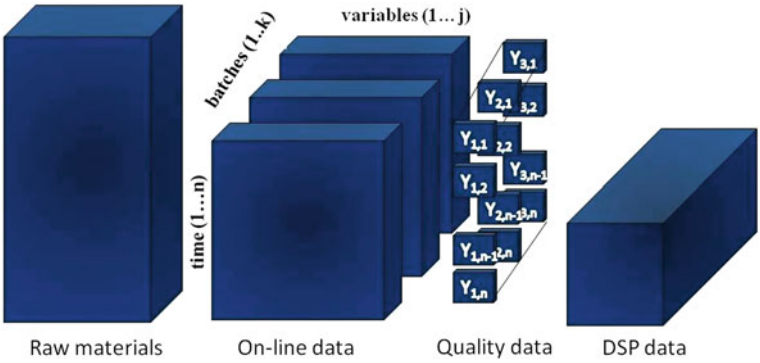

Fig. 1 Typical data structures collected during bioprocess operation, highlighting the different frequencies of data collection and discrete character of the quality data as well as the online data array structure resulting from the collection of measurements for a number of variables over time from a number of batches

The batch character of the data typically collected from bioprocesses, as indicated in Fig. 1, introduces additional non-linearities that a number of researchers claimed must be addressed by modified PCA approaches. There are a range of non-linear variants of PCA, including kernel PCA [50] and principled curves and surfaces [52], the mathematical principles of which are reviewed in Yin and Huang [64].

An alternative approach to addressing the issue of non-linearity is the batch transformation, termed multiway PCA, initially proposed by Wold et al. [61]. Since then, other authors have adopted the approach and applied the method to a number of processes [40]; for example, Gregersen and JØrgensen [16] investigated the detection of faults in a fed-batch fermentation process, and Kompany-Zareh [24] used this methodology for online monitoring of a continuous pharmaceutical process.

The multiway technique re-arranges the data into a two-dimensional matrix as demonstrated in Fig. 2. As also indicated in the figure, a number of possibilities exist when unfolding the array, with batch-wise decomposition (horizontal slice in Fig. 2) being the more traditional method.

The case study demonstrated in this chapter is based on this alternative unfolding approach, resulting in a matrix of size ($i \times kj$), where each row represents one batch (and thus is represented by a single symbol in the principal component plots, see Figs. 3 and 4). The first j columns represent the time trajectory of the first variable. The next j columns represent the second variable, and so on. Thus when the data are normalised by subtracting the mean and dividing by the standard deviation at each sample point, the deviations from the mean trajectory are investigated and the major source of the non-linearity (due to the batch mode of culture growth not attaining a steady state) is eliminated.

It should be noted that there are a range of alternative methods, such as parallel factor analysis (PARAFAC) and trilinear decomposition (TLD) [24], that have been successfully used in the analysis of data array structures illustrated in Fig. 2, but these are outside the scope of this text.

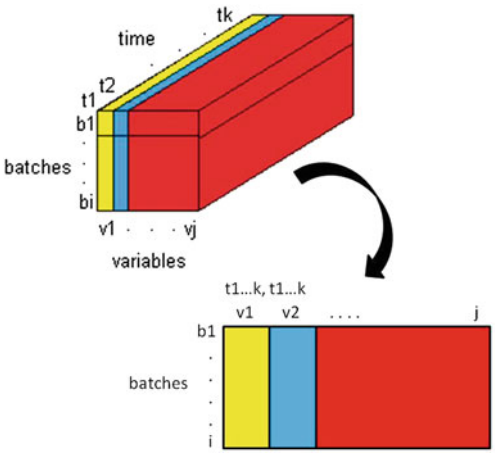

Fig. 2 Multiway decomposition of a typical 3D bioprocess data array consisting of time trajectories of various variables for a range of batches resulting in a 2D matrix with indicated dimensions

3.2.2 PCA-Based Feature Extraction Case Study

In an unsupervised mode, PCA can reveal similarities between individual bioprocessing batches, although a more frequent application is the detection of deviation from 'nominal' or expected process behaviour. In this type of application, historical examples of data representing 'nominal' or desired behaviour (e.g. high concentration or quality/purity of desired product) are used together with examples of process data deviating from this behaviour. Interrogation of score plots may reveal the reason for the deviation between the batches, thus furthering process understanding and enabling more effective process control and operation.

Figure 3 illustrates an example of a bivariate plot of the scores resulting from a multivariate PCA analysis of historical process data collected during a typical recombinant monoclonal antibody fragment production cultivation, with typical online and offline measurements collected during the cultivations. In this particular model 7 online variables, including pH, dissolved oxygen concentration and temperature, and 13 design variables (whose values are changed during process development) were used. Note that each batch is represented by a single symbol on the plot. Given that the results, in terms of product concentration, are known at the time of the analysis, it is possible to represent each high-producing batch in one colour (in this case red) and low-producing batches in a different colour (green in this case).

Figure 3 illustrates potential issues with the interpretation of the results. A researcher may believe they achieved a very good clustering and be tempted to draw arbitrary clusters A and B as indicated in Fig. 3. However, when the same figure is re-drawn with a different colour scheme, this time using black colour for feed composition 1 (one of the design variables) and red for feed composition 2, there is a much better separation, with one type of batch (high and low producing) contained at the left-hand part of Fig. 4 while cultivations carried out with feed composition 2 lie entirely at the right-hand side of the figure. Note that the

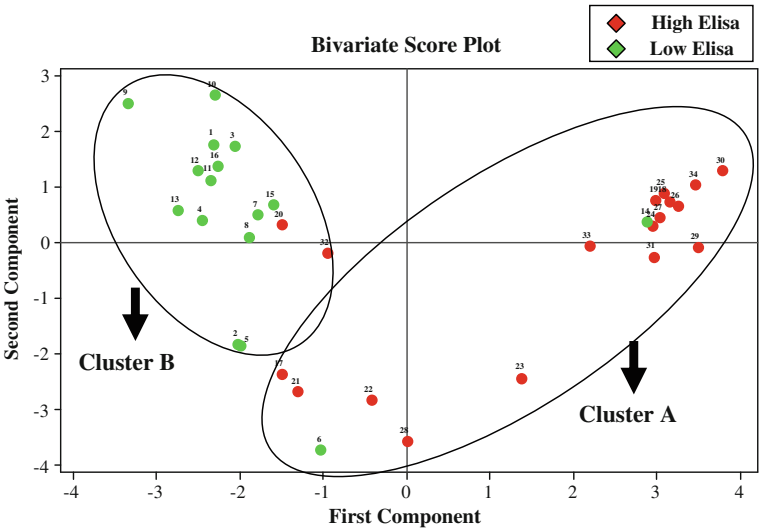

Fig. 3 Example bivariate plot of PC1 versus PC2 for a PCA model of recombinant monoclonal antibody production cultivation, indicating high (*red*) and low (*red*) productivity

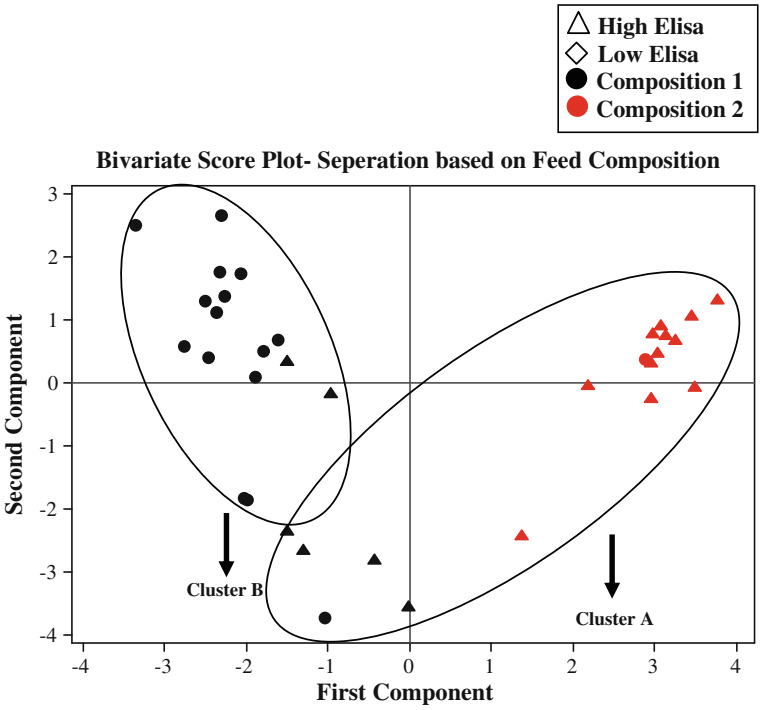

Fig. 4 Bivariate scores plot from Fig. 3, re-drawn on the basis of different feed composition (composition 1, *black colour*; composition 2, *red colour*)

arbitrary clusters A and B are re-drawn for easy comparison with Fig. 3. It is also obvious that feed composition 1 appears to result in cultivations with much more variable outcomes. Such analysis can lead to further investigations as to the causes of variability where the loadings of process variables used in the model can indicate possible causes of deviation/variability.

When Multiwat Principal Componetnt Analysis (MPCA) is used within a MSPC scheme, the issues with cluster boundaries are addressed typically by constructing confidence limits around the cluster of nominal behaviour and using Hotteling's T^2 statistics to identify any deviation from such behaviour. For an example of MSPC application to bioprocess monitoring see e.g. Nucci et al. [41], where PCA was used for monitoring of penicillin G acylase production.

3.3 Neural Networks and Non-Linear Approaches

Neural networks represent an alternative non-linear feature extraction approach that has been successfully applied in biosciences in the past. Although traditional types of neural networks, such as feedforward or radial basis function (RBF) networks (see Sect. 4.3.1) can be used to predict correct class membership on the basis of input data, there are types of neural networks that were specifically developed for feature extraction. These include probabilistic neural networks (PNNs) with Bayesian decision strategies used as a basis of classification boundaries, which were used for example for growth phase classification [29], pharmaceutical applications [47] or biosystem reverse engineering [30].

Kramer [26] developed autoassociative neural networks as an alternative to PCA, claiming that the bottleneck layer in this neural network structure (Fig. 5) extracts essential non-linear features contained in the data in order to perform an accurate unity mapping of process data onto the same space. Such networks were shown to yield important information in fault detection for bioprocess monitoring [15, 21].

The category of non-linear approaches and neural networks utilising competitive training includes learning vector quantisation (LVQ), self-organising maps (SOM) and support vector machines (SVM). LVQ is based on the principle of a competitive learning algorithm defining a reduced set of reference vectors that cover the same space as the original training set patterns and has been shown to be effective in the interpretation of sensor array data [48]. The SOM network uses a 'best matching unit' (BMU, similarly to the reference vectors of LVQ) to map each new training data point onto one of the nodes in the map [23, 63]. The BMU (Fig. 6) is assigned on the basis of appropriate distance metrics, and the network parameters (weights) are adjusted to match the presented training examples as closely as possible. In this process, the original regular, usually two-dimensional grid warps to map the topological characteristics of the training data.

This non-linear mapping of high-dimensional bioprocess data enables the SOM to be used as a visualisation tool, as reported for example by Nikhil et al. [39], where they were used to detect three distinct metabolic states during biohydrogen

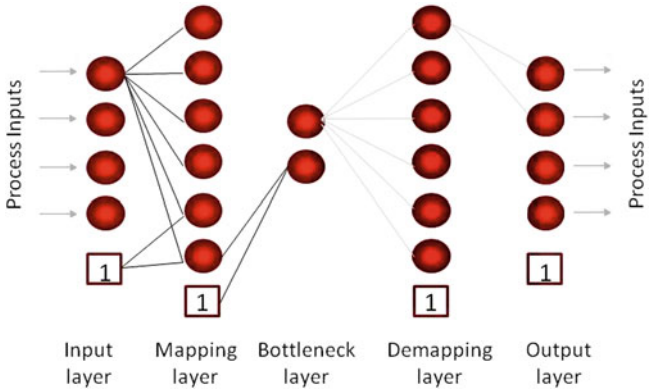

Fig. 5 Autoassociative neural network structure, indicating five layers performing unity mapping; only a selection of network weights are illustrated to reduce the complexity of the figure

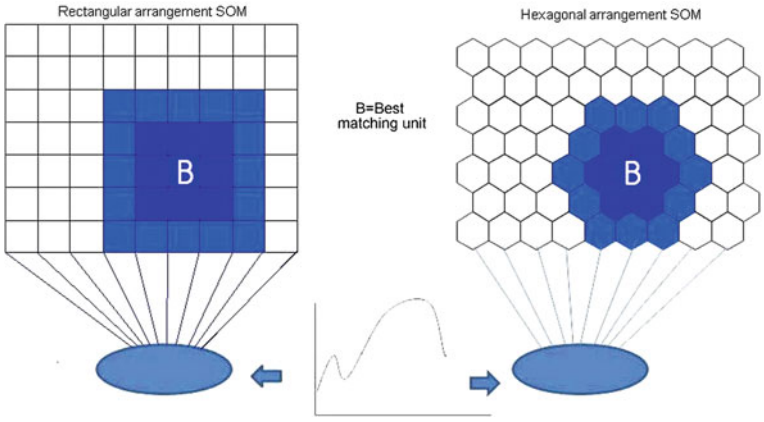

Fig. 6 Two different self-organising map arrangements with best matching unit (*B*) and neighbouring nodes, whose weights are adjusted upon matching the input pattern to *B*

production through dark fermentation. However, SOMs were also used to analyse 2D fluorescence data collected during the cultivation of recombinant *E. col* and *Saccharomyces cerevisiae*, where they successfully captured the relationships between the spectra and process parameters [45].

3.4 Neural Network-Based Feature Extraction Case Study

Sections 3.2 and 3.3 concentrated on linear and non-linear MVDA methods, respectively, although it is important to stress that these two categories of methods are often used in combination to increase the accuracy of the resulting

representations. The following case study demonstrates how PCA and canonical variate analysis (CVA) can be combined with PNNs to predict physiological states (PS) during *E. col* cultivations producing an industrially relevant recombinant protein at laboratory scale.

Pyrolysis mass spectrometry (PyMS,) [60] was used to reflect the changes in the composition of the offline biomass samples taken during the cultivation. PyMS represents a fingerprinting method where the changes in composition of the sample are detected without necessarily indicating the component responsible for such a change. This kind of analysis, similarly to NIR spectroscopy, can be highly beneficial in bioprocess monitoring where measurements of individual metabolites may not fully reflect the PS of the culture even if they were technically and economically feasible.

Since PyMS yields a large multivariate matrix of measurements, it is necessary to reduce and cluster the data into individual PSs. PCA-CVA has been applied successfully to identify patterns in Fourier-transform infrared (FTIR) spectra [53] and is suitable for this purpose. In most analytical studies including PyMS analyses, samples are generally analysed in duplicate or triplicate in order to assess the reproducibility of sample preparation and analytical conditions. A subset of spectra, in principle from identical sample material, is referred to as a group. Most PyMS studies are concerned with identifying the chemical components which account for the differences between the groups. CVA is able to account for the information contained in the replicate samples and highlights the differences between the groups.

In several respects, CVA is similar to PCA. Both result in linear combinations of variables chosen to maximise a particular quantity. The difference is that PCA maximises the variance of the derived variables whilst CVA maximises the covariance or correlation between corresponding members of a pair of derived variables. The solution in both algorithms is provided by singular value decomposition (SVD).

CVA can be described briefly as shown in Eq. (2).

$$\begin{aligned} x &= a_k u_k \\ y &= b_k v_k \end{aligned} \tag{2}$$

where x and y are the observed variables; a_k and b_k are the coefficients which maximise the correlation between u_k and v_k; vectors u_k and v_k are the resulting derived new variables. CVA seeks to maximise the dimensionless correlation coefficient r_k between u_k and v_k. In PyMS data analysis, CVA is used to maximise the ratio of between-group to within-group variance.

Figure 7 illustrates the PyMS data resulting from analysing two different samples from the same cultivation. Samples of consecutive time points (2.5 and 3 h post-inoculation) are shown to demonstrate the differences between individual samples within the same cultivation. Figure 7 also clearly demonstrates the multivariate character of the data and indicates the difficulties faced in identifying changes in the sample composition.

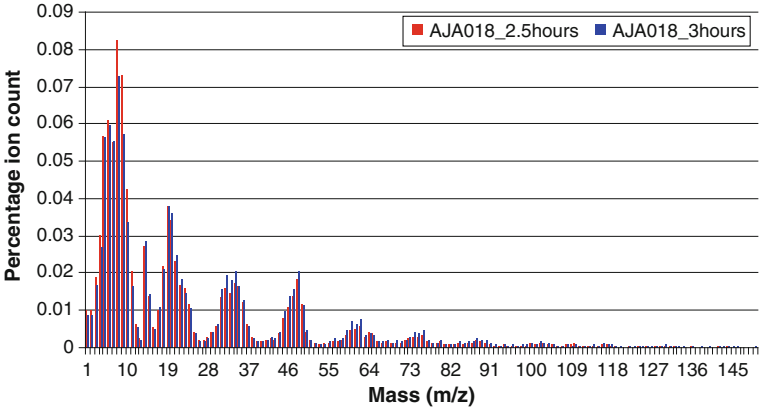

Fig. 7 Comparison of PyMS data from the same cultivation using two consecutive time-point samples in *blue* and *red* colour

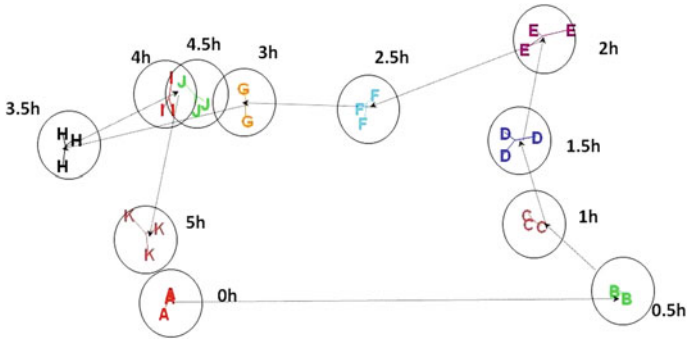

Fig. 8 Clustering of data from a recombinant *E. col* cultivation. Triplicates of individual time-point samples are represented by full circles and connected by lines. Each of the triplicates is additionally labelled to indicate the fermentation and time post-inoculation

Each PyMS data file consists of 150 mass to ion charge (m/z) data reflecting the composition of the sample. Triplicates of each of the samples were analysed using PCA-CVA to minimise the variance between the triplicates (within-group variance) and to maximise the variance between the individual time-point samples (between-group variance). The results are shown in Fig. 8.

Finally, for an online application of PS inferential estimation and control, as proposed in Fig. 9, methods capable of assigning online data into individual PSs without relying upon the time-consuming and often un-economical offline analyses have to be developed.

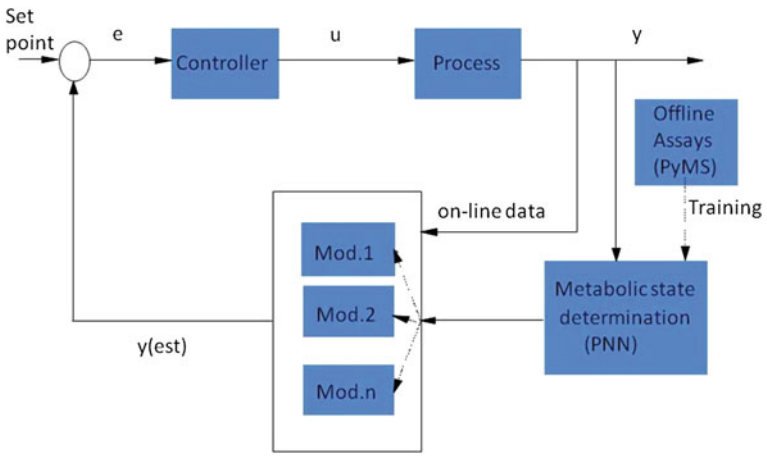

Fig. 9 Physiological state (PS)-based inferential bioprocess control scheme indicating a number of PS-specific models used for estimating the process variable of interest

In an inferential control scheme as proposed in Fig. 9, a classification method is required to allow offline and online prediction of the physiological state of the culture and therefore allow the appropriate estimation model (Mod 1, Mod 2 or Mod *n* in Fig. 9) to be used to estimate the process variable of interest, y(est), and thus drive the controller to maintain the desired set-point.

The PNN approach, described in Sect. 3.3, was used in this particular case study to predict three PSs, assigned on the basis of the PCA-CVA, using online data typically measured during cultivation (e.g. dissolved oxygen, pH, and CO_2 and O_2 concentration in the exhaust gas). Whilst the network successfully predicted the PS for most of the cultivations throughout the duration of the experiment, further improvement was required in assigning data into a particular PS based on the PyMS data.

4 Regression Methods

Exploratory data analysis, clustering and classification are important during bioprocess development and monitoring as highlighted in Sect. 3. For a range of applications, in particular for software sensors (Chap. 1), it is very important to use regression methods which allow the prediction of a desired variable from process data presented to the model. Given the issues with the measurement of important biological variables during bioprocessing highlighted in Chap. 1, it is not surprising that MVDA applications in this area are abundant in the literature. The most pertinent of these are mentioned below.

4.1 Application areas in Bioprocessing

Luttmann et al. [33] provide a status report on the use of software sensors in the bioprocess area, highlighting industrial implications in particular. All aspects of bioprocessing, from raw material and seed quality assessment, through bioreactor monitoring for a range of microbial and cell culture processes, to downstream process monitoring, are discussed in the report with relevant examples drawn from literature sources.

A range of modelling methods are used in software sensors, although from the perspective of this chapter, those of most interest are the MVDA regression methods. These can be broadly divided into linear and non-linear methods, and the most frequently used methods are described in Sects. 4.2 and 4.3 with reported bioprocess applications of each technique.

4.2 Linear Regression Methods

Although a range of simple linear regression models, including multiple linear regression (MLR) and principal component regression (PCR) [14], have been used to correlate cause (**X**) and effect (**Y**) variables for a range of processes, the partial least squares (PLS) method and its variations are arguably the most frequently used MVDA regression tools in this application area. The PLS algorithm operates by projecting the cause and effect data onto a number of latent variables and then modelling the relationships between these new variables (the so-called inner models) by single-input single-output linear regression as described by Eqs. (3) and (4):

$$\mathbf{X} = \sum_{k=1}^{np<nx} \mathbf{t_k p_k^T} + \mathbf{E} \text{ and } \mathbf{Y} = \sum_{\mathbf{k=1}}^{\mathbf{np<nx}} \mathbf{u_k q_k^T} + \mathbf{F}^* \tag{3}$$

where **E** and **F** are residual matrices, *np* is the number of inner components that are used in the model and *nx* is the number of causal variables,

$$u_k = b_k t_k + \varepsilon_k \tag{4}$$

where b_k is a regression coefficient, and ε_k refers to the prediction error.

Whilst the PLS algorithm is developed as a method of dealing with large-dimensional data sets, the multianalyte measurement methods, such as NIR or 2D fluorescence spectroscopy (Chap. 1), being introduced more frequently into bioprocess monitoring, often lead to data sets with a very large number of variables, particularly when combined with a number of traditional process measurements or with data collected from other unit operations up- or downstream of the investigated unit. Building separate models representing individual unit operations and even individual measurement techniques is an approach that can be used to address

the issues of large numbers of PLS model parameters and their interpretation. However, there are also alternative approaches that can be applied, and these are discussed in Sect. 4.2.1 with relevant bioprocessing examples.

4.2.1 Variants of PLS

Hierarchical PLS modelling, where causal (and sometimes also effect) variables are split into blocks of related variables, has been reported to result in simplified interpretation of PLS models if not in more accurate predictions [62]. The temptation with MVDA methods is typically to include all available process data in the analysis. However, it is now widely accepted and reported in literature that prediction quality and the complexity of the multivariate models can be significantly improved by eliminating variables with low informational content prior to model building. Methods such as genetic algorithms, the jack-knife method or bootstrapping have been used successfully [28]; For example Wold et al. [62] argue that an interactive variable selection (IVS) strategy is not only simple and straightforward, but more robust than other methods in the sense that it 'does not forget the eliminated variables and thus does not distort the interpretation of the model'. Ödman et al. [43] compared four different methods of variable selection, namely genetic algorithms, interval PLS, principal variable (PV) selection and three-way stepwise variable elimination, for predicting biomass and substrate concentrations in fed-batch cultivations of *Streptomyces coelicolor* producing the antibiotic actinorhodin. They observed that the variable elimination methods yielded improved PLS models for both effect variables, although the methods did not pre-select the same wavelength combination for biomass prediction.

Another modification of the PLS algorithm developed for spectral filtering (similar to that mentioned in Sect. 3.2.1) is orthogonalised PLS (O-PLS), where elimination of orthogonal variation with respect to effect variables **Y** from a given causal variable set **X** is performed to improve the model predictions; For example Guebel et al. [18] used this methodology to analyse the data from a continuous *E. col* culture under glycerol pulse experiments, and stressed and successfully demonstrated increased understanding of the metabolic pathways and responses of the organism under these conditions.

A particular class of PLS model variations aims to address the issues associated with the non-linear character of the data, in particular in bioprocessing. These include incorporating polynomial relationships into the PLS structure [62], using artificial neural networks (ANNs) as inner PLS models [31] or hybrid structures incorporating mass balance equations based on first-principles understanding of the process [55].

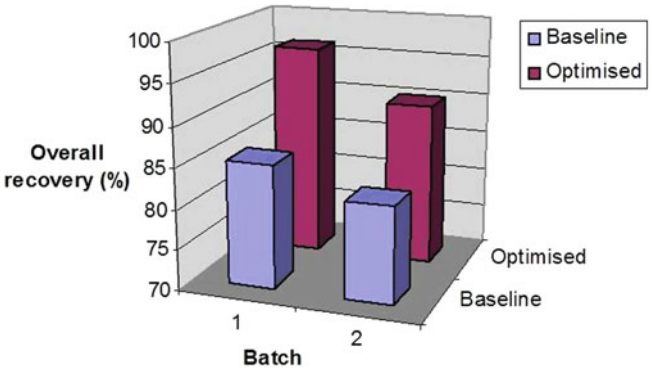

Fig. 10 Comparison of optimised (*red*) and standard (*blue*) settings of operating conditions for three chromatography columns in the recovery of an industrially relevant recombinant protein

4.2.2 Process Improvement Case Study

The importance of overall process understanding and optimisation is growing. In the case study reported here, manufacturing process data from a series of downstream processing units of an industrially relevant recombinant protein process were used to demonstrate that real-time optimisation of operating conditions in response to the process behaviour in upstream units can lead to significant improvements in overall product yield and recovery.

A series of three chromatographic columns with typical process data collected from such processes were used to develop PLS and O-PLS models predicting either product recovery or quality. The predictions of the best performing models were subsequently used in an optimisation scheme where the operational settings of the subsequent columns were adjusted within a pre-specified operating window to maximise the recovery of the product whilst maintaining the required levels of product quality.

Following offline development of the modelling and optimisation scheme using historical process data, pilot-scale experiments with online monitoring and optimisation were performed. Figure 10 illustrates the comparison of two validation batches that used optimised settings for the manipulated variables for each of the columns with two batches running with standard operating condition settings for each of the columns.

Clearly, significant improvements can be observed in the recovery of the product in each of the batches used for comparison. The benefit of using a well-established modelling methodology, on which this optimisation scheme is based, is that in the highly regulated biopharmaceutical industry such an approach provides a more straightforward route to implementation within a validated environment.

A similar approach of overall process optimisation based on soft sensors combined with mechanistic models of individual unit operations has been reported by Gao et al. [13]. The multi-agent system reported by the authors comprised a

process knowledge base, MVDA and first-principles process models and a group of functional agents. The role of the agent components was to cooperate with each other in 'describing the whole process behaviour, evaluating process operating conditions, monitoring of the operating processes, predicting critical process performance, and providing guidance to decision-making when coping with process deviations'. This demonstrated that, for the protein model system used in this case, the overall agent-based framework provided superior process performance compared with the traditional approach of optimising individual processing steps.

4.3 Non-Linear Regression Methods

Section 4.2.1 introduced non-linear variants of PLS with ANNs as one of the means of building non-linear capability into a standard PLS model. ANNs, in particular feedforward (sometimes referred to as multilayer perceptrons, MLPs, or back propagation) and RBF networks, have been reported extensively in the literature in the last two decades as providing an effective means of predicting a range of important biological variables during bioprocessing. The basic principles of these techniques are described in Sect. 4.3.1 with appropriate examples of application.

However, it should be noted that there are also successful bioprocess applications of alternative non-linear methods reported in the literature. These include methods such as genetic algorithms [10] and support vector regression [11] and Petri nets [8] amongst others. These applications cover optimisation of operating conditions, predictions of various process variables, such as biomass and product/metabolite concentrations and representation of biological systems.

4.3.1 Neural Networks

Initially developed in the 1940s from research to model the operation of a biological neuron by McCulloh [36], their applications increased significantly with the introduction of the back propagation learning algorithm in the 1980s [59]. ANNs share many of the characteristics of biological systems such as parallel computation, robustness, insensitivity to noise, adaptability and good generalization properties i.e. the ability to predict data different from that contained in the training data set [4]. They also have demonstrated an ability to generate acceptable models from limited data sets [34].

A typical neural network consists of a number of neurons arranged in layers as indicated for example in Fig. 11. The neurons in the input layer distribute the process data through the network of weighted connections to the hidden layer(s), which perform a non-linear transformation of the data before passing the output through another set of weights to the output neurons.

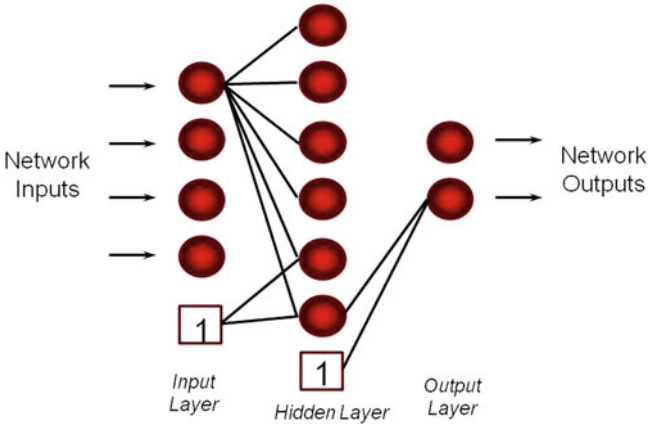

Fig. 11 A typical artificial neural network structure, indicating layers of neurons; only a selection of network weights is illustrated to reduce the complexity of the figure

A typical non-linear transformation of MLPs is a sigmoidal function, although other non-linear functions can also be applied. The algorithm for adjusting the weighted connections is typically based on the 'back-propagation' of the error between the actual output and the network estimation.

In RBFs the data in the input space are non-linearly transformed from the input layer space to the hidden layer space, typically using a form of a Gaussian function, and then the transformation from the hidden layer space to the output layer space is linear. This type of mapping allows a non-linearly separable classification problem to be transformed into a linearly separable one, which is easier to solve. RBF networks are thus capable of faster learning and are less sensitive to the order in which the training data are presented to the model. However, a very large number of RBF may be required to span the input space adequately.

An added benefit of using an RBF network is the indication of the reliability of the network estimations, based on the data density estimation. During model development, the number of radial basis units is specified and the centre of each function is found by *k*-means clustering, where the data matrix is arranged into a number of clusters, the average positions of the data points in each cluster are taken to be the cluster centres, and the data included within each cluster are optimised so that the distance between the data points and the centre of the cluster is minimised. The final cluster centres represent the centres of the RBF of the trained network. Once these centres have been found, the width of each function is defined based on a *p*-nearest-neighbour heuristic [57]. The number of nearest neighbours (*NN*) is user-specified, and the distance between each of the function centres is calculated. The width of each function is adjusted so that it overlaps with the centres of *NN* functions around it. This structure allows an indication of the reliability of the model prediction, as it indicates when new data points are presented to the network outside the data space used for the model development.

The applications of ANNs in process industries range from multisensor data interpretation in chemical processes [65], through NIR spectra calibration with RBF [56] to enzyme engineering [32]. Most of these publications demonstrate the applicability of ANNs as a non-linear modelling tool in the bioprocess area, but often also raise issues with model parsimony and the ability to deal with unseen data. The latest developments thus combine these 'black box' approaches with first-principles modelling (see Chap. 6). In such hybrid schemes, biological understanding of the first principles is complemented by MVDA models capturing the dynamic characteristics of the system to improve the overall performance of the hybrid modelling framework [5, 13, 55].

5 Conclusions

This chapter set out to provide a brief overview of MVDA methods used to advance the interpretation of measurement and monitoring data from a bioprocess. Methods used for exploratory data analysis, clustering and classification together with regression methods were described with a brief description of the fundamental characteristics of the most frequently used linear and non-linear methods in each category. Readers are referred to relevant literature for more details on any particular technique. The bioprocess case studies used in this chapter have been selected to demonstrate the areas of successful application throughout bioprocess development and monitoring and control across typical unit operations encountered in bioprocess manufacturing.

Issues with regards to industrial applicability were highlighted, where relevant, and it is important to note that significant progress in the industrial implementation of MVDA techniques within the PAT and QbD frameworks remains to be realised. However, continuous developments in the robustness and reliability of sensors and data analysis techniques provide the necessary requirements for much wider take-up and implementation of such frameworks within the (biopharmaceutical) industry. Whilst the highly regulated nature of this particular sector may appear to preclude the application of certain types of MVDA methods, it is important to emphasise that the regulatory environment has advanced in this respect over the past decade and fundamental process understanding and risk-based approaches to process development and operation are actively encouraged. The MVDA methods described above represent a useful tool for achieving this goal and have already been proven to enhance bioprocess performance at varying scales of manufacturing process. More sophisticated measurement techniques will push further the boundaries of MVDA method development, which in turn will lead to wider acceptance of these techniques by the industry.

References

1. Alexandrakis D (2012) NIRS in an industrial environment. Euro Pharmaceut Rev 17(1):27–30
2. Arnold SA, Crowley J, Woods N, Harvey LM, McNeil B (2003) In-situ near infrared spectroscopy to monitor key analytes in mammalian cell cultivation. Biotechnol Bioeng 84(1):13–19
3. Arora N, Biegler LT (2001) Redescending estimators for data reconciliation and parameter estimation. Comput Chem Eng 25(11–12):1585–1599
4. Balestrassi PP, Popova E, Paiva AP, Marangon Lima JW (2009) Design of experiments on neural network's training for nonlinear time series forecasting. Neurocomputing 72:1160–1178
5. Basheer IA, Hajmeer M (2000) Artificial neural networks: fundamentals, computing, design and application. J Microbiol Methods 43:3–31
6. Brereton R (2009) Chemometrics for pattern recognition. Wiley, Chichester
7. Chatfield C, Collins AJ (1980) Introduction to multivariate analysis. Chapman and Hall, London
8. Chen M, Hu M, Hofestädt R (2011) A systematic petri net approach for multiple-scale modeling and simulation of biochemical processes. Appl Biochem Biotechnol 164:338–352
9. Cunha CCF, Glassey J, Montague GA, Albert S, Mohan P (2002) An assessment of seed quality and its influence on productivity estimation in an industrial antibiotic fermentation. Biotechnol Bioeng 78:658–669
10. David F, Westphal R, Bunk B, Jahn D, Franco-Lara E (2010) Optimization of antibody fragment production in *Bacillus megaterium*: the role of metal ions on protein secretion. J Biotechnol 150(1):115–124
11. Desai K, Badhe Y, Tambe SS, Kulkarni BD (2006) Soft-sensor development for fed-batch bioreactors using support vector regression. Biochem Eng J 27(3):225–239
12. Fielding AH (2007) Cluster and classification techniques for the biosciences. Cambridge University Press, Cambridge
13. Gao Y, Kipling K, Glassey J, Willis M, Montague G, Zhou Y, Titchener-Hooker N (2010) Application of agent-based system for bioprocess description and process improvement. Process Biochem 26:706–716
14. Geladi P, Kowalski BR (1986) Partial least-squares regression: a tutorial. Anal Chimica Acta 185:1–17
15. Glassey J, Ignova M, Montague GA, Morris AJ (1994) Autoassociative neural networks in bioprocess condition monitoring. In: ADCHEM'94, Kyoto, pp 447–451
16. Gregersen L, JØrgensen SB (1999) Supervision of fed-batch fermentations. Chem Eng J 75:69–76
17. Gu H, Pan Z, Xi B, Asiago V, Musselman B, Raftery D (2011) Principal component directed partial least squares analysis for combining nuclear magnetic resonance and mass spectrometry data in metabolomics: application to the detection of breast cancer. Anal Chim Acta 686(1–2):57–63
18. Guebel DV, Cánovas M, Torres NV (2009) Analysis of the *Escherichia coli* response to glycerol pulse in continuous, high-cell density culture using a multivariate approach. Biotechnol Bioeng 102(3):910–922
19. Han-Ming W (2011) On biological validity indices for soft clustering algorithms for gene expression data. Comput Stat Data Anal 55(5):1969–1979
20. Haseltine EL, Rawlings JB (2005) Critical evaluation of extended Kalman filtering and moving horizon estimation. Ind Eng Chem Res 44(8):2451–2460
21. Huang JH, Shimizu H, Shioya S (2002) Data preprocessing and output evaluation of an autoassociative neural network model for online fault detection in virginiamycin production. J Biosci Bioeng 94(1):70–77

22. Igne B, Zacour BM, Shi Z, Talwar S, Anderson CA, Drennen JK III (2011) Online monitoring of pharmaceutical materials using multiple NIR sensors—Part I: blend homogeneity. J Pharm Innov 6:47–59
23. Kohonen T (1997) Self-organizing maps, springer series in information sciences, 2nd edn. vol 30. Springer, Heidelberg
24. Kompany-Zareh M (2011) On-line monitoring of a continuous pharmaceutical process using parallel factor analysis and unfolding multivariate statistical process control representation. J Iran Chem Soc 8(1):209–222
25. Kong CS, Yu J, Minion FC, Rajan K (2011) Identification of biologically significant genes from combinatorial microarray data. ACS Comb Sci 13(5):562–571
26. Kramer NA (1991) Nonlinear principal component analysis using autoassociative neural networks. AIChE J 37:233–243
27. Kuehn DR, Davidson H (1961) Computer control II. Mathematics of control. Chem Eng Prog 57:44–47
28. Landgrebe D, Haake C, Höpfner T, Beutel S, Hitzman B, Scheper T, Rhiel M, Reardon KF (2010) On-line infrared spectroscopy for bioprocess monitoring. Appl Microbiol Biotechnol 88:11–22
29. Laurent S, Karim MN (2001) Probabilistic neural networks using Bayesian decision strategies and a modified Gompertz model for growth phase classification in the batch culture of *Bacillus subtilis*. Biochem Eng J 7(1):41–48
30. Lee D (2005) Component-based software architecture for biosystem reverse engineering. Biotechnol Bioprocess Eng 10(5):400–407
31. Lee DS, Lee MW, Woo SH, Kim YJ, Park JM (2006) Nonlinear dynamic partial least squares modeling of a full-scale biological wastewater treatment plant. Process Biochem 41:2050–2057
32. Linko S, Zhu YH, Linko P (1999) Applying neural networks as software sensors for enzyme engineering. Trends Biotechnol 17:155–162
33. Luttmann R, Bracewell DG, Cornelissen G, Gernaey KV, Glassey J, Hass VC, Kaiser C, Lindström IM, Preusse C, Striedner G, Mandenius CF (2012) Soft Sensors in Bioprocessing. Biotechnol J 7, 1040–1047
34. Maier HR, Dandy GC (1998) The effect of internal parameters and geometry on the performance of back-propagation neural networks: an empirical study. Environ Model Softw 13:193–209
35. Miao Y, Su HY, Chu J (2009) A support vector regression approach for recursive simultaneous data reconciliation and gross error detection in nonlinear dynamical systems. Acta Automatica Sinica 35(6):708–716
36. McCulloh WP (1943) A logical calculus of the ideas immanent in nervous activity. Bull Math Biophys 5:115–133
37. MØller SF, von Frese J, Bro R (2005) Robust methods for multivariate data analysis. J Chemometrics 19:549–563
38. Naes T, Isaksson T, Fearn T, Davies T (2002) A user-friendly guide to multivariate calibration and classification. NIR, Chichester
39. Nikhil , Koskinen PEP, Visa A, Kaksonen AH, Puhakka JA, Yli-Harja O (2008) Clustering hybrid regression: a novel computational approach to study and model biohydrogen production through dark fermentation. Bioprocess Biosyst Eng 31(6):631–640
40. Nomikos P, MacGregor JF (1994) Monitoring of batch processes using multi-way principal component analysis. AICHE J 40:1361–1375
41. Nucci ER, Cruz AJG, Giordano RC (2010) Monitoring bioreactors using principal component analysis: production of penicillin G acylase as a case study. Bioprocess Biosyst Eng 33:557–564
42. O'Malley CJ, Montague GA, Martin EB, Liddell JM, Kara B, Titchener-Hooker NJ (2012) Utilisation of key descriptors from protein sequence data to aid bioprocess route selection. Food Bioprod Process (in press). doi: 10.1016/j.fbp.2012.01.005

43. Ödman P, Johansen CL, Olsson L, Gernaey KV, Lantz AE (2010) Sensor combination and chemometric variable selection for online monitoring of *Streptomyces coelicolor* fed-batch cultivations. Appl Microbiol Biotechnol 86:1745–1759
44. Pate ME, Turner MK, Thornhill NF, Titchener-Hooker NJ (2004) Principal component analysis of nonlinear chromatography. Biotechnol Prog 20:215–222
45. Rhee JI, Kang TH, Lee KI, Sohn OJ, Kim SY, Chung SW (2006) Application of principal component analysis and self-organizing map to the analysis of 2D fluorescence spectra and the monitoring of fermentation processes. Biotechnol Bioprocess Eng 11(5):432–441
46. Roger JM, Chauchard F, Williams P (2008) Removing the block effects in calibration by means of dynamic orthogonal projection. Application to the year effect correction for wheat protein prediction. J Near Infrared Spectrosc 16(3):311–315
47. Roggo Y, Chalus P, Maurer L, Lema-Martinez C, Edmond A, Jent N (2007) A review of near infrared spectroscopy and chemometrics in pharmaceutical technologies. J Pharmaceut Biomed Anal 44(3):683–700
48. Shaffer RE, Rose-Pehrsson SL, McGill A (1999) A comparison study of chemical sensor array pattern recognition algorithms. Anal Chim Acta 384:305–317
49. Shen D, Kiehl TR, Khattak SF, Li ZJ, He A, Kayne PS, Patel V, Neuhaus IM, Sharfstein ST (2010) Transcriptomic responses to sodium chloride-induced osmotic stress: a study of industrial fed-batch CHO cell cultures. Biotechnol Prog 26(4):1104–1115
50. Schölkopf B, Smola A, Müller KR (1998) Nonlinear component analysis as a kernel eigenvalue problem. Neural Comput 10:1299–1319
51. Tarazona S, Prado-López S, Dopazo J, Ferre A, Conesa A (2012) Variable selection for multifactorial genomic data. Chemometrics Intell Lab Syst 110(1):113–122
52. Tenenbaum JB, de Silva V, Langford JC (2000) A global geometric framework for nonlinear dimensionality reduction. Science 290:2319–2323
53. Tewari J, Vivechana D, Kamal M (2011) On-line monitoring of residual solvent during the pharmaceutical drying process using non-contact infrared sensor: a process analytical technology (PAT) approach. Sens Actuators B Chem 144(1):104–111
54. Varmuza K (2009) Introduction to multivariate statistical analysis in chemometrics. Taylor & Francis, CRC, New York
55. von Stosch M, Oliveira R, Peres J, Feyo de Azevedo S (2011) A novel identification method for hybrid (N) PLS dynamical systems with application to bioprocesses. Expert Syst Appl 38(9):10862–10874
56. Walczak B, Massart DL (2000) Local modelling with radial basis function networks. Chemometrics Intell Lab Syst 50:179–198
57. Warnes MR, Glassey J, Montague GA, Kara B (1998) Application of radial basis function and feedforward artificial neural networks to the *Escherichia coli* fermentation process. Neurocomputing 20:67–82
58. Weiss GH, Romagnoli JA, Islam KA (1996) Data reconciliation—an industrial case study. Comput Chem Eng 20:1441–1449
59. Widrow B, Lehr MA (1990) 30 years of adaptive neural networks: perceptron, Madaline, and backpropagation. In: Proceeding of the IEEE, vol 78(9). p 1415
60. Wilkes JG, Rushing L, Nayak R, Buzatu DA, Sutherland JB (2005) Rapid phenotypic characterization of *Salmonella enterica* strains by pyrolysis metastable atom bombardment mass spectrometry with multivariate statistical and artificial neural network pattern recognition. J Microbiol Methods 61(3):321–334
61. Wold S, Geladi P, Esbensen K, Ohman J (1987) Multi-way principal components and PLS-analysis. J Chemom 1:41–56
62. Wold S, Trygg J, Berglund A, Antti H (2001) Some recent developments in PLS modelling. Chemom Intell Lab Syst 58:131–150
63. Yin H (2008) The self-organizing maps: background, theories, extensions and applications. In: Fulcher J, Jain LC (eds) Computational intelligence: a compendium. Springer, Heidelberg, pp 715–762

64. Yin H, Huang W (2010) Adaptive nonlinear manifolds and their applications to pattern recognition. Inform Sci 180(14):2649–2662
65. Yu DL, Gomm JB, Williams D (1999) Sensor fault diagnosis in a chemical process via RBF neural networks. Control Eng Pract 7:49–55

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Design of Pathway-Level Bioprocess Monitoring and Control Strategies Supported by Metabolic Networks

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Abstract In this chapter we explore the basic tools for the design of bioprocess monitoring, optimization, and control algorithms that incorporate a priori knowledge of metabolic networks. The main advantage is that this ultimately enables the targeting of intracellular control variables such as metabolic reactions or metabolic pathways directly linked with productivity and product quality. We analyze in particular design methods that target elementary modes of metabolic networks. The topics covered include the analysis of the structure of metabolic networks, computation and reduction of elementary modes, measurement methods for the envirome, envirome-guided metabolic reconstruction, and macroscopic dynamic modeling and control. These topics are illustrated with applications to a cultivation process of a recombinant *Pichia pastoris* X33 strain expressing a single-chain antibody fragment (scFv).

Keywords Bioprocess control · Dynamic modeling · Elementary modes · Envirome measurement · Metabolic networks

Contents

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1 Introduction

Historically, process control for cell culture has relied on empirical models with cells treated as "black boxes." Purely descriptive empirical models based on measurements of the concentrations of biomass and normally only a few extracellular compounds, which completely neglect the structure of the intracellular compartment, have been widely used for bioprocess optimization and control [1]. With the advances in systems biology, molecular biology data and mechanistic models for microorganisms of industrial interest are becoming available. Systems biology is expected to have a great impact on biotechnological processes including process control, enough to justify the coining of the term "industrial systems biology" [2].

Cell factories consist of complex, intricate networks of a large number of genes, proteins, and metabolites. At a higher hierarchical level, cells are part of larger networks comprising the environment as well as other cells or organisms [3]. As we learn more from genome-scale network reconstruction projects, it becomes apparent that the number of molecular interactions between the extracellular and intracellular environments is very large. Borenstein et al. [4] estimated that 8–11 % of the metabolites in the metabolic networks of prokaryotic species originate from the environment. Indeed, cells take a large number of compounds from the environment to carry out their metabolic activity. These include inorganic ions and a large array of low-molecular-weight organic molecules such as sugars, vitamins, fatty acids, and amino acids. As a consequence, cells leave a complex and informative metabolic footprint in the environment, which in yeast cultures may account for more than 100 metabolites [5]. Moreover, experiments with single-gene deletion mutants have shown that the metabolic footprint was sufficiently informative to classify the different mutants [6]. Larger macromolecules present in the environment, such as proteins, carbohydrates, and lipids, also play an important role in signal transduction pathways. Both the low- and high-molecular-weight extracellular molecules form a natural extension of the intracellular biochemical networks of considerable complexity. Understanding the molecular interplay between extra- and intracellular components is essential to

engineer the environment of cells more efficiently, namely for optimization of culture medium composition and design of process monitoring and control strategies that target intracellular control variables.

Metabolic networks can be used to interpret metabolic footprinting data and to study how the extracellular environment can be manipulated to control intracellular processes [7]. A few studies have addressed the development of dynamic macroscopic models for process control derived from metabolic networks. Haag et al. [8] showed that, for a class of macroscopic dynamic models, systems with complex intracellular reaction networks can be represented by macroscopic reactions relating extracellular components only with equivalent "input–output" behavior. Following a similar approach, Provost and Bastin [9] have developed macroscopic dynamic models for Chinese hamster ovary (CHO) cultures wherein the reaction mechanism is defined by the elementary modes (EMs) of the metabolic network. An elementary mode can be defined as a minimal set of metabolic reactions able to operate at steady-state, with the enzymes weighted by the relative flux they need to carry for the mode to function [10]. As a result, each elementary mode can be viewed as a metabolic subnetwork, which, under the steady-state assumption, can be equivalently represented by a macroscopic reaction involving only extracellular substrates and end-products.

The main difficulty in macroscopic dynamic modeling based on elementary modes lies in the definition of the elementary mode weighting factors. As discussed later, any particular set of metabolic fluxes, or fluxome (i.e., phenotypic state), can be represented as a weighted sum of elementary modes. The magnitude of a weighting factor thus quantifies the contribution of the particular elementary mode to the overall phenotypic state. In Provost and Bastin [9], the elementary mode weighting factors were modeled by Michaelis–Menten kinetic laws as functions of extracellular concentrations. The analogy between Michaelis–Menten kinetics and elementary mode weighting factors is, however, not founded on mechanistic principles. Moreover, this approach gives rise to very complex nonlinear systems, which are difficult to identify. In Teixeira et al. [11] we developed hybrid macroscopic models structured by elementary flux modes for baby hamster kidney (BHK) cells. Instead of Michaelis–Menten kinetic laws, empirical modeling, namely artificial neural networks, was employed to model the elementary mode weighting factors as functions of extracellular physicochemical variables.

Another difficulty in macroscopic dynamic modeling based on elementary modes lies in the typically very high number of elementary modes. Indeed, the number of elementary modes increases exponentially with the size and complexity of the network [12]. However, most of these elementary modes are not active at preset environmental conditions [13]. It is thus not necessary to use the full set of elementary modes for a specific application. Of particular interest is the subset of elementary modes describing a collection of measured phenotypic data. The importance of this lies in the fact that the internal fluxes are not independently distributed but strictly constrained by external fluxes through the pathways at steady-state [14]. Therefore, the challenge is how to select the subset of elementary modes that describe a physiological state of interest. Effective reduction of

elementary modes is mandatory to reduce the complexity of the final model and facilitate the design of process control.

In this chapter we explore the basic tools to design bioprocess modeling, monitoring, and control algorithms based on metabolic networks. We start by reviewing basic properties of metabolic networks, metabolic modeling, and elementary modes. The envirome layer of information affects critical bioprocess monitoring and control challenges. The envirome consists of the total quantitative collection of physicochemical properties that define the extracellular environment. These are the properties that can be individually or collectively manipulated in a process and also the ones that are more easily measured in real time. We thus dedicate a section to the measurement of the envirome. In a recent paper we explored the possibility of metabolic reconstruction from envirome dynamic data. We have named this technique "cell functional enviromics" [7]. We show here how this methodology can be used to design bioprocess control algorithms that target intracellular control variables such as fluxes or pathways.

2 Genome-Scale Networks Lay the Foundation

In January 2012, the genome online database (GOLD) recorded 3,065 completed bacterial genome sequences and 7,755 more sequencing projects underway [15]. Furthermore, the metagenomes (genome of mixed cultures) of 340 sample communities were also recorded in the same database, with 9 % of them from engineered mixed microbial systems (wastewater, solid waste, or bioremediation) [16]. Genome-scale networks are constructed on the basis of the complete genome annotation. Identified genes may be associated with metabolic enzymes, membrane transporters, signal transduction, or regulatory control. Combining genome annotation with basic biochemical information currently available in several databases (e.g., KEGG [17] and BioCyc [18] databases), it is possible to reconstruct the majority of the metabolic reactions network and also the associated exometabolome [19]. At least 62 genome-scale metabolic models have been reconstructed for single organisms, representing 37 genera [20, 21], including organisms of industrial relevance such as *Escherichia coli* [22], *Saccharomyces cerevisiae* [23], *Pichia pastoris* [24, 25], and many others. Metabolic networks convey critical information about the interaction between the extra- and intracellular phases, which is essential for design of advanced process control strategies that target intracellular control variables.

2.1 Structure of Metabolic Networks

Studies on the architecture of metabolic networks of microorganisms from the different domains of life (Eukarya, Bacteria, and Archaea) have shown that cellular metabolism has a scale-free topology, which means that most metabolites

participate in only one or two reactions, while a few, such as adenosine triphosphate (ATP) or pyruvate, are metabolic hubs participating in dozens of metabolic reactions [26]. In the context of bioprocess control it is particularly relevant to analyze how metabolic networks interact with the extracellular environment. Bernhardsson et al. [27] have analyzed the metabolic networks of 134 bacterial species and concluded that common reactions are found at the center of the network and decrease as we move to the periphery of the metabolic network, i.e., closer to the metabolites that cross the cellular membrane. Borenstein et al. [4] have determined the seed set compounds (i.e., exogenously acquired compounds) for each of the 478 prokaryotic species with metabolic networks available in the KEGG database. They found that about 8–11 % of the compounds in the whole metabolic network correspond to the seed set and that each organism possesses a characteristic seed set. Moreover, comparing the seed set of the different organisms enabled them to trace the evolutionary history of both metabolic networks and growth environments across the tree of life, supporting the "reverse ecology" principle. These structural features are pivotal for the design of process control strategies based on metabolic networks. On the one hand, given the high number and specificity of metabolites that cross the cellular membrane, the measurement of the metabolic footprint, i.e., the complete set of extracellular metabolites, might carry sufficient information to reconstruct a large number of intracellular metabolic processes. On the other hand, the concentrations of many such extracellular metabolites can be manipulated in order to control intracellular processes linked to product yield and quality.

2.2 Material Balances

The list of metabolic reactions identified in a genome-scale reconstruction project can be translated into a stoichiometric matrix, $\mathbf{A}$, with $\dim(\mathbf{A}) = m \times q$, where m is the number of intracellular metabolites and q is the number of metabolic reactions. The material balances over the intracellular metabolites take the following general form:

$$\frac{d\mathbf{c}^i}{dt} = \mathbf{A} \times \mathbf{v} - \mu \cdot \mathbf{c}^i, \tag{1}$$

where $\mathbf{c}^i$ is the vector of intracellular concentrations [$\dim(\mathbf{c}^i) = m$], $\mathbf{v}$ is the vector of intracellular fluxes, and μ is the specific growth rate. Under the pseudo-steady-state hypothesis, intracellular metabolites do not accumulate and the dilution term is much smaller than the net turnover of metabolites, thus Eq. (1) simplifies to

$$\begin{cases} \mathbf{0} = \mathbf{A}\mathbf{v} \\ \mathbf{v}_j \geq 0 \end{cases} \tag{2}$$

The inequality constraints in Eq. (2) refer to the subset j of irreversible reactions with nonnegative flux values. Equation (2) expresses an undetermined system of algebraic equations because $q >> m$, and thus it has no unique solution. The universe of solutions of Eq. (2) forms a polyhedral cone in the fluxome solution space whose edges correspond to independent elementary modes (elementary modes are discussed in more detailed in the next section).

Equation (2) applies only to balanced intracellular metabolites. For extracellular metabolites the net accumulation is nonzero and the following equation applies:

$$\begin{cases} \mathbf{b} = \mathbf{A}'\mathbf{v} \\ \mathbf{v}_j \geq 0 \end{cases} \tag{3}$$

with $\mathbf{b}$ the vector of fluxes of extracellular metabolites across the cellular membrane and $\mathbf{A}'$ the stoichiometric matrix of such extracellular metabolites.

2.3 Elementary Modes

Elementary mode analysis has become a widespread technique for systems-level metabolic pathway analysis [28, 29]. An elementary mode can be defined as a minimal set of enzymes able to operate at steady state, with the enzymes weighted by the relative flux they need to carry for the mode to function [10]. The universe of elementary modes of a given metabolic network defines the full set of nondecomposable steady-state flux distributions that the network can support. Any particular steady-state flux distribution can be expressed as a nonnegative linear combination of elementary modes.

As such, the phenotype of a cell, as defined by its fluxome, $\mathbf{v}$, can be expressed as a weighted sum of the contribution of each elementary mode

$$\mathbf{v} = \lambda_1 \cdot \mathbf{e}_1 + \lambda_2 \cdot \mathbf{e}_2 + \ldots + \lambda_k \cdot \mathbf{e}_k = \sum_{i=1}^{K} \lambda_i \cdot \mathbf{e}_i, \tag{4}$$

where $\mathbf{e}_i$ is an elementary mode vector with $\dim(\mathbf{e}_i) = q$, λ_i is the weighting factor of $\mathbf{e}_i$, K is the number of elementary modes, and $\dim(\mathbf{v}) = \dim(\mathbf{e}_i) = q$ is the number of metabolic reactions of the metabolic network. Geometrically the elementary modes correspond to the edges of the polyhedral cone in the fluxome solution space (Fig. 1).

The elementary mode matrix, $\mathbf{EM}$, is obtained by concatenating all the $\mathbf{e}_i$ vectors into a $q \times K$ matrix

$$\mathbf{EM} = [\,\mathbf{e}_1 \quad \mathbf{e}_2 \quad \cdots \quad \mathbf{e}_K\,]. \tag{5}$$

Multiplying the $\mathbf{EM}$ matrix by the stoichiometric matrix of the extracellular metabolites, $\mathbf{A}'$, one obtains the elementary mode stoichiometric matrix

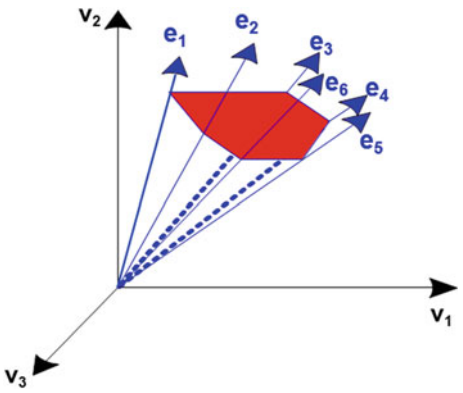


Fig. 1 Fluxome solution space of a metabolic network in steady state obeying the material balances of Eq. (2). The solution space has the shape of a polyhedral cone whose edges are the elementary modes

$$\mathbf{A}_{\mathrm{EM}} = \mathbf{A}' \times \mathbf{EM}. \tag{6}$$

The dimension of $\mathbf{A}_{\mathrm{EM}}$ is $m' \times K$, where m' is the number of extracellular metabolites. Each column of $\mathbf{A}_{\mathrm{EM}}$ contains the stoichiometry of extracellular metabolites for the particular elementary mode. This matrix holds critical information for process control, since it defines the theoretical metabolic footprint of each elementary biochemical state of the cell. The specific reaction rates of extracellular compounds are given by

$$\mathbf{b} = \mathbf{A}_{EM} \times \boldsymbol{\lambda}. \tag{7}$$

As shown later, these rates can be used to formulate macroscopic dynamic models of extracellular compounds.

2.4 Example: Elementary Modes of P. pastoris

To illustrate the elementary mode concept, we have built a *P. pastoris* metabolic network based on the KEGG database and papers by Chung et al. [24] and Çelik et al. [30]. The genes associated with each reaction are in most cases known and can be found in [24]. The network included the following processes/pathways: uptake reactions (glycerol, sulfate, phosphate, and ammonia), glycolysis/gluconeogenesis, pentose phosphate pathway, tricarboxylic acid cycle (TCA), biosynthesis of amino acids, biosynthesis of macromolecular components of biomass (nucleotides, lipids, carbohydrates, and proteins), and biosynthesis of a single-chain variable fragment (scFv), interconversion of folate compounds, oxidative phosphorylation, and energy interconversions. The metabolic network was further simplified by lumping together in single reactions the consecutive reactions in the pathways for synthesis and degradation of biomass and product precursors . The stoichiometry of ATP, nicotinamide adenine dinucleotide ($NADH_2$), nicotinamide adenine dinucleotide

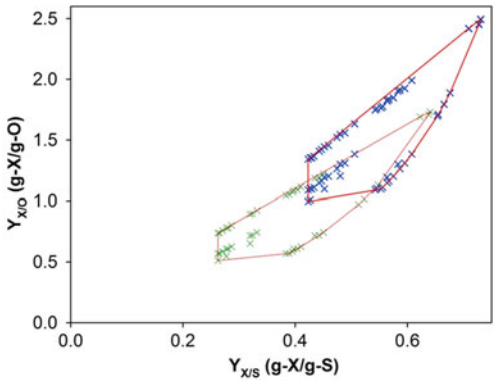


Fig. 2 Bounded convex hull in the space of yields of biomass (*blue crosses*) and product (*green crosses*) on glycerol and oxygen

phosphate ($NADPH_2$), flavin adenine dinucleotide ($FADH_2$), and H_2O were also accounted for in the metabolic reactions in order to close the balance of oxygen, hydrogen, and phosphorus. It was assumed a fixed P/O ratio of 2 mol-ATP/mol-$NAD(P)H_2$ and of 1 mol-ATP/mol-$FADH_2$. The resulting metabolic network for glycerol as carbon source has 104 reactions (thus 104 fluxes), 90 intracellular metabolites, and 16 extracellular metabolites (17 % of all metabolites).

The open-source bioinformatics software METATOOL 5.0 [31] was used to compute the elementary modes of the *P. pastoris* metabolic network. The total number of elementary modes was 4,119. Figure 2 shows a representation of the yields of biomass and product on glycerol and oxygen obtained through stoichiometric analysis of elementary modes.

The number of elementary modes for glycerol feeding was 2,520, 960, 600, and 39 for biomass growth, scFv synthesis, simultaneous biomass growth and scFv synthesis, and catabolism, respectively. From Fig. 2 it can be seen that the yields on glycerol for biomass growth and scFv synthesis vary from 0.42 to 0.73 g-X/g-S and 0.26 to 0.64 g-X/g-S, respectively, and that the yields on oxygen increase with the yields on glycerol. The elementary modes with lowest yields on glycerol and oxygen are those that include the metabolic reactions involved in the secretion of organic acids from TCA, namely succinate for biomass growth and citrate for scFv synthesis. On the other hand, the elementary modes with the highest yields on glycerol and oxygen involve the metabolic reaction of the pentose phosphate pathway.

3 Measuring the Envirome

The whole set of physical and chemical properties that define the environment of cells is known as the "envirome". The envirome is the critical layer of information for bioprocess monitoring and control, since it can be readily measured and/or manipulated in real time. The vast majority of envirome components are also

Table 1 Different levels of metabolome analysis

Metabolite target analysis	Identification and quantification focused on one or a few metabolites related to a specific pathway [34]
Metabolite profiling (or metabolic profiling)	Identification and quantification of a selected group of metabolites, e.g., metabolites from a specific metabolic pathway or a specific compound class, such as amino acids, organic acids, or carbohydrates [34]
Metabolomics	Identification and quantification of all metabolites in a biological system. Sample preparation method must retain all metabolites. Analytical technique must be suited to measure metabolites over a broad range of concentrations and needs high discriminatory power
Metabonomics	Analysis of tissues and/or biological fluids to detect changes caused by disease or therapeutic treatments [35]
Metabolic fingerprinting	Fast, high-throughput analysis of intracellular metabolites to provide a characterization of the cells for sample classification. Analytical technique must allow sample discrimination, but it is not required to identify and quantify all the metabolites individually [34]
Metabolic footprinting	Fast, high-throughput analysis of the surrounding medium to characterize the cells based on their exometabolome. As with fingerprinting, it is not necessary to identify and quantify all the metabolites individually to allow sample discrimination [6]

metabolites, of which some are provided by the culture medium and many others are produced inside the cells and then secreted or excreted into the environment.

The "metabolome" was defined by Oliver et al. [32] as the qualitative and quantitative collection of all metabolites, that is, all the low-molecular-weight molecules present in a cell, which are also participants in general metabolic reactions and that are required for the maintenance, growth, and normal function of the cell. The metabolome can be subdivided into the endometabolome (intracellular metabolites) and exometabolome (extracellular metabolites) [33]. Depending on whether the analysis is being targeted for the endo- or exometabolome and depending on the analytical detail and quantitative power desired, there are several measurement strategies available (Table 1); some such strategies have the potential for real-time monitoring and are therefore suitable for process control.

Measuring the endometabolome (metabolic fingerprinting) is not as straightforward as measuring the exometabolome (metabolic footprinting) given the complex sample preparation protocols and the higher number of intracellular metabolites. Endometabolome analysis requires separation of cells from extracellular medium followed by cell breakage. In addition, the rapid turnover inherent to intracellular metabolites, which can be under one second for microbial systems [36], results in the need for a rapid quenching step to halt metabolism. In contrast, the turnover rates of exometabolites are much lower given the low volume ratio between the intracellular and extracellular phases.

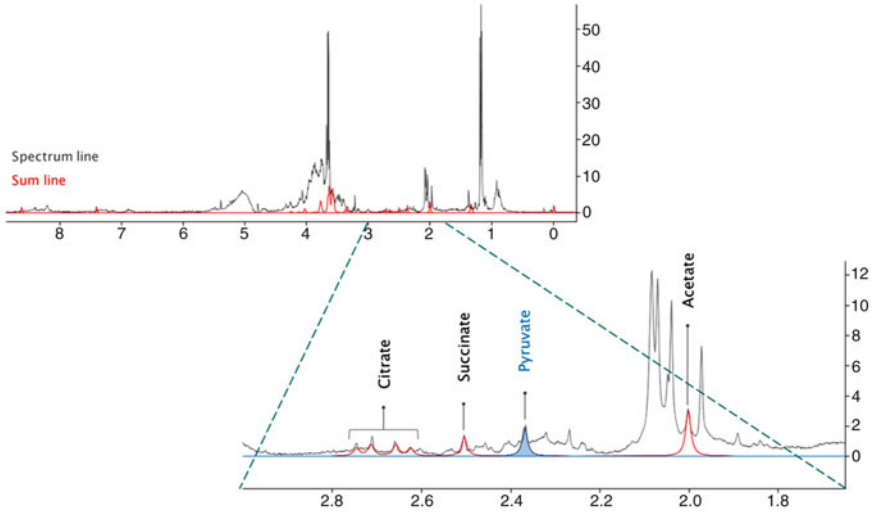


Fig. 3 ^{1}H-NMR spectrum for a *P. pastoris* supernatant sample. The *black line* is the acquired spectrum, whereas the *red line* is the estimated sum of the individual spectra for identified metabolites. Looking more closely into specific regions of the spectrum it is possible to identify key metabolites

Current analytical techniques for exo- or endometabolome analysis include nuclear magnetic resonance (NMR) spectrometry [37, 38] and mass spectrometry (MS) [39]. Either of them can be coupled to separation methods for higher resolution. These hyphenated methods include, for instance, capillary electrophoresis coupled to mass spectrometry (CE-MS) [40], gas chromatography mass spectrometry (GC–MS) [41], and liquid chromatography coupled to nuclear magnetic resonance spectrometry (LC-NMR) [42]. For a detailed review on the application of such methods to metabolomics refer to [5, 43, 44].

A fast and low-cost technique is ^{1}H-NMR. The time of spectral acquisition ranges from 2 to 10 min per sample, and automatic samplers can be used. Figure 3 shows a ^{1}H-NMR spectrum of the supernatant of *P. pastoris* culture samples. In preliminary offline tests with this technique we detected over 20 metabolites in the extracellular phase. Bundy and coworkers [45] have detected over 80 metabolites in the extracellular medium of *P. pastoris* cultures using ^{1}H-NMR and GC–MS as complementary techniques.

Knowledge of the metabolome is useful since it is very closely related to cellular phenotype. Because changes upstream accumulate downstream, changes in the transcriptome and proteome are found amplified in the metabolome. As a result, the metabolome allows the detection of changes that have a very small effect on metabolic fluxes [37, 46]. Metabolic fluxes, which can be regarded as the phenotype of a cell, are regulated not only at transcription and translation levels, but also by means of posttranslational events, and as such the metabolome is considered closer to the phenotype than the transcriptome or proteome [34, 47].

Moreover, metabolites are not organism specific, which means the techniques are equally applicable to prokaryotic, fungal, plant and animal cells.

Even though a lot of progress has been made towards enabling whole metabolome quantification, these techniques still face challenges related to the inherent characteristics of the metabolome. The size of the metabolome varies greatly, depending on the organism studied. The nature of the metabolites, whether they are polar or nonpolar, volatile or nonvolatile, also influences the analysis, and most methods are biased towards some group of metabolites. In addition, the concentration of different metabolites extends over several orders of magnitude [48], thus adding difficulty to the task of quantifying all metabolites with a single technique. However, quantification of the whole metabolome is not essential for the purposes of process monitoring and control, as a subset of key metabolites is enough to infer cell function.

4 Elementary Mode Reduction

The number of elementary modes increases geometrically with the size of the network. The typically very high number of elementary modes denotes the innate adaptability and robustness of biological networks. As a consequence, the computation of elementary modes suffers from combinatorial explosion, particular for genome-scale networks. The central carbon metabolism of a genome-scale reconstructed *E. coli* metabolic network has approximately 26 million EMs [49]. It is essential to reduce such large numbers of elementary modes according to some criterion in order to decrease the computational power requirements. Indeed, not all calculated elementary modes are thermodynamically feasible or even physiologically reachable [50]. Several methods have been developed to reduce the number of elementary modes, founded on different principles. In what follows we review some of them.

4.1 Reduction Based on Network Structural Properties

Elementary modes can be reduced on the basis of structural information of the metabolic network without the use of experimental data. de Figueiredo et al. [51] presented a method based on the ranking of elementary modes in increasing order of number of reactions. This approach enables identification of the K shortest elementary modes, which are in principle energetically more efficient. Song and Ramkrishna [14] proposed a reduction algorithm based on the effect of elementary modes on the convex hull volume. The principle consists in removing the elementary modes with negligible contribution to the convex hull volume of the original network. This allowed a priori reduction from the initial 369 to a final set

of 35 elementary modes for a yeast metabolic network fermenting both glucose and xylose without using experimental data.

4.2 Reduction Based on Thermodynamic Properties

Elementary modes can also be discriminated and reduced on the basis of metabolic reaction thermodynamics. The main assumption is that metabolic networks have evolved over time in the sense that cellular regulatory mechanisms were created that favor efficient pathways with low entropy generation. Wlaschin et al. [52] demonstrated with experimentally determined intracellular fluxes that elementary mode weighting factors are inversely correlated with the entropy generated by the involved metabolic reactions. Zhao and Kurata [53] proposed a method for correlating enzyme activity and flux distribution which uses Shannon's maximum-entropy principle, a measure of system complexity, as an objective function to estimate the enzyme control flux.

4.3 Reduction Based on Flux Data

Several methods have been proposed to eliminate elementary modes on the basis of measured flux data. The equation that applies here is Eq. (4); however, the number of elementary mode weighting factors is in general much larger than the number of metabolic fluxes, thus the system is largely undetermined. Palsson and coworkers [54, 55] suggested linear optimization methods to determine how extreme pathways (the systemically independent subset of elementary modes) contribute to a given (measured) steady-state flux distribution. There is a range of possible nonnegative weighting values associated to extreme pathways that produce a given steady-state flux distribution. This range was calculated by maximizing and minimizing the extreme pathway weighting factors, resulting in the so-called α-spectrum. The allowable ranges for the values of λ_i were computed as

$$\begin{aligned} &\max \lambda_i \\ &\text{subject to Eq.}(4), \quad i = 1, \ldots, K \quad 0 \leq \lambda_i \leq 1 \\ &\min \lambda_i \\ &\text{subject to Eq.}(4), \quad i = 1, \ldots, K \quad 0 \leq \lambda_i \leq 1 \end{aligned} \tag{8}$$

Wang et al. [56] presented a method to calculate the elementary mode coefficients for a large set of elementary modes by devising a quadratic program to explore the possibility and performance of using a subset of the elementary modes to reconstruct flux distributions. Alternatively, a framework based on elementary mode analysis and the convex properties of elementary modes was developed to calculate flux regulation coefficients (FRC) corresponding to an appropriate

fractional operation of this mode within the complete set of elementary modes [57].

Schwartz and Kanehisa [58] showed that a combination of structural and kinetic modeling in yeast glycolysis significantly constrains the range of possible behaviors of a metabolic system. All elementary modes are not equal contributors to physiological cellular states, and this approach may open a direction towards a broader identification of physiologically relevant elementary modes among the very large number of stoichiometrically possible modes.

Very recently, Bastin et al. [59] developed a methodology to compute a decomposition of admissible flux vectors in a minimal number of elementary modes without explicitly enumerating all of them. They demonstrated that the vector of admissible weighting vectors (λ) rewritten as

$$\boldsymbol{\lambda} = \sum_k \beta_i \cdot \mathbf{h}_i \quad \beta_i \geq 0 \quad \sum_k \beta_i = 1 \tag{9}$$

is necessarily an admissible λ satisfying Eq. (7). In this case, the convex polytope, $H = [\, h_1 \quad h_2 \quad \cdots \quad h_k \,]$, contains a number of solutions equal to the number of measurements p. Each polytope solution represents a minimal flux distribution given by $\hat{\mathbf{v}}_i = \mathbf{EM} \cdot \mathbf{h}_i$ and may be viewed as the simplest pathways that satisfy the pseudo-steady-state assumption and the constraints imposed by the extracellular measurements defined in Eq. (7).

4.4 Example: Reduction of the Elementary Modes by Weighting Factor Minimization

Here we illustrate the method proposed by Schwartz and Kanehisa [58] for elementary mode reduction. This method identifies a subset of elementary modes by minimizing the sum of weighting factors (λ):

$$\begin{aligned} &\min \sum_{i=1}^{k} \lambda_i \\ &\text{subject to Eq. (4)} \end{aligned} \tag{10}$$

This method was applied to the previously described *P. pastoris* metabolic network including 4,119 elementary modes. The results are shown in Table 2 for three distinct time points. Only 17 elementary modes were obtained with nonzero weighting factors. Several of these are selected at least twice in the three different phases of the culture. Note that this method basically selects the elementary modes which are closest to the actual biological state by minimizing the sum of weighting factors.

Table 2 Elementary mode reduction results for three distinct culture time points

$t = 30.7$ h		$t = 71$ h		$t = 119$ h	
#EM	λ	#EM	λ	#EM	λ
EM29	0.5324	EM228[a]	0.9691	EM228[a]	0.0745
EM153	0.2148	EM193[a]	0.4303	EM193[a]	0.0370
EM128	0.1480	EM219	0.1038	EM144	0.0110
EM189[a]	0.1259	EM185[a]	0.0773	EM189[a]	0.0075
EM159	0.0021	EM189[a]	0.0554	EM18[a]	0.0067
EM51	0.0002	EM290	0.0449	EM290[a]	0.0052
		EM206	0.0028	EM185[a]	0.0010
		EM18[a]	0.0002	EM45	0.0007
				EM120	0.0006
				EM177	0.0003

[a] Elementary modes that are selected at least twice

5 Pathway-Level Process Control

Upon the identification of the most significant elementary modes, macroscopic dynamic models can be derived with implicit intracellular structure, which can then be used for process monitoring and control. For a stirred tank bioreactor, such material balance equations take the following general form:

$$\frac{\mathrm{d}\mathbf{c}}{\mathrm{d}t} = \mathbf{b}X - D(\mathbf{c} - \mathbf{c}_{\mathrm{in}}) + \mathbf{Q}. \tag{11}$$

In Eq. (11), the state space vector, $\mathbf{c}$, is formed by the concentrations of extracellular compounds, X is the biomass concentration, D is the dilution rate, $\mathbf{c}_{\mathrm{in}}$ is the concentration of extracellular compounds in the inlet stream, and $\mathbf{Q}$ is the vector of gas–liquid transfer rates of volatile extracellular compounds. Note that Eq. (11) is analogous to the state-space equation proposed by Dochain and Bastin to design adaptive state estimation and control algorithms [1]. The main difference lies in the fact that the extracellular fluxes, $\mathbf{b}$, and the intracellular fluxes, $\mathbf{v}$, are functions of the elementary flux mode weighting factors instead of the traditional reaction kinetics:

$$\begin{bmatrix} \mathbf{v} \\ \mathbf{b} \end{bmatrix} = \begin{bmatrix} \mathbf{EM} \\ \mathbf{A}' \times \mathbf{EM} \end{bmatrix} \times \boldsymbol{\lambda} \tag{12}$$

An important implication is that any state-space solution of Eqs. (11, 12) obeys the steady-state stoichiometric constraints imposed by the metabolic network.

The main difficulty in deriving these models is the definition of the elementary mode weighting factors as functions of environmental properties. Provost and Bastin [9] employed Michaelis–Menten kinetic laws, resulting in very complex nonlinear systems, which are very difficult to identify. Teixeira et al. [11] have developed hybrid macroscopic models structured by elementary modes using neural networks to model the respective weighting factors. In any case, the

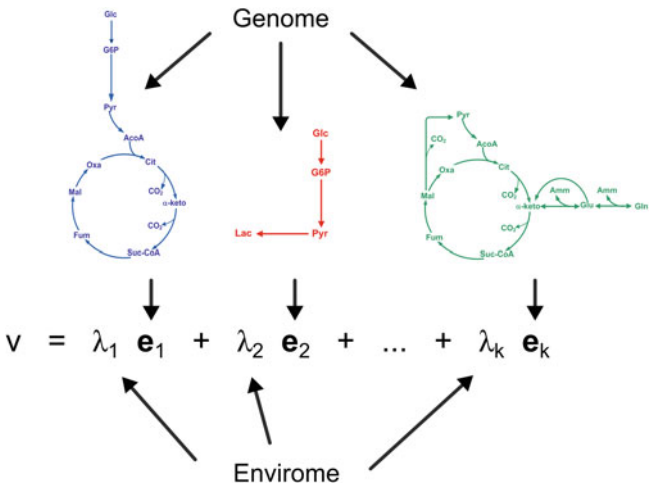


Fig. 4 Functional genomics versus functional enviromics. The genome sets the structure of elementary modes. The activation of elementary modes is controlled by the environment

effective reduction of the initially very large number of elementary modes is critical to decrease the complexity and to obtain a final parsimonious model.

In a set of recent studies [11, 13] we investigated the systematic reconstruction of metabolic processes based on regression analysis of elementary mode weighting factors against measured environmental effectors. We have called this technique "cell functional enviromics" [7]. The principle is depicted in Fig. 4. While the genome sets the structure of elementary modes, the envirome sets the relative contribution of each elementary mode to a given flux phenotype observation. While functional genomics studies genome-wide cellular function reconstruction through the collection and analysis of transcriptome or proteome data over time, functional enviromics studies the reconstruction of cellular function through the collection and analysis of dynamic envirome data. Functional enviromics applies the following main steps:

(i) Compute the elementary mode matrix, **EM**, from the microorganism metabolic network.
(ii) Acquire informative envirome data over time and organize it in the form of a envirome data matrix $\mathbf{X} = \{c_{i,j}\}$, a $M \times N$ matrix of M envirome factors, $c_{i,j}$, and respective measured flux data, $\mathbf{R} = \{\mathbf{b}_i\}$, a $M \times q'$ matrix of measured fluxes.
(iii) Apply systems-level analysis of dynamical envirome data **X** and **R** to find relationships between environmental variables, $c_{i,j}$, and elementary modes weighting factors, $\lambda_{i,j}$.

In what follows, we describe a possible functional enviromics algorithm based on the previous work by Ferreira et al. [13].

5.1 Functional Enviromics Algorithm

Among the whole set of elementary modes, the subset that is tightly linked to the envirome can be effectively determined by regression analysis of flux data, $\mathbf{R} = \{\mathbf{b}_i\}$, against envirome data, $\mathbf{X} = \{c_{i,j}\}$, satisfying the following criteria:

(a) Maximize the captured variance of envirome data $\mathbf{X} = \{c_{i,j}\}$ and of flux data $\mathbf{R} = \{\mathbf{b}_i\}$.
(b) Maximize the correlation between elementary mode weighting factors and envirome variables.
(c) Minimize the number of elementary modes required to capture a given variance of $\mathbf{R} = \{\mathbf{b}_i\}$ and $\mathbf{X} = \{c_{i,j}\}$, i.e., minimize redundancy.

These criteria can be fulfilled by maximizing the covariance between envirome data, $\mathbf{X} = \{c_{i,j}\}$, and respective measured flux data, $\mathbf{R} = \{\mathbf{b}_i\}$, according to the formula

$$\begin{array}{ll} \underset{\mathbf{I}}{\text{Maximize}} & \text{cov}(\mathbf{X}, \mathbf{R}) \\ \text{s.t.} & \begin{cases} \mathbf{R} = \mathbf{\Lambda} \times \mathbf{EM}^T \\ \mathbf{\Lambda} = \mathbf{X} \times \mathbf{I}^T \end{cases} \end{array} \tag{13}$$

with $\mathbf{EM} = \{\mathbf{e}_i\}$ a $q \times K$ matrix of K elementary cellular functions, $\mathbf{e}_i[\dim(\mathbf{e}_i) = q]$, $\mathbf{\Lambda} = \{\boldsymbol{\lambda}_i\}$ a $M \times K$ matrix of weight vectors $\boldsymbol{\lambda}_i$ of elementary modes $[\dim(\boldsymbol{\lambda}_i) = M]$, and $\mathbf{I} = \{I_{i,j}\}$ a $K \times N$ matrix of intensity parameters, which are the degrees in Eq. (13). Several methods can be used to solve Eq. (6). One efficient method consists in one-by-one decomposition of elementary modes according to Eqs. (14–16)

$$\mathbf{X} = \mathbf{T} \times \mathbf{W}^T + \mathbf{EF}_X \tag{14}$$

$$\mathbf{R} = \mathbf{\Lambda} \times \mathbf{EM}^T + \mathbf{EF}_R \tag{15}$$

$$\mathbf{\Lambda} = \mathbf{T} \times \mathbf{B}^T + \mathbf{EF}_\Lambda \tag{16}$$

with $\mathbf{EF}_i$ residuals matrices that are minimized, $\mathbf{W}$ a matrix of loading coefficients, and $\mathbf{B}$ a matrix of regression coefficients. Finally, the intensity matrix $\mathbf{I}$ is given by

$$\mathbf{I} = \mathbf{B} \times \mathbf{W}^T \tag{17}$$

The result of this procedure is the discrimination of a minimal set of elementary modes that are tightly linked with medium composition. The information can finally be organized into an $N \times K$ data array, called a functional enviromics map:

$$\text{Functional enviromics map} = \mathbf{I}^T = \{I_{j,i}\}; j = 1, \ldots, N; i = 1, \ldots, K \tag{18}$$

The rows represent envirome factors, the columns represent elementary modes, and $I_{j,i}$ is the relative "intensity" of up- or downregulation of elementary cellular function i by medium factor j.

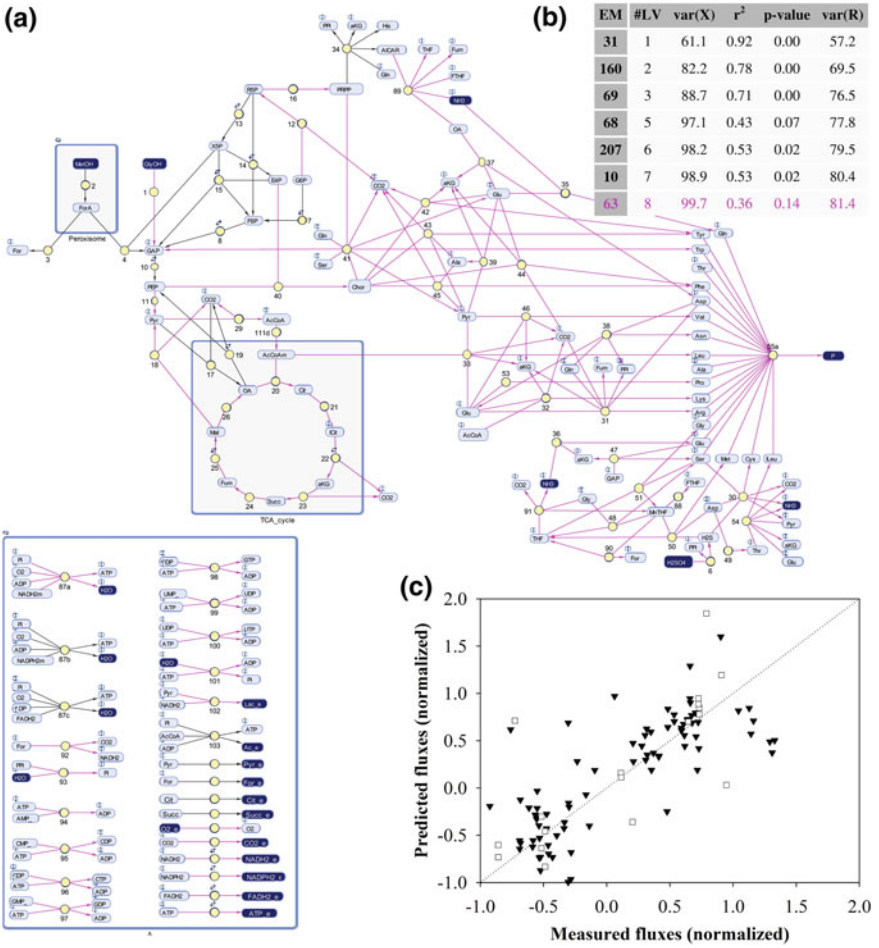
5.2 Example: Metabolic Process Control of P. pastoris Cultures

We study here the optimization and control of a pilot 50-L fermentation of a constitutive *P. pastoris* X33 strain expressing a single-chain antibody fragment; for details see [60]. The reactor was inoculated at a starting volume of 15 L. Cultivation temperature was controlled at 30 °C, and pH was controlled at 5.0 with addition of ammonium hydroxide 25 %, which was also the main nitrogen source for the culture. The airflow rate was kept constant at 1,800 L/h throughout the fermentation. Overhead pressure was controlled at 200 mbar. Glycerol feeding and dissolved oxygen (DO) control was divided into three phases:

(i) *Glycerol batch phase*—The reactor was operated initially in batch mode, starting with a glycerol concentration of 40 g/L. DO drops very slowly and remains close to saturation levels.
(ii) *Glycerol fed-batch phase*—An exponential feeding program is initiated once the concentration of biomass reaches the level of 18 g DCW/L. It is in this phase that cell density increases significantly and DO decreases more rapidly. Once the DO reaches 50 %, it is kept at that level by automatic closed-loop control, manipulating the stirrer speed between 300 and 1,000 rpm.
(iii) *Oxygen transfer limitation phase*—Once the stirrer speed reaches the maximum level of 1,000 rpm, DO decreases very rapidly and the glycerol feeding program is aborted. From this point on, the DO is kept constant at a low level (e.g., 3–5 %) by closed-loop manipulation of the glycerol feeding rate (DO-stat feeding control strategy).

We have investigated how the calculated elementary modes in the example in Sect. 2.4 correlate with the environmental parameters by applying the previously described functional enviromics algorithm. Measured environmental parameters comprised the temperature (T), pH, stirrer speed (Ni), pressure (Press), and the concentrations of dissolved oxygen (DO), glycerol (Gly), biomass (X), product (scFv), and inorganic compounds ($[NH_4^+]$, $[K^+]$, $[Ca^{2+}]$, $[Mg^{2+}]$, [S], and [P]). From the concentrations of inorganic salts we have calculated the ionic strength (IS) and the osmolarity (Osm). The measured rates were those of biomass (μ), glycerol (v_{Gly}), oxygen (v_{O2}), carbon dioxide (v_{CO2}), and product (v_{scFv}).

The initial number of elementary modes was 4,119, of which a small set of six elementary modes were discriminated by functional enviromics (Fig. 5b). The total explained variance of measured fluxes was 81.4 %, and the correlation between predicted and measured fluxes was acceptable (Fig. 5c) given the high level of noise in the measured rates.



EM	#LV	var(X)	r^2	p-value	var(R)
31	1	61.1	0.92	0.00	57.2
160	2	82.2	0.78	0.00	69.5
69	3	88.7	0.71	0.00	76.5
68	5	97.1	0.43	0.07	77.8
207	6	98.2	0.53	0.02	79.5
10	7	98.9	0.53	0.02	80.4
63	8	99.7	0.36	0.14	81.4

Fig. 5 Functional enviromics of a *P. pastoris* X33 strain expressing a single-chain antibody fragment (scFv): **a** metabolic network, **b** subset of elementary modes with highest correlation with the environmental variables. Elementary mode 63 (marked in *pink* in the network and in the table) refers to the scFv biosynthesis. **c** Predicted versus measured fluxes by the method of Eq. (11)

Of the six identified elementary modes, three make by far the largest contribution. Elementary modes 68 and 69 describe the biomass growth, and elementary mode 63 describes the product synthesis (marked in pink in the network of Fig. 5). We have built a macroscopic model with these three elementary modes.

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \left(\sum_i a_X e_{i,X} \lambda_i\right) X - DX \tag{19}$$

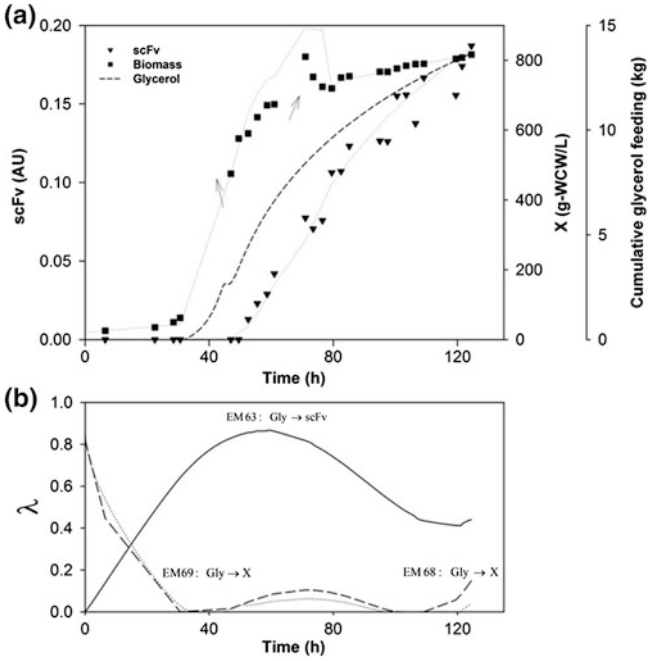


Fig. 6 Simulation of biomass and product dynamic profiles on the basis of three elementary modes (EM 63, EM 68, and EM 69) identified by functional enviromics: **a** biomass and product concentration over time, and **b** elementary mode weighting factors over time

$$\frac{\mathrm{d}P}{\mathrm{d}t} = \left(\sum_i a_P e_{i,P} \lambda_i\right) X - DP \tag{20}$$

A comparison between the model simulation and experimental measurements is shown in Fig. 6.

From the initial 4,119 elementary modes, 2,520, 960, 600, and 39 are for biomass growth, scFv synthesis, simultaneous biomass growth and scFv synthesis, and catabolism, respectively. It is interesting to note that the functional enviromics algorithm identified EM 63 for product synthesis, which belongs to the second group of 960 elementary modes. This suggested that the product is cell growth dissociated, which is in agreement with our previous study [60]. Analysis of the weighting factor dynamics (Fig. 6b) clearly shows that the product synthesis elementary mode peaks when the weighting factors for cell growth are almost zero. The analysis of three additional fermentations further confirmed that product synthesis is cell growth dissociated and that the final product titer increases with the biomass concentration time integral. The maximum product titer and productivity could be achieved by maximizing the glycerol feeding rate by applying an accurate DO-stat glycerol feeding controller at very low DO set-points in the range of 3–5 % [60].

6 Conclusions

In a typical cell culture process there is a large number of environmental variables that shape cellular physiology. One important implication is that the design space for process development, namely culture medium optimization and process control, is potentially very large. Current process development methodologies in the industry are essentially of empirical nature. Empirical methods are not well suited to handle high-dimensional design spaces unless a substantial level of reductionism is applied, and even then with potential reduction of performance.

With the advances in systems biology, accurate genome-scale metabolic networks are becoming available for several microorganisms used in industry. Such metabolic networks contain the required information to enumerate all the operational modes of cells (i.e., elementary modes). With adequate systems biology tools such as functional enviromics, one can learn how such operational modes are controlled by the environment and/or how they modify the environment. This paves the way for pathway-level process development strategies, which are much more efficient than traditional empirical methods.

Here, we have laid out a process development methodology that can be summarized in the following main steps

(i) Formulation of an accurate (genome-scale) metabolic network
(ii) Computation of the elementary modes and pre-reduction of the same
(iii) Discrimination of elementary modes with high correlation with environmental variables by functional enviromics
(iv) Formulation of macroscopic material balances with explicit envirome-correlated elementary modes
(v) Process optimization oriented to the manipulation of elementary mode weighting factors

Such design tools can be used to optimize culture media and for advanced process control. A main advantage is the significant reduction of the number of experiments for very large design spaces. This is possible because the structure of the metabolic network constrains the manipulation of the environment. Another big benefit is the possibility to target intracellular control variables such as metabolic reactions or metabolic pathways directly linked with productivity and product quality. All in all, such techniques have the potential to considerably accelerate process development speed, to improve the mechanistic interpretability, and to increase process performance.

References

1. Bastin G, Dochain D (1990) On-line estimation and adaptive control of bioreactors. Elsevier, Amsterdam
2. Otero JM, Nielsen J (2010) Industrial systems biology. Biotechnol Bioeng 105:439–460

3. Talarek N, Cameroni E, Jaquenoud M et al (2010) Initiation of the TORC1-regulated G(0) program requires Igo1/2, which license specific mRNAs to evade degradation via the 5′-3′ mRNA decay pathway. Mol Cell 38:345–355
4. Borenstein E, Kupiec M, Feldman MW, Ruppin E (2008) Large-scale reconstruction and phylogenetic analysis of metabolic environments. Proc Natl Acad Sci U S A 105:14482–14487
5. Kell DB, Brown M, Davey HM et al (2005) Metabolic footprinting and systems biology: the medium is the message. Nat Rev Microbiol 3:557–565
6. Allen J, Davey HM, Broadhurst D et al (2003) High-throughput classification of yeast mutants for functional genomics using metabolic footprinting. Nat Biotechnol 21:692–696
7. Teixeira AP, Dias JML, Carinhas N et al (2011) Cell functional enviromics: unravelling the function of environmental factors. BMC Syst Biol 5:92
8. Haag JE, Wouwer AV, Bogaerts P (2005) Dynamic modeling of complex biological systems: a link between metabolic and macroscopic description. Math Biosci 193:25–49
9. Provost A, Bastin G (2004) Dynamic metabolic modelling under the balanced growth condition. J Process Contr 14:717–728
10. Schuster S, Fell DA, Dandekar T (2000) A general definition of metabolic pathways useful for systematic organization and analysis of complex metabolic networks. Nat Biotechnol 18:326–332
11. Teixeira AP, Alves C, Alves PM et al (2007) Hybrid elementary flux analysis/nonparametric modeling: application for bioprocess control. BMC Bioinform 8:30
12. Feist AM, Palsson BO (2008) The growing scope of applications of genome-scale metabolic reconstructions using *Escherichia coli*. Nat Biotech 26:659–667
13. Ferreira A, Dias J, Teixeira A et al (2011) Projection to latent pathways (PLP): a constrained projection to latent variables (PLS) method for elementary flux modes discrimination. BMC Syst Biol 5:181
14. Song H-S, Ramkrishna D (2009) Reduction of a set of elementary modes using yield analysis. Biotechnol Bioeng 102:554–568
15. Pagani I, Liolios K, Jansson J et al (2012) The genomes online database (GOLD) v. 4: status of genomic and metagenomic projects and their associated metadata. Nucleic Acids Res 40:D571–D579
16. Tajparast M, Frigon D (2012) Genome-scale metabolic modeling to provide insight into the production of storage compounds during feast-famine cycles of activated sludge. In: Paper presented at the 3rd IWA/WEF wastewater treatment modeling seminar, Mont-Sainte-Anne, Quebec, 26–28 February 2012
17. Kanehisa Laboratories (2010) Kyoto encyclopedia of genes and genomes (KEGG). http://www.genome.jp/kegg/
18. SRI International (2010) BioCyc database collection. http://biocyc.org/
19. Janga SC, Babu MM (2008) Network-based approaches for linking metabolism with environment. Genome Biol 9:239
20. Feist AM, Herrgard MJ, Thiele I et al (2009) Reconstruction of biochemical networks in microorganisms. Nat Rev Microbiol 7:129–143
21. Orth JD, Palsson BO (2010) Systematizing the generation of missing metabolic knowledge. Biotechnol Bioeng 107:403–412
22. Feist AM, Henry CS, Reed JL et al. (2007) A genome-scale metabolic reconstruction for *Escherichia coli* K-12 MG1655 that accounts for 1260 ORFs and thermodynamic information. Mol Syst Biol 3:121
23. Herrgard MJ, Swainston N, Dobson P et al (2008) A consensus yeast metabolic network reconstruction obtained from a community approach to systems biology. Nat Biotechnol 26:1155–1160
24. Chung BKS, Selvarasu S, Andrea C et al (2010) Genome-scale metabolic reconstruction and in silico analysis of methylotrophic yeast *Pichia pastoris* for strain improvement. Microb Cell Fact 9:50

25. Sohn SB, Graf AB, Kim TY et al (2010) Genome-scale metabolic model of methylotrophic yeast *Pichia pastoris* and its use for in silico analysis of heterologous protein production. Biotechnol J 5:705–715
26. Barabasi AL, Oltvai ZN (2004) Network biology: understanding the cell's functional organization. Nat Rev Genet 5:U101–U115
27. Bernhardsson S, Gerlee P, Lizana L (2011) Structural correlations in bacterial metabolic networks. BMC Evol Biol 11:20
28. Schuster S, Dandekar T, Fell DA (1999) Detection of elementary flux modes in biochemical networks: a promising tool for pathway analysis and metabolic engineering. Trends Biotechnol 17:53–60
29. Papin JA, Stelling J, Price ND et al (2004) Comparison of network-based pathway analysis methods. Trends Biotechnol 22:400–405
30. Celik E, Calik P, Oliver SG (2010) Metabolic Flux Analysis for recombinant protein production by *Pichia pastoris* using dualcarbon sources: effects of methanol feeding rate. Biotechnol Bioeng 105:317–329
31. von Kamp A, Schuster S (2006) Metatool 5.0: fast and flexible elementary modes analysis. Bioinformatics 22:1930–1931
32. Oliver SG, Winson MK, Kell DB, Baganz F (1998) Systematic functional analysis of the yeast genome. Trends Biotechnol 16:373–378
33. Nielsen J, Oliver S (2005) The next wave in metabolome analysis. Trends Biotechnol 23:544–546
34. Fiehn O (2002) Metabolomics—the link between genotypes and phenotypes. Plant Mol Biol 48:155–171
35. Nicholson JK, Lindon JC, Holmes E (1999) 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 29:1181–1189
36. de Koning W, van Dam K (1992) A method for the determination of changes of glycolytic metabolites in yeast on a subsecond time scale using extraction at neutral pH. Anal Biochem 204:118–123
37. Raamsdonk LM, Teusink B, Broadhurst D et al (2001) A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. Nat Biotechnol 19:45–50
38. Duarte I, Barros A, Belton PS et al (2002) High-resolution nuclear magnetic resonance spectroscopy and multivariate analysis for the characterization of beer. J Agric Food Chem 50:2475–2481
39. Castrillo JI, Hayes A, Mohammed S et al (2003) An optimized protocol for metabolome analysis in yeast using direct infusion electrospray mass spectrometry. Phytochemistry 62:929–937
40. Soga T, Ohashi Y, Ueno Y et al (2003) Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. J Proteome Res 2:488–494
41. Fiehn O, Kopka J, Dormann P et al (2000) Metabolite profiling for plant functional genomics. Nat Biotechnol 18:1157–1161
42. Wolfender JL, Ndjoko K, Hostettmann K (2003) Liquid chromatography with ultraviolet absorbance-mass spectrometric detection and with nuclear magnetic resonance spectroscopy: a powerful combination for the on-line structural investigation of plant metabolites. J Chromatogr 1000:437–455
43. Hollywood K, Brison DR, Goodacre R (2006) Metabolomics: current technologies and future trends. Proteomics 6:4716–4723
44. Oldiges M, Lutz S, Pflug S et al (2007) Metabolomics: current state and evolving methodologies and tools. Appl Microbiol Biotechnol 76:495–511
45. Tredwell GD, Edwards-Jones B, Leak DJ, Bundy JG (2011) The Development of metabolomic sampling procedures for *Pichia pastoris* and baseline metabolome data. PLoS One 6:e16286
46. Urbanczyk-Wochniak E, Luedemann A, Kopka J et al (2003) Parallel analysis of transcript and metabolic profiles: a new approach in systems biology. EMBO Rep 4:989–993

47. ter Kuile BH, Westerhoff HV (2001) Transcriptome meets metabolome: hierarchical and metabolic regulation of the glycolytic pathway. FEBS Lett 500:169–171
48. Dunn WB, Ellis DI (2005) Metabolomics: current analytical platforms and methodologies. Trends Anal Chem 24:285–294
49. Terzer M, Stelling J (2008) Large-scale computation of elementary flux modes with bit pattern trees. Bioinformatics 24:2229–2235
50. Edwards JS, Palsson BO (2000) The *Escherichia coli* MG1655 in silico metabolic genotype: its definition, characteristics, and capabilities. Proc Natl Acad Sci U S A 97:5528–5533
51. de Figueiredo LF, Podhorski A, Rubio A et al (2009) Computing the shortest elementary flux modes in genome-scale metabolic networks. Bioinformatics 25:3158–3165
52. Wlaschin AP, Trinh CT, Carlson R, Srienc F (2006) The fractional contributions of elementary modes to the metabolism of *Escherichia coli* and their estimation from reaction entropies. Metab Eng 8:338–352
53. Zhao Q, Kurata H (2009) Maximum entropy decomposition of flux distribution at steady state to elementary modes. J Biosci Bioeng 107:84–89
54. Wiback SJ, Mahadevan R, Palsson BO (2003) Reconstructing metabolic flux vectors from extreme pathways: defining the alpha-spectrum. J Theor Biol 224:313–324
55. Wiback SJ, Mahadevan R, Palsson BO (2004) Using metabolic flux data to further constrain the metabolic solution space and predict internal flux patterns: the *Escherichia coli* spectrum. Biotechnol Bioeng 86:317–331
56. Wang Q, Yang Y, Ma H, Zhao X (2007) Metabolic network properties help assign weights to elementary modes to understand physiological flux distributions. Bioinformatics 23:1049–1052
57. Nookaew I, Meechai A, Thammarongtham C et al (2007) Identification of flux regulation coefficients from elementary flux modes: a systems biology tool for analysis of metabolic networks. Biotechnol Bioeng 97:1535–1549
58. Schwartz J-M, Kanehisa M (2006) Quantitative elementary mode analysis of metabolic pathways: the example of yeast glycolysis. BMC Bioinform 7:186
59. Jungers RM, Zamorano F, Blondel VD et al (2011) Fast computation of minimal elementary decompositions of metabolic flux vectors. Automatica 47:1255–1259
60. Ferreira AR, Ataide F, von Stosch M et al (2012) Application of adaptive DO-stat feeding control to *Pichia pastoris* X33 cultures expressing a single chain antibody fragment (scFv). Bioprocess Biosyst Eng 35:1603–1614

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Knowledge Management and Process Monitoring of Pharmaceutical Processes in the Quality by Design Paradigm

Anurag S. Rathore, Anshuman Bansal and Jaspinder Hans

Abstract Pharmaceutical processes are complex and highly variable in nature. The complexity and variability associated with these processes result in inconsistent and sometimes unpredictable process outcomes. To deal with the complexity and understand the causes of variability in these processes, in-depth knowledge and thorough understanding of the process and the various factors affecting the process performance become critical. This makes knowledge management and process monitoring an indispensable part of the process improvement efforts for any pharmaceutical organization.

Keywords Process monitoring · Quality by design · Knowledge management

Abbreviations

BDS	Bulk Drug Substance
BPR	Batch production record
CAPA	Corrective And Preventive Action
cGMP	Current good manufacturing practices
CFR	Code of Federal Regulations
CPP	Critical process parameters
CQA	Critical quality attributes
DO	Dissolved oxygen
ERP	Enterprise resource planning
FDA	Food and Drug Administration
HCD	Host cell DNA

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ICH	International Conference on Harmonisation
LIMS	Laboratory information management system
OOS	Out of specification
PAT	Process analytical technology
PCA	Principal component analysis
PLS	Partial least squares
PQ	Process qualification
QMS	Quality management system
QbD	Quality by design
RVLP	Retrovirus Like Particles
SOP	Standard operating procedure
SPE	Squared prediction error
SCADA	Supervisory control and data acquisition
TPP	Target Product Profile

Contents

1 Process Data and Knowledge Management

1.1 Background

Commercial Current good manufacturing practices (cGMP) manufacturing of a therapeutic product has to continuously go through various checks in terms of process, people, raw materials, equipment, facility and processing environment. Availability of and instant access to information regarding past and current records

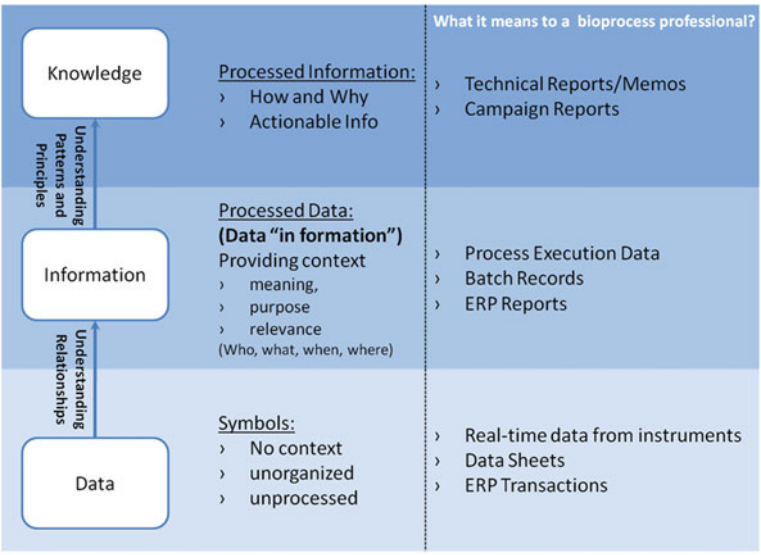

Fig. 1 Relationship between data, information and knowledge in the bioprocess context [6]

from these would lead to faster and better decision making, since the final product quality is a complex interaction of all these interdependent functional areas. For a data-intensive industry such as pharmaceuticals, access to data and information enables organizations to understand and streamline operational and business processes. However, most organizations fail to leverage this information for better decisions due to the lack of proper means of managing information. The next few sections of this chapter explore the challenges and complexities associated with capture, storage and retrieval of information and current best practices.

1.2 D–I–K Hierarchy

Data, information and knowledge are often referred to interchangeably, however in practice they have different meanings [1] and follow a defined hierarchy as shown in Fig. 1. *Data* usually means raw numbers that have no context. To a bioprocess professional, this means data entered into enterprise resource planning (ERP) systems, datasheets or data captured in real time by sensors on process equipment. *Information* provides meaning or context to data. To a bioprocess professional this means the ERP reports, batch records and process execution reports and trends. *Knowledge* is processed information that results in action. To a bioprocess professional this means technical and investigation reports that contain process knowledge, based on which process execution schemes, corrective and preventative

actions are defined. Each of these requires different skills and tools for effective storage, retrieval and management of its lifecycle.

1.3 Understanding Bioprocess Data

1.3.1 Data Sources

Processes involved in commercial manufacture of therapeutic proteins are mostly batch processes that involve process data generated from diverse sources, often isolated by data ownership across various departments and functions within an organization. Information regarding a single process batch is located within the following business systems:

- *Batch production record (BPR)*: A BPR is a record of process execution, providing a step-by-step account of product-specific processing. BPRs are usually paper based, but many organizations are adopting electronic BPR systems.
- *Log books and operator notebooks*: The information within these notebooks and logs provides important contextual information regarding the chronological sequence of events, activities, material exchanges, equipment status etc. which may prove to be vital information in identifying root causes and resolving process non-conformances.
- *Supervisory control and data acquisition (SCADA) system*: SCADA systems on process equipment monitor and control the processes in real time. The system captures streaming data from the sensors [e.g. temperature, pH, dissolved oxygen (DO) etc.] installed on this equipment. The raw data are usually stored within these systems, where the raw data storage format varies for each vendor supplying the process equipment. Due to limited storage capacity in these systems and the sheer volume of data generated, organizations sometime deploy data historians to extract the data from these SCADA systems, and compress and store the raw data.
- *Laboratory information management systems (LIMS)*: In a pharmaceutical manufacturing organization, LIMS is typically utilized to capture and manage information from analytical tests performed on test samples of product intermediates, bulk drug substances and all raw materials utilized in a manufacturing process. Along with the test results, the LIMS also tracks the logistics of the test samples (who, what, when, and where) throughout the sample lifecycle. If LIMS is not deployed in test laboratories inside organizations, this information usually resides in various spreadsheets and paper test reports.
- *ERP systems*: An ERP system within an organization captures all supply-chain and inventory-related information. This is important in providing useful logistics information regarding quality of raw materials, intermediate products and process equipment. Organizations that do not have an ERP system deploy manual procedural control, workflow and paper records to achieve this goal.

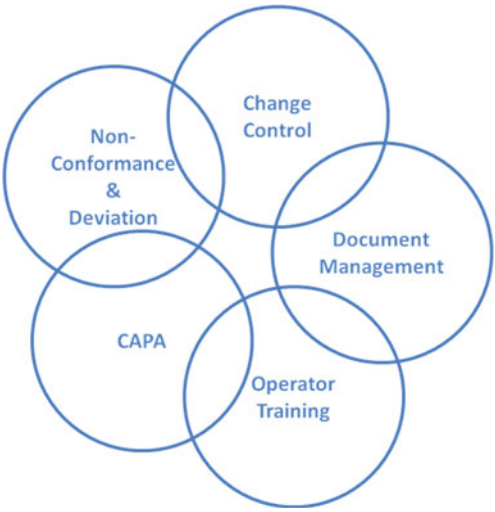

Fig. 2 Elements of a quality management system

- *Quality management systems (QMS)*: Records of controlled documents such as BPRs, standard operating procedures (SOP), non-conformance investigation reports and operator training records are all maintained for the full lifecycle of the product. The main process execution compliance systems are interlinked to each other as shown in Fig. 2 and are an integral part of the complete knowledge infrastructure. These carry useful information including batch reports, non-conformances, investigational and technical reports that contain vital process knowledge that is important for continuous improvement initiatives.

1.3.2 Data Capture Modes

Bioprocess execution data are typically captured in three modes: offline, at-line, and online. These modes are defined by the data sampling frequency, the time it takes to obtain the results and the distance the test sample has to travel. The modes are explained as follows:

- *Offline data capture*: Offline data comprise mainly test results on samples taken during processing that need to be sent for testing to a location outside of the processing room. Typically these data are captured and maintained by quality control laboratories within an organization or off-site testing agencies. The data are recorded in paper records, spreadsheets, custom databases or LIMS systems.
- *At-line data capture*: At-line data are manual data captured near the process equipment on the processing floor, being recorded in paper records, spreadsheets, custom databases or electronic batch records (e.g. cell count data, metabolite data, processing times etc.). These data are typically recorded by scientists or operators executing the process batch.

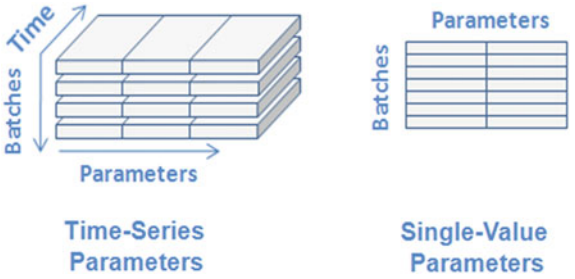

Fig. 3 Dimensionality of time-series and single-value parameters

- *Online data capture*: Online data comprise real-time streaming data coming from sensors installed on process equipment that captures process parameters [e.g. pH, DO, temperature, conductivity etc.]. This capture mode has the highest data volume and is recorded every few seconds or according to a set, periodic data sampling frequency. Due to their high volume, these data are usually retrieved from the native SCADA system storage (since there is limited native data storage capacity), being compressed and stored by plant-wide data historians. The data from the historians are then decompressed on demand.

1.3.3 Data Types and Dimensionality

Captured process data can be in numeric, alphanumeric or purely text form. Numeric data comprise parameter values that are either recorded manually or captured automatically by the automation system of process equipment. Alphanumeric data are typically metadata comprising a numbering system used to encode identification of materials (e.g. raw materials, product intermediate batches, equipment etc.). Finally, pure text data comprise all events and observations related to the process execution. Metadata such as parameter names, product names and equipment names also comprise full-text data.

The data for parameters (also referred to as time-series parameters) that are captured at regular time intervals while the batch is progressing are three dimensional in nature. The Batch IDs constitute the first dimension, the process time the second dimension and the parameter names (e.g. daily viable cell density, daily lactate concentration, daily temperature etc.) constitute the third dimension, as shown in Fig. 3. In contrast, parameters (usually referred to as single value) that are captured once per process execution (e.g. step yield, harvest titre etc.) are two dimensional in nature, with Batch IDs as the first dimension and parameters as the second dimension, as shown in Fig. 3.

1.3.4 Multi-Unit Batch Processing Involving Splits and Combinations

Bioprocesses are typically batch processes comprising many unit operations, usually executed in series (Fig. 4a). Processes that involve multiple cycles of chromatography operations also involve batches running in parallel for that unit

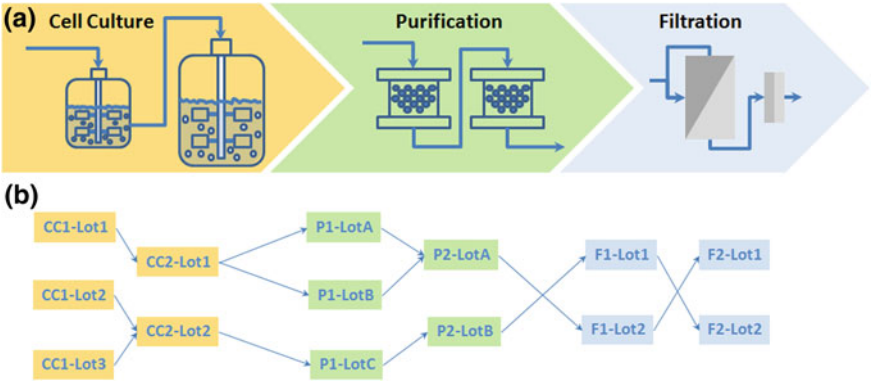

Fig. 4 **a** Example execution sequence of bioprocess unit operations. **b** Lot genealogy, involving multiple splits and combinations

operation. Due to biological uncertainty and inherent process variability, the upstream titres at the cell culture stage dictate the overall process sequence and hence the need for flexible downstream processing. This flexibility usually results in various splits, combinations and process step cycles, as shown in Fig. 4b, and poses a significant challenge in data management and root cause resolution.

1.4 Data Flow Map

Biomanufacturing involves leveraging a large amount of information for successful process execution and continuous improvement. This information set consists of not only process data from the current batch/run but also data from historical batches and execution-related information, including:

- Data from process development runs and technology transfer reports.
- Previously executed commercial batches.
- Equipment usage and maintenance records.
- Clean-room environment data.
- Personnel training data.
- Current and historical process non-conformances and deviations.

Figure 5 shows various data, information and knowledge repositories and how the content flows from one to another during normal execution of the commercial manufacturing process lifecycle of a product batch. The information is maintained in systems owned by various functional groups such as process development, engineering, quality control, quality assurance and manufacturing.

As shown in Fig. 5, *process development* engineers and scientists conduct experiments to understand and develop a robust production process for manufacturing. The outcome of these experiments is development and technology transfer reports. These reports form the basis of process validation activities for

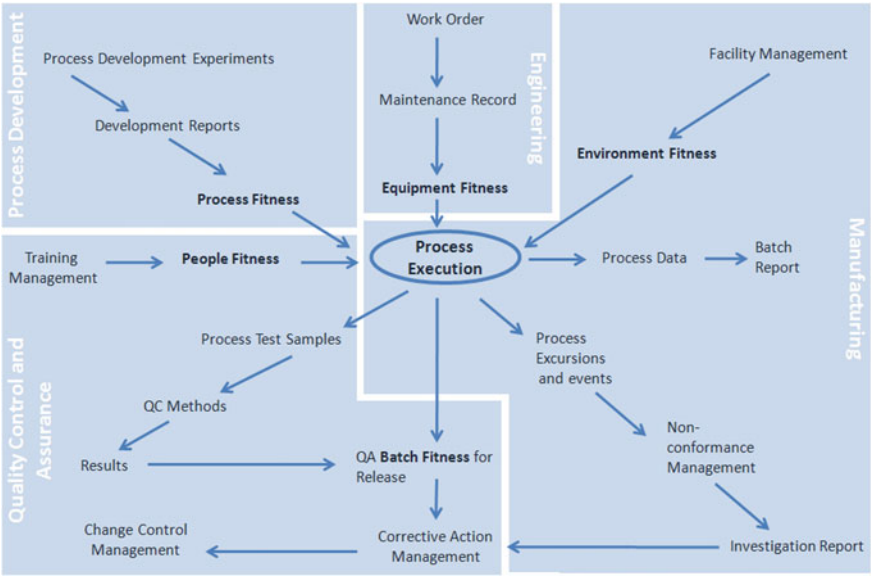

Fig. 5 Process knowledge repositories and the flow of information during process execution

"process fitness" on the manufacturing floor. After a successful validation exercise, BPRs are created to capture process execution data.

Engineering groups in an organization are involved in ensuring that utilities (air, gases and water) that are supplied and the process equipment that is used are fit to execute the process. To achieve this goal, engineering teams maintain metrology records and maintenance logs/schedules for all the equipment and instruments used during process execution.

The *facility management* group ensures that the clean-room environment is maintained according to cGMP standards and that the batch is not jeopardized due to contamination issues. This group also maintains a cleaning log/schedule for all the clean rooms used during the process execution.

Test samples are regularly collected to check for in-process controls, and these are sent to an internal *quality control* laboratory for testing. The test results are compared against the specification limits set in the process development technology transfer reports. Any out of specification (OOS) or deviations are reported to the execution and *quality assurance* teams.

The *manufacturing team* investigates all batch OOS, non-conformances and deviations observed during the process execution. Such root cause investigation draws upon information captured at all stages by various groups (process development, engineering, facility, quality) as shown in Fig. 6. The corrective and preventative actions derived from these investigation reports form the basis of process change control. The change control system is logged and maintained by the quality assurance group, which then feeds information for improvement initiatives back into various systems across various functional groups as shown in Fig. 6.

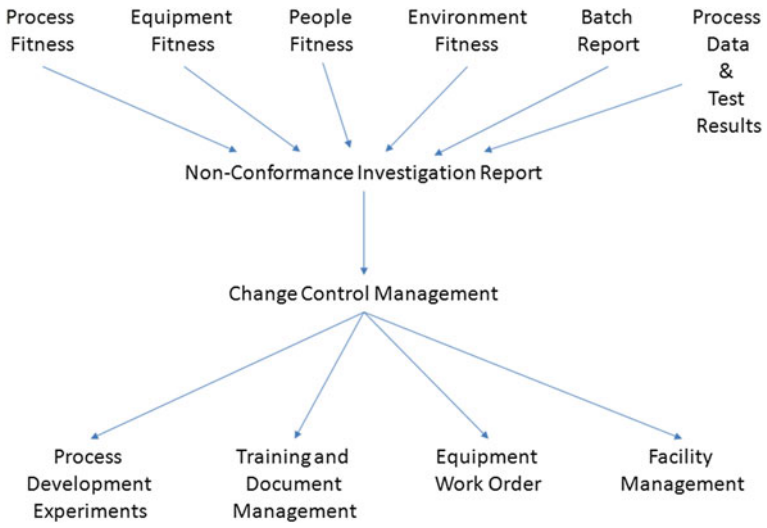

Fig. 6 Flow of information during the root cause investigation process

Figures 5 and 6 shows the lifecycle of various data, information and knowledge repositories used in bioprocess manufacturing. An open and unified data management approach across all functional groups involved in process execution within an organization will significantly help in efficient bioprocess knowledge management.

2 Process Monitoring

2.1 Introduction

Process monitoring can be defined as an approach involving collection of process data and statistical evaluation of parameters to verify or demonstrate that the process is operating in a state of control, identify possible process changes and shifts and promote continuous improvement. Consistency in product quality each time the batch is executed requires maintaining process variables within the specified limits for each batch. In pharmaceutical manufacturing, where the processes are complex and have interdependent multistage operations, each of which has numerous variables that affect the process, achieving consistent quality becomes a challenging goal. This makes it even more important to monitor these variables from batch to batch.

Generally, the process monitoring program starts with identifying the parameters relevant to the process and then recording the behaviour of those parameters

every time the process is executed. Once the important parameters are identified and captured, the data from these parameters are represented in the form of various charts and visualizations that can help in detection of trends and deviations occurring in the process. This type of regular monitoring helps in decision making, fault detection and root cause investigations whenever unusual process activity is encountered [11].

2.2 Tools and Approaches

The approaches for process monitoring can be mainly divided into two broad levels. Under each of these levels there are various tools, charts and graphs that help to assess the process performance from various angles and provide useful insights regarding areas of improvement. These two levels are:

- Level 1: basic visual monitoring.
- Level 2: statistics-based monitoring.

2.2.1 Basic Visual Monitoring

Basic visual monitoring mainly consists of basic displays of data that are easily understood by a wider audience without having advanced knowledge of statistics. The main focus of tools/displays at this level is communication of process trends to easily identify unusual events and anomalies that occurred during the process execution. This makes the process stakeholders (operators, manufacturing supervisors) aware of and efficient at handling and troubleshooting of day-to-day operational issues. Another benefit gained from the usage of these basic displays of data is that they provide standard views to represent parameter trends, parameter-to-parameter relationships and comparison of batches. This standardisation provides a single language to discuss data and process issues and thus enables healthy communication among all stakeholders.

Some of the charts/displays that are useful at this level for monitoring of pharmaceutical data are as follows:

- Parameter profiles.
- Bubble charts (scatter plots).
- Parallel coordinates.
- Treemaps.

Parameter profiles display the behaviour of a particular parameter over a period of time. As pharmaceutical data consist of many parameters that follow a particular trajectory for a process, to achieve consistent output it is important to monitor these parameters to ensure that they always follow the same trajectory for a given process. There are two approaches to show this kind of data:

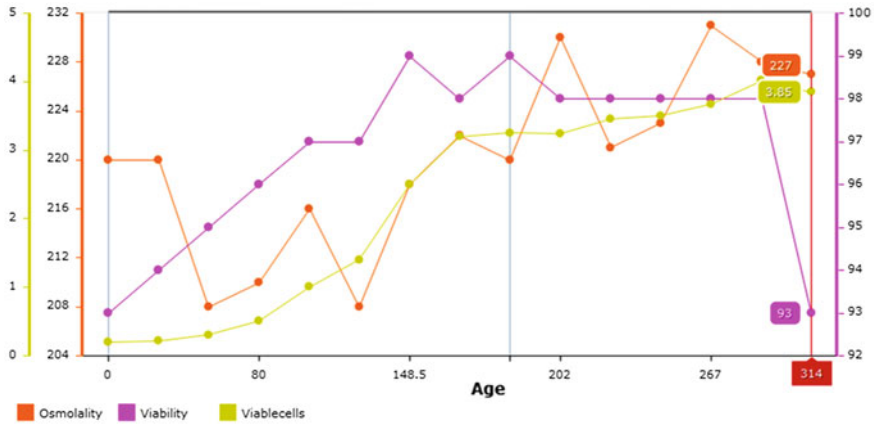

Fig. 7 Profiles of three parameters for a single batch of a cell culture process

- To monitor multiple time-series parameters belonging to the same unit operation for a single batch in one view, parameters of interest can be displayed on multiple *Y*-axes, one for each parameter, whereas the *X*-axis can show the process time. This gives a complete picture of the parameter profiles for one batch (Fig. 7).
- Another way of visualizing this kind of data is to compare profiles for a single parameter for different batches in one view. The *X*-axis again shows the process time, while the *Y*-axis shows the parameter of interest and the various graphs on the chart correspond to different batches (Fig. 8).

A *bubble chart* is a variation of a scatter plot where the markers of the scatter plot are replaced by bubbles and a third dimension of "bubble size" is plotted along with the two dimensions (*X* and *Y*) of the scatter plot. It can therefore be used to study three parameters at a time, each represented by one dimension (*X*-axis, *Y*-axis and bubble size). Such a chart provides a visual representation of the relationship that exists among three parameters and how the change in one affects the other two. Also, by plotting the values of three parameters for different batches, it conveys useful information about the region where most of the values lie for the three parameters for the majority of the batches and may help in easily detecting outliers; For example, the chart in Fig. 9 shows time-series data for three cell culture parameters: lactate concentration, ammonia concentration and product titre. Data from 15 historical batches are plotted on the chart, each represented by a different marker colour. Two patterns quickly emerge from this chart:

- At lower titres (small bubble size) both lactate and ammonia concentrations in the culture broth are increasing. This indicates the early phase of the cell culture process.
- The end of the cell culture process is indicated by higher titres (larger bubble size). It can be seen that, in this portion of the process, the lactate concentration

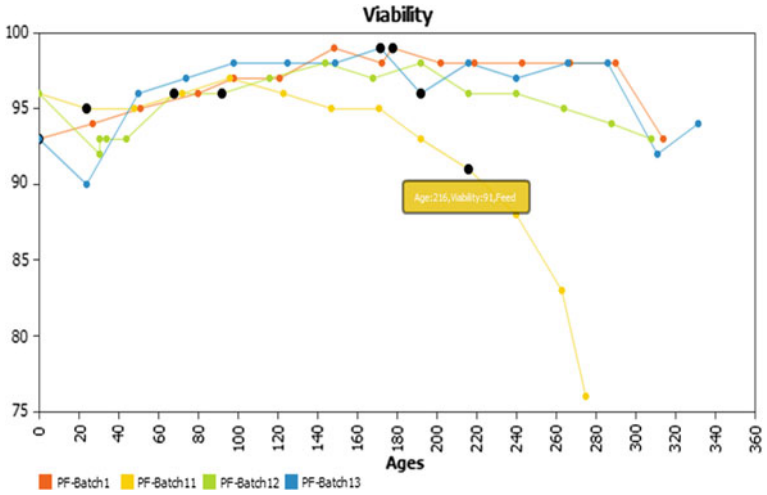

Fig. 8 Chart comparing the profile of the same parameter for different batches

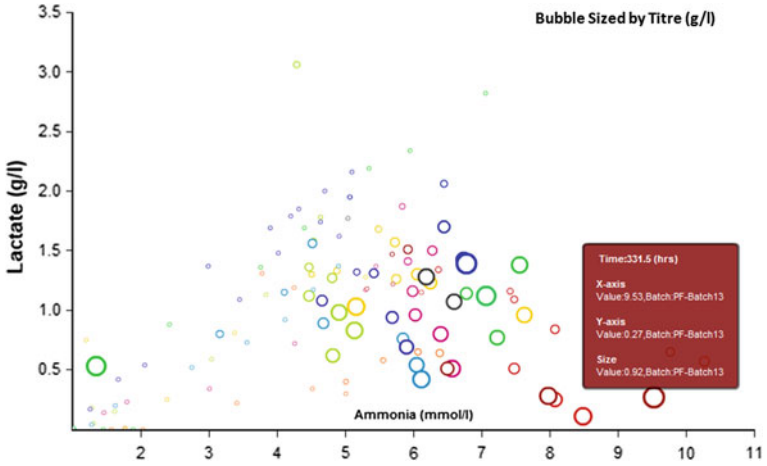

Fig. 9 Scatter plot for three time-series parameters: lactate concentration, ammonia concentration and titre values, represented by the *Y*-axis, *X*-axis, and bubble size, respectively. For each batch, there are a series of values for all three parameters according to culture age. Different batches are represented in different *colours*

decreases. This is indicative of lactate consumption by the cells. The ammonia concentration, however, continues to increase in the culture broth with time.

A *parallel coordinates* chart is a useful way of presenting data that involve multiple parameters and where the aim is to study the effect of those parameters on the final process output (e.g. yield, recovery etc.). In this chart, each parameter is represented on the horizontal axis and a vertical reference line is drawn for each

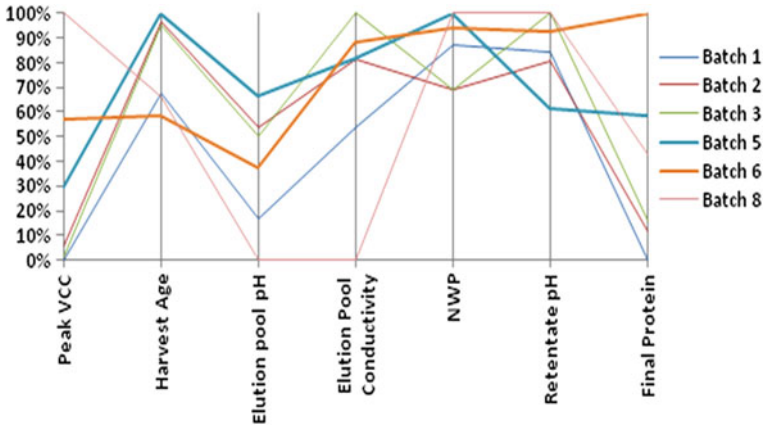

Fig. 10 Parallel coordinates chart of various parameters for different batches on a scale of 0–100 %, where 0 % denotes the minimum value and 100 % denotes the maximum historical value of each parameter

parameter to indicate its scale. One batch is taken at a time, and all the parameters of interest are plotted for that batch on the vertical axis. All the points for that batch on these vertical reference lines are then connected with a line. Multiple batches can be similarly plotted on the chart, with each line representing one batch. In this way, each line represents the profile of one batch and can be compared with the profiles of other batches. Thus, this allows comparison of different batches across various dimensions (parameters), which helps to detect patterns, correlations or exceptional profiles.

Figure 10 shows a parallel coordinate chart for multiple parameters relating to different stages of the process. As the parameter scales are different, the scales have been normalized, with 0 % representing the minimum value of a parameter and 100 % representing its maximum value for a given dataset. The highlighted lines represent the batches with exceptional profiles (batch 5 and batch 6 in this example).

A *treemap* is used mainly to visualize large datasets. It provides a useful means of displaying data with hierarchical structure. It represents the hierarchy in the dataset by forming a hierarchical structure of rectangles inside rectangles. The rectangles at the lowest level mainly represent the metrics that need to be tracked. The size and colour of the rectangles can be used to represent various dimensions of data.

Figure 11 shows a representation of the step recovery for various batches belonging to different unit operations of the process. The first level of the hierarchy consists of four rectangles, each representing a different unit operation. The second level of the hierarchy consists of 14 rectangles inside each unit operation, representing the executed batches of that particular unit operation. The colour of these rectangles represents the parameter " % step recovery" for that batch and unit operation. This can provide a useful visualization to quickly answer questions such as:

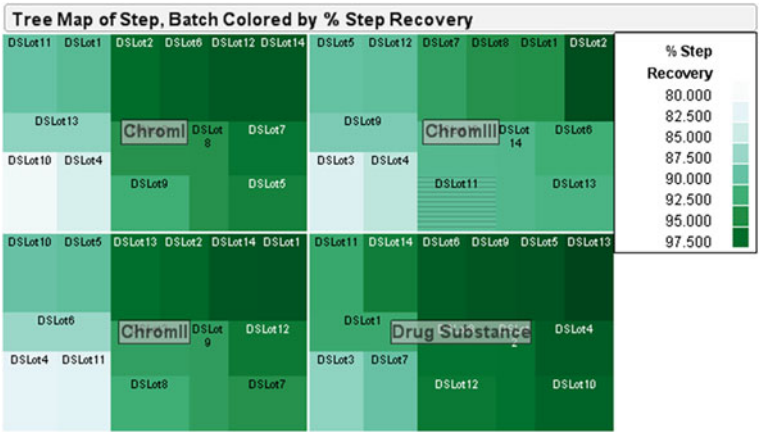

Fig. 11 Treemap of batches for four steps (Drug Substance, ChromI, ChromII and ChromIII) of a process, coloured according to the "% step recovery" value obtained in those batches; *darker colour* indicates higher recovery

- Which were the batches where the step recovery was highest?
- Which were the batches that had low step recovery?
- Which unit operation had highest step recovery?

On the one hand such quick answers to these questions can provide employees with faster process knowledge, while at the same time they serve as a powerful tool to trigger root cause investigations and process improvement initiatives by representing the process in a concise and easy-to-understand format.

Many types of view can be generated for a process based on a treemap; another example is presented in Fig. 12. This view can help a process engineer/scientist to visually compare all important parameters for a single batch against its respective specification limits, providing a quick snapshot of all the parameters that are approaching specification limits. In this example, each step is represented by larger rectangles, within which the parameters for that step are represented by smaller rectangles. Since the values of parameters differ in scale, these have been normalized based on the specification limits for the parameter, where 0 % denotes the lower limit and 100 % represents the upper limit. The colour shade of the smaller rectangles (parameters) is scaled according to the value obtained after this normalization, where parameters approaching upper limits are coloured red and those approaching lower limits are coloured green.

2.2.2 Statistics-Based Monitoring

The next level of process monitoring involves various tools and charts that are based on principles of statistics and need a specialised skill-set and expertise to understand and infer useful conclusions. Most of these apply statistical process

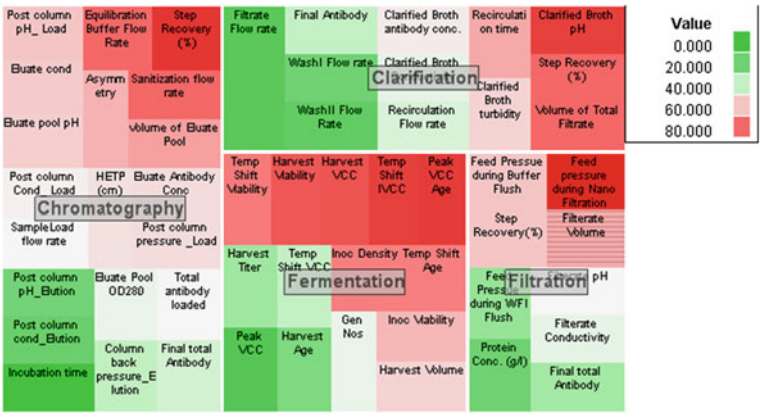

Fig. 12 Treemap representing parameter values of four steps of a process for a single batch. Each *coloured rectangle* represents a parameter. The colour is scaled from *red* to *green*, where *red* indicates that the parameter value is approaching the upper alert limit and *green* indicates that the value is approaching the lower alert limit

control and the multivariate analysis approach to understand the bioprocess variability and relationships between different parameters. In basic visual monitoring tools (described in the previous subsection) the focus is easier detection of unusual trends and patterns in the data to enable troubleshooting, whereas the focus for statistical monitoring tools is to provide a quantitative measure of the inherent variability in the process, assess the current state of control and enable process improvement.

The various tools that are used for statistical monitoring are:

- Univariate control charts.
- Multivariate control charts.

Among such statistical monitoring tools, *univariate control charts* are the most basic and widely used tool for monitoring process performance. These charts are used to monitor whether or not the process is in control (where the sources of variation are common). Analysis of such charts can provide an indication regarding when a particular cause occurred (if any) resulting in the process going out of control.

Univariate control charts plot a single parameter in one display where the *Y*-axis represents the value of the parameter for the corresponding batches on the *X*-axis. The data points for all the batches are then connected with a line that shows the trend of the values for that parameter. A centreline is drawn based on the average of the parameter values. Two lines (one above and one below the average line) represent the statistical limits of the process that are calculated based on ± 3 standard deviations (SDs). The statistical limits are the bounds for the parameter, where a value inside this region signifies that the variability in the process is due to inherent variability of the process (i.e. the process is in control)

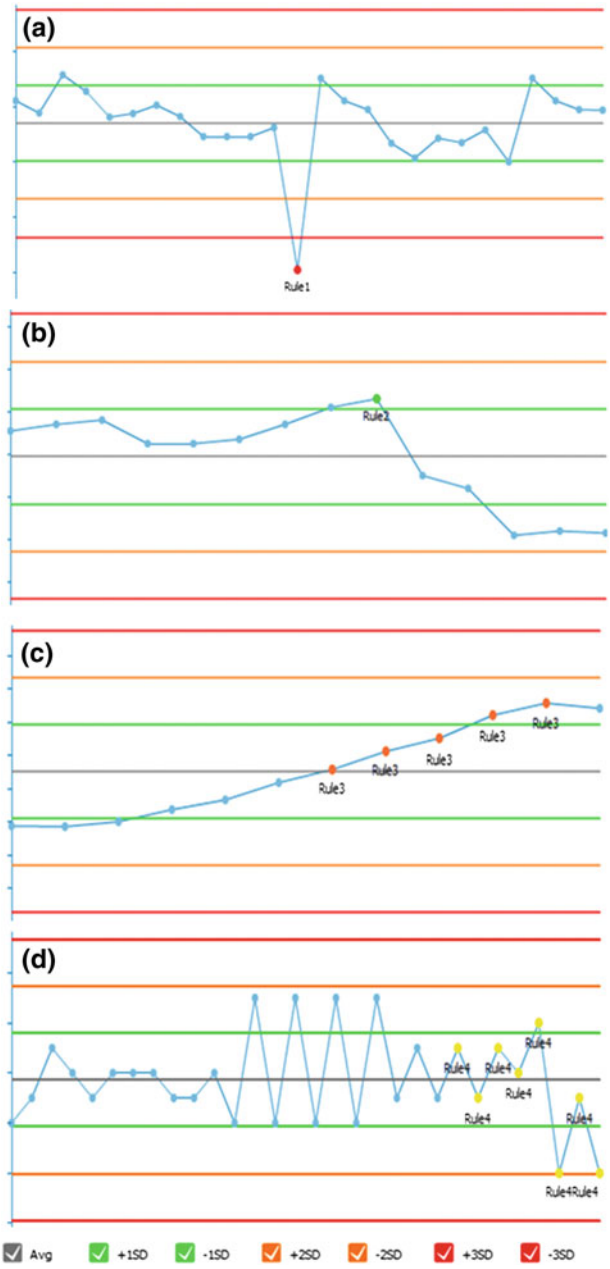

Fig. 13 Control chart showing the first four violations of the Nelson rules: **a** Nelson rule 1: one point is more than 3 SDs from the mean, **b** Nelson rule 2: nine (or more) points in a row are on the same side of the mean, **c** Nelson rule 3: six or more points in a row are continually increasing or decreasing, **d** Nelson rule 4: fourteen or more pints in a row alternate in direction

and there are no special causes involved. A value lying outside this region signifies the presence of special causes for the resulting process variability, making the corresponding batch an outlier among the rest of the batches. There are various rules, such as the Nelson rules and Western Electric rules, that when applied to the control chart give an indication about the trends and systematic patterns that can ultimately lead to an out-of-control situation. This makes the control chart an important aid in preventing loss of future batches caused by some special cause of variation.

Figure 13 shows a control chart. There is one horizontal line each for the average, ±1SD, ±2SD, and ±3SD. The various data points that violate the Nelson rules are marked according to the rule that they violate.

Though univariate Statistical Process Control (SPC) charts are easy to generate and interpret, they are susceptible to giving false positives if the monitored variables are highly interrelated. For this reason, the bioprocessing industry is rapidly adopting more sophisticated tools such as multivariate statistics for deeper insights into the process. These tools, however, require advanced algorithms for data preprocessing and modelling [4, 13, 20]. This has enabled analysis of multidimensional data in an efficient way in the form of various multivariate control charts.

Multivariate control charts are used to monitor several related parameters at the same time. In univariate control charts, the focus is on one parameter at a time, whereas multivariate control charts take into account the fact that most of these parameters are related to each other and therefore it is essential to investigate these interactions and correlations to benchmark the process performance. These charts combine the principles of multivariate analysis and statistical process control to trend and analyse the process data. Most of these are based on principal component analysis (PCA) and partial least squares (PLS) techniques. These are projection methods that project the data into lower-dimensional spaces to facilitate analysis. PCA is mainly used to reduce the number of variables that require monitoring by combining the variables to generate new variables called principal components that are fewer in number than the original variables and are less correlated compared with the original variables. In this way, it focuses on deriving a model based on the data where the variance between the projection coordinates is maximum. PLS, on the other hand, analyses the covariance between a set of variables with the focus on finding the directions that represent variation in process variables as well as how such variation in process variables affects quality variables.

Most of these methods operate by first constructing a process model based on historical data. Subsequent batches are then studied based on that model to detect shifts and deviations in the process. There are several charts based on these principles that can be used for multivariate process monitoring:

- T^2 *control charts* based on Hotelling's statistics are used to detect shifts and deviations of principal components from the normal behaviour defined by the model;
- *Score plots* are used to detect out-of-control behaviour of the latent variables from the control limits defined by the reference data;

- *Squared prediction error (SPE) charts* detect deviations from normal behaviour that are not defined by the model. These are based on the error between raw data and the PCA model fitted to those data;
- *Contribution plots* provide an indication about the process variables that are responsible for a particular deviation;
- *Loading plots* represent the relative contribution of each process variable towards a particular principal component;
- *Variable importance for the projection (VIP) plots* provide a method to quantify the relative importance of various variables used in the PLS model;
- *Batch control charts* are used to identify the time point at which the process starts deviating from the normal behaviour on the basis of the ±3SD limits.

These charts are used in combination to detect variation and for predictive modelling of the process. A combination of Hottelings T^2 charts and contribution plots provides a powerful method for fault detection and diagnosis, where the T^2 chart provides an indication of the shift and the contribution plots are then used to determine the cause of the deviation [14]. These charts also prove useful for real-time monitoring of bioprocesses. This is illustrated by two case studies, where this approach could save future batches by identification of equipment issues related to lower viability in a particular bioreactor and probe failure in a chromatography column as the cause of the out-of-control trend [23]. Score plots have been used as a preliminary aid in identifying abnormal batches based on a model developed from historical batches that can take into account multiple input parameters [23]. These plots also act as a fingerprint for the process and help to identify batch evolution and progression trends [10].

Over the years, the term "process monitoring" has evolved from being not just a basic review of process trends and has come to include various types of advanced statistics. The applications of "process monitoring" are not limited to fault detection and diagnosis but have proliferated into real-time monitoring, feedback and control as well. However, the success of these advanced tools requires instant access to process information, which mandates efficient process knowledge management as a prerequisite.

3 Knowledge Management and Process Monitoring in the Quality by Design Paradigm

3.1 Quality by Design

Quality by design (QbD) is defined in the ICH Q8 guideline as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management" [18].The publication of the Food and Drug Administration's (FDA) *PAT—A Framework for Innovative Pharmaceutical Manufacturing*

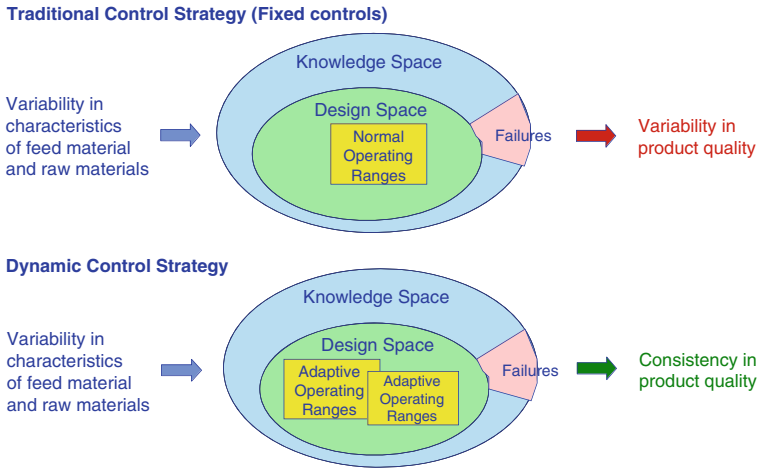

Fig. 14 Illustration of different control strategy approaches for a pharmaceutical process. Adapted from Rathore [17]

and Quality Assurance initiated an effort that eventually evolved into QbD [15]. The underlying principles of science and risk-based process and product development and commercialization are also reflected in the contents of the International Conference on Harmonization (ICH) guidelines: ICH Q8 *Pharmaceutical Development*, ICH Q9 *Quality Risk Management* and ICH Q10 *Pharmaceutical Quality System* (ICH Guideline Q8, Q9, Q10) [7, 8, 9]. The recently issued Guidance on Process Validation from the US Food and Drug Administration (US FDA) also imbibes these principles [5]. The last 5 years have seen QbD gaining widespread adoption in the pharmaceutical industry, with several publications attempting to elucidate a path forward for implementation of QbD and resolving the various issues that otherwise serve as detriments to successful implementation [12, 17, 18].

In the traditional approach to pharmaceutical production (Fig. 14), manufacturers define a process and run it consistently such that the process parameters are controlled within a narrow range to maintain consistent product quality. This has been the approach adopted for the vast majority of pharmaceutical products on the market today. The major downside of this approach is that, since the process controls are fixed, variability in raw materials and process manifests as variability in product quality and sometimes results in lot failures. Since variability is not eliminated at any step, it accumulates as the process advances from one step to the next, with the final product quality varying significantly. In contrast, in the QbD paradigm, the control strategy is dynamic and allows the process to be run differently to deal with incoming variability in characteristics of feed material and raw materials. The net result of this is that the incoming variability is either reduced or eliminated and the resulting product quality is very consistent. This is best shown by the data presented in a recent publication [19]. As seen in Table 1, when a process analytical technology (PAT)-based control scheme was used for

Table 1 Data demonstrating the effectiveness of a process analytical technology-based control scheme in eliminating process variability. Adapted from Rathore et al. [19]

Run	Load purity (%)	PAT pooling	
		Pool purity (%)	Yield (%)
1	62.8	91.6	81.9
2	72.2	91.1	83.8
3	81.6	90.2	87.8

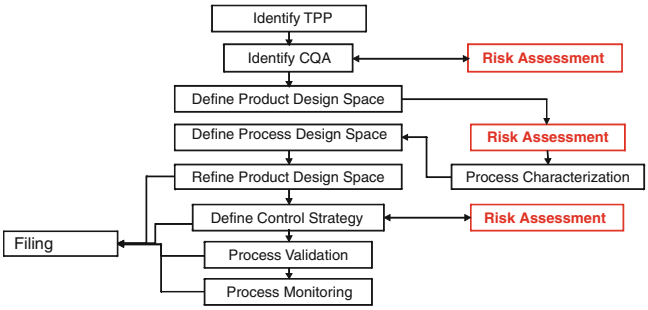

Fig. 15 Steps that need to be taken for implementation of QbD for pharmaceutical product development. Adapted from Rathore and Winkle [18] and Rathore [17]

pooling of a process chromatography column, up to 20 % variability in the quality of the incoming feed material results in less than 1 % variability in the product quality of the resulting pool. If the same occurs at every step of the process, variability can systematically be reduced or eliminated and the quality of the final product will be very consistent.

Figure 15 illustrates the roadmap for QbD implementation and shows the key steps that need to be taken for successful implementation of QbD for a pharmaceutical product [17, 18]. Key steps are: identification of the product attributes that are of significant importance to the product's safety and/or efficacy [target product profile and critical quality attributes (CQA)]; design of the process to deliver these attributes; a robust control strategy to ensure consistent process performance; validation and filing of the process, demonstrating the effectiveness of the control strategy; and finally ongoing monitoring to ensure robust process performance over the lifecycle of the product. Furthermore, risk assessment and management, raw material management, use of statistical approaches and process analytical technology (PAT) provide a foundation for these activities.

3.2 Knowledge Management and Process Monitoring in the QbD Paradigm

3.2.1 Knowledge Management

The role of quality control in traditional pharmaceutical manufacturing was limited to doing end product testing. Most such analysis was performed retrospectively, after the product had already been manufactured, thus leaving the manufacturer the choices of either accepting or rejecting a product lot. Lot rejection results in an increase in the cost of manufacturing the product. In the QbD/PAT paradigm, there is an emphasis of putting in place a more holistic approach for determination of product quality and establishment of process control to ensure consistent product quality.

While the need for efficient knowledge management in a complex and knowledge-intensive industry such as the pharmaceutical industry was always there, the need for efficiency and thereby the cost of inefficiency of managing knowledge within a company have become critical in the QbD/PAT paradigm. At present, knowledge transfer within companies is somewhat limited across the different departments, namely clinical, commercial, quality and regulatory. A lot of information is documented in the form of technical reports which may not even be accessible outside the function that created them. Even if such reports are accessible, raw data may not be easy to obtain. This is especially true if the author of the technical report is no longer an employee of the company. It is clear that companies that are successful in the future will be characterized by efficient knowledge management networks that ensure that the right knowledge reaches the right person at the right time.

The following are some salient points of knowledge management in the QbD paradigm:

1. Companies are spending a lot more effort upfront to meet the higher expectations from QbD activities such as identification of CQA and of critical process parameters (CPP). With the known complexity of pharmaceutical products, budgetary and timeline constraints require a company to leverage knowledge from multiple sources. These include:
   (a) Prior experience in process and product development that the company has in developing the product in the earlier stages of product development (discovery, toxicity etc.).
   (b) Prior experience in process and product development that the company may have in developing similar kinds of products (monoclonal antibody, globular protein etc.), the clinical indication and the mechanism of action of the product.
   (c) Published literature from other companies that may be working on similar products and clinical indications

A. Collect and Archive	B. Routinely Monitor	C. PAT Control - Gas Transfer	C. PAT Control - Cell count
N2	pCO2	pCO2	pH
RPM	pO2	pO2	Temperature
Air FLow	pH	RPM	Cell Count
O2 Flow	Temperature	Air Flow	RPM
CO2 Flow	Integrated Viable Cell Density		Air Flow
Feed Age	Titer		Feed flow rate
Temperature shift Age	Cell Count		
Feed Quantity	%Viability		
Generation No.	pH(offline)		
Hold Times	Glucose		
Inoculation Density	Lactate		
RVLP	Osmolality		
Mycoplasma	RPM		
Glutamate	Air Flow		
Glutamine	Feed flow rate		
Ammonia			
HCO3			
Galactose			
Sodium			
Potassium			
Media Quantity			
Media Blend Ratio			
Harvest Age			
Harvest Volume			

Fig. 16 Outcome of risk analysis for prioritization of various input and output parameters that are typically encountered when performing mammalian cell culture processes. Adapted from Bansal et al. [2] with copyright permission from Advanstar Communications

2. The lag between the time when the manufacturing process is designed and the time when routine manufacturing of the product is started in the commercial facility can be anywhere from 5 to 10 years. A deviation at the time of manufacturing may result in non-conformance, which in turn may require an investigation to find the root cause and take the necessary preventive and/or corrective action. If the data created during the development of the process are available after such a gap of time, they can be used for closure of the non-conformance. Otherwise, as is typical in the pharmaceutical industry today, data need to be recreated so that the necessary investigation can be completed, resulting in spending resources for recreating process data that already exist in the company. Further, release of the lot will be delayed until such data have been created and the investigation has been completed.
3. The significant advancements in analytical/bioanalytical chemistry with respect to development and implementation of new tools, combined with a similar increase in our ability to collect and store data, have resulted in a very significant increase in the amount of data that is routinely generated in a pharmaceutical company. This has, however, resulted in a challenge with respect to timely analysis of the data as well as the sharing of the results of the analysis with the broader organization. This issue has been dealt with in a recent publication, and the following recommendations were made [2]:

   (a) We need to apply risk management principles towards data analysis. It is not optimal to collect and analyse all available information with uniform rigor. For all parameters, a company may continue to record all data on

paper (such as batch records) or in electronic format. However, more sophisticated forms of data archiving, visualization and analysis may be reserved for parameters that have been prioritized. An example of such an approach is illustrated in Fig. 16.

(b) Create domain-specific visualizations that can help to monitor processes and serve as a standard way of communication of process data across the organization. Such visualizations will represent the relationship between different types of data (e.g. batch performance data, time profiles, batch events etc.) and would be accessible to all who are associated with the process and the product.

(c) Perform advanced data capture and statistical analysis to gain deeper insights into the process for chosen parameters (Fig. 16). These advanced tools should be used (or piloted) by a smaller subset of the organization.

3.2.2 Process Monitoring

As defined earlier, process monitoring involves collection of process data and statistical evaluation of parameters to serve multiple purposes ranging from demonstration of process control to identification of opportunities for process improvements. In the USA, this is a requirement in the Code of Federal Regulations (21CFR Part 211) specifying "application of suitable statistical procedures where appropriate", with in-process specifications "derived from previous acceptable process average and process variability estimates" [16].

In the QbD paradigm, process monitoring has gradually evolved from just being a retrospective analysis of data for the annual product review to a more dynamic process involving more real-time analysis of data and linkages to the quality systems of the company [11]. The latter include systems related to non-conformances, corrective and preventive actions, lot release as well as other key activities associated with GMP manufacturing of pharmaceuticals. This thinking is also reflected in recently published documents from the different regulatory agencies and the International Conference on Harmonisation (ICH). The ICH Q8 guideline states that "Collection of process monitoring data during the development of the manufacturing process can provide useful information to enhance process understanding." [7]. The PAT guidance further states that "Process monitoring and control strategies are intended to monitor the state of a process and actively manipulate it to maintain a desired state. Strategies should accommodate the attributes of input materials, the ability and reliability of process analyzers to measure critical attributes, and the achievement of process end points to ensure consistent quality of the output materials and the final product." (FDA's PAT Guidance). The ICH Q10 guidance identifies monitoring as a key element of the pharmaceutical quality system and states that "Pharmaceutical companies should plan and execute a system for the monitoring of process performance and product quality to ensure a state of control is maintained. An effective monitoring system provides assurance of the continued capability of

processes and controls to meet product quality and to identify areas for continual improvement." [9].

More recently, the newly issued Draft Guidance on Process Validation by the US FDA makes several references to process monitoring. Some excerpts in this regard are as follows:

1. Section 211.110(a), *Sampling and testing of in-process materials and drug products*, requires that control procedures "… be established to monitor the output and to validate the performance of those manufacturing processes that may be responsible for causing variability in the characteristics of in-process material and the drug product".
2. Special attention to control of the process through operational limits and in-process monitoring is essential (1) where the product attribute is not readily measurable due to limitations of sampling or detectability (e.g., viral clearance or microbial contamination), or (2) when intermediates and products cannot be highly characterized and well-defined quality attributes cannot be identified.
3. In most cases, process qualification (PQ) will have a higher level of sampling, additional testing, and greater scrutiny of process performance. The level of monitoring and testing should be sufficient to confirm uniform product quality throughout the batch during processing. This greater scrutiny accompanied by a higher level of sampling should continue through the process verification stage, as appropriate.
4. We recommend continued monitoring and/or sampling at the level established during the PQ stage until sufficient data is available to generate significant variability estimates. Once the variability is known, sampling and/or monitoring should be adjusted to a statistically appropriate and representative level. Process variability should be periodically assessed and sampling and/or monitoring adjusted accordingly.
5. Maintenance of the facility, utilities, and equipment is another important aspect of ensuring that a process remains in control. Once established, qualification status must be maintained through routine monitoring, maintenance, and calibration procedures and schedules (21 CFR part 211, subparts C and D).
6. Process flow diagrams should describe each unit operation, its placement in the overall process, monitoring and control points, and the component, as well as other processing material inputs (e.g., processing aids) and expected outputs (i.e., in-process materials and finished 611 product).

It is clear from the above references that both the industry and regulators are opting for implementation of a holistic approach where process characterization, process validation and process monitoring are closely intertwined, being capable of initiating one another as needed. The future of process monitoring lies in combined use of powerful analytical tools capable of supporting real-time decision making and sophisticated statistical tools that can analyse complex datasets in an efficient and effective manner [3]. Several examples in the literature demonstrate the benefits of such monitoring schemes [10, 21, 22, 24].

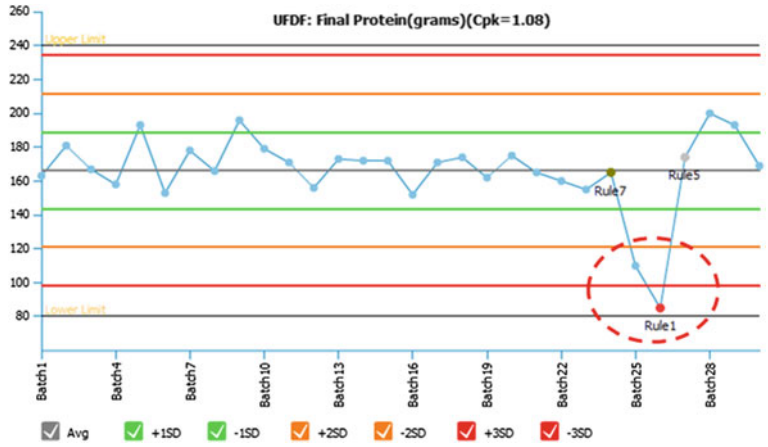

Fig. 17 Statistical process control chart for a downstream process parameter

4 Case Studies

Any cGMP campaign involves resolution of deviations and non-conformances that occur during process execution. Resolution of these process issues requires a detailed root cause investigation so that effective corrective actions can be taken to prevent future occurrences. A typical root cause investigation starts with answering some of the following questions:

- What was the observation?
- At what stage (process step) was it observed in the process?
- Was any other abnormal behaviour observed for this process step?
- Was everything OK with raw materials or intermediate products that feed this process step?
- Does a similar trend or observation exist in earlier steps that feed the current process step?

An efficient process monitoring and knowledge management program (as discussed in earlier sections) will equip the organization with the information required to answer these questions. The cases discussed next detail the investigative procedure usually followed for a root cause investigation or process improvements.

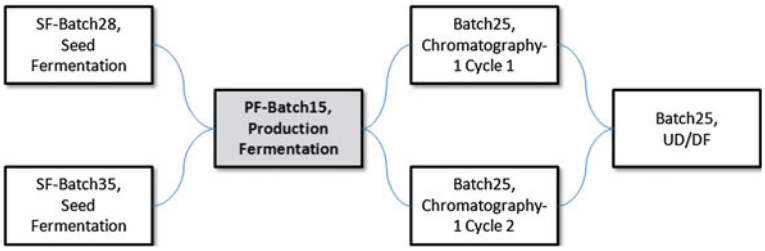

Fig. 18 Lot genealogy for batch 25 of the UF/DF step

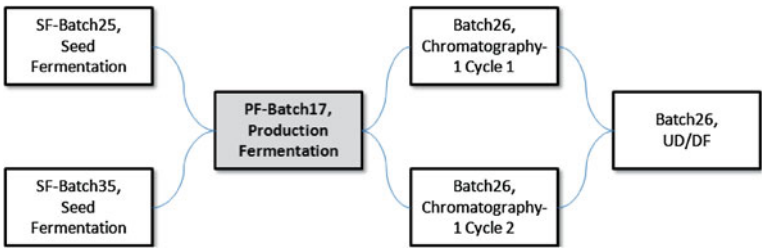

Fig. 19 Lot genealogy for batch 26 of the UF/DF step

4.1 Case Study 1: Root Cause Investigation

4.1.1 Problem Statement

Company A has a process monitoring program in place that mandates it to perform periodic process reviews. During one such review it was observed on a run control chart that the final protein yield for the Ultrafiltration/ Diafiltration (UF/DF) step for two batches (batch 25 and batch 26) was unusually lower than for other batches, as shown in Fig. 17. Since these batches met all batch release criteria, these observations went unnoticed during regular batch release activity.

4.1.2 Investigation

Tracing the lot genealogy (Figs. 18 and 19) for batches 25 and 26, the related batches that fed these outlier batches in the UF/DF step were investigated.

Trends for various parameters in these steps involving these related batches were investigated, and it was observed that PF-Batch 15 and PF-Batch 17 at the fermentation step had abnormal titre at harvest (Fig. 20) and slower cell growth profiles (Fig. 21) compared with other batches.

Going back to the lot genealogy (Figs. 18 and 19), it was observed that a common batch SF-Batch 35 (at the seed fermentation stage) fed both the abnormal

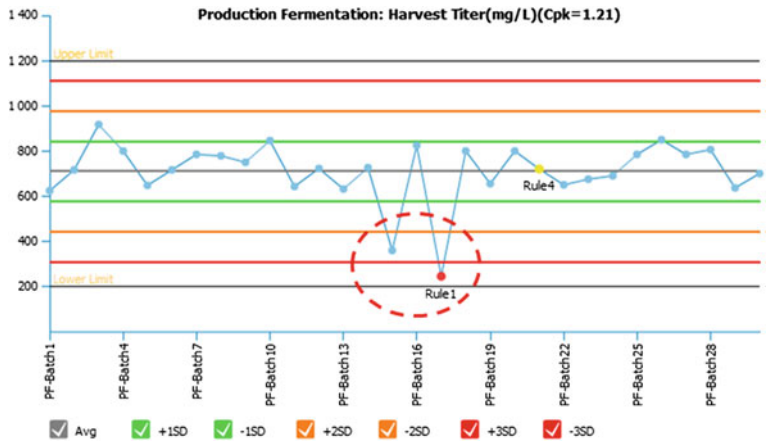

Fig. 20 Statistical process control chart for a cell culture process parameter

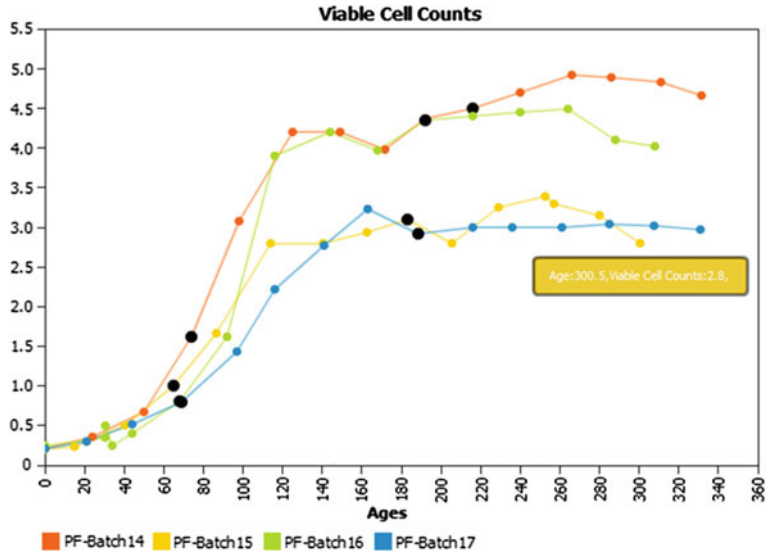

Fig. 21 Viable cell density profiles for various batches for a cell culture process

fermentation batches. Scanning through various process parameters for the seed fermentation step, it was found that the SF-Batch 35 transfer age was unusually higher than for other batches, as shown in Fig. 22. A review of the batch record of SF-Batch 35 indicated that the root cause was delay due to scheduling issues that caused the delay in transfer by 1 day.

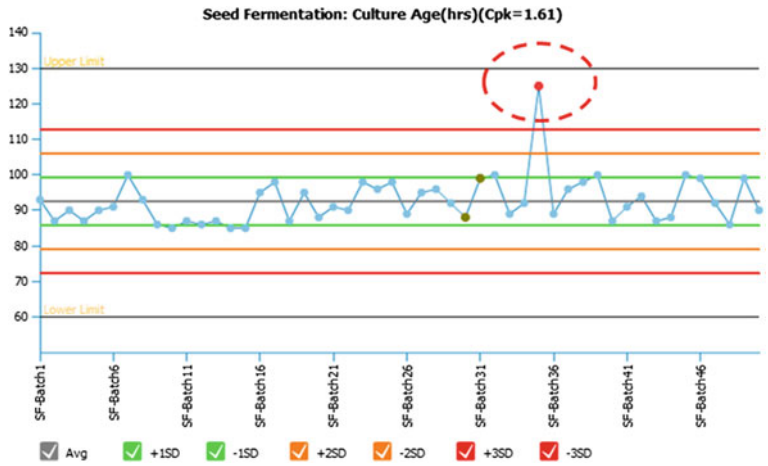

Fig. 22 Statistical process control chart for a seed culture process parameter

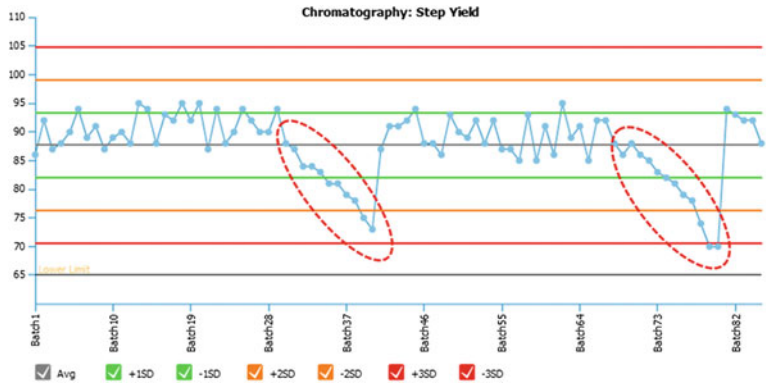

Fig. 23 Statistical process control chart of % step yield of a chromatography process

4.2 Case Study 2: Process Improvement

During another process review, a cyclic trend of gradual decrease in step yields after 30 batches was observed for a chromatography step in the process (Fig. 23). As shown in the control chart (Fig. 23), yields returned to normal after every 40 batches.

Plotting the step yields against the number of column cycles (Fig. 24) clearly differentiated the batches processed with the same column pack up to 30 cycles from those processed after 30 cycles. Also, the host cell DNA (HCD) was plotted using the marker size, clearly indicating a loss in column performance for columns used after 30 cycles, as indicated by a loss in yield and increase in output HCD

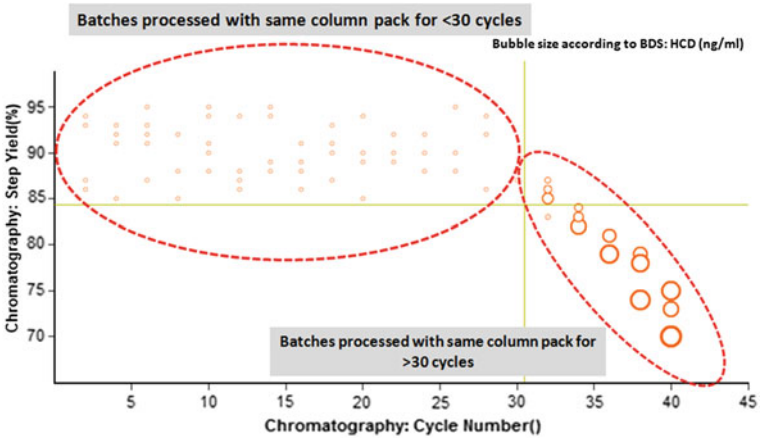

Fig. 24 Bubble chart showing the relationship between cycle number and % step yield of a chromatography step (*X*- and *Y*-axes) compared with HCD concentration (marker size) at BDS step

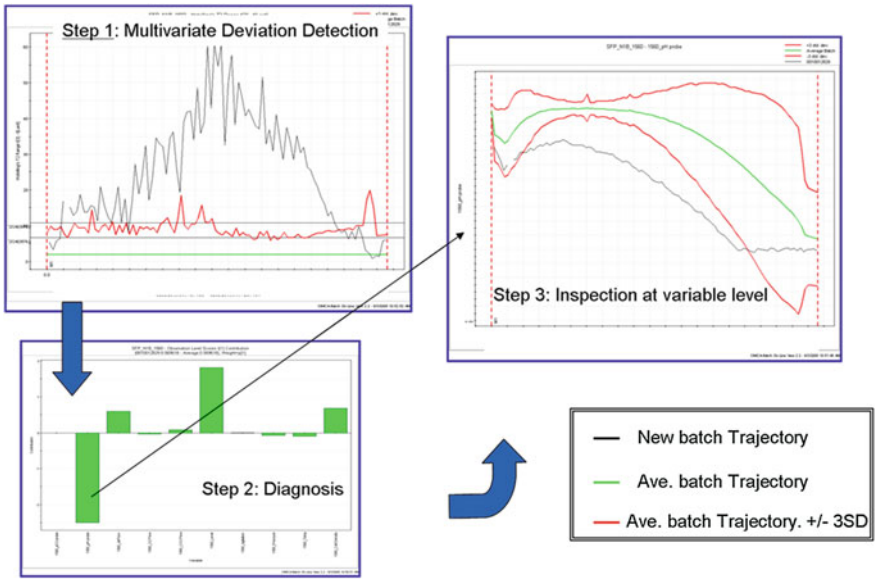

Fig. 25 Real-time multivariate monitoring of a seed bioreactor (detection, diagnosis and inspection steps). Published with copyright permission from Advanstar Communications from Konold et al. [11]

(increase in marker size). On the basis of this analysis, a process change was initiated to repack the column every 30 cycles, replacing the current practice of packing after every 40 cycles.

4.3 Case Study 3: Real-Time Process Monitoring

As an example of real-time multivariate statistical process monitoring (RT-MSPM), we present a case study involving development of a PCA-based model for monitoring of a mammalian cell culture bioreactor at commercial scale [11]. Data were collected during operation of a seed bioreactor train in a manufacturing plant. The dataset consisted of 11 process variables that were measured online for 30 batches. The resulting model was able to explain overall process variability with three principal components. New production batches were then monitored against this model in real time. The outcome of the analysis is illustrated in Fig. 25. In step 1, a T^2 chart was used to detect a deviation. Step 2 involved diagnosis at the variable level, which indicated that the pH probe is reading less than historical averages, i.e. outside of ± 3 SDs. Finally, in step 3, inspection of the pH trace was performed. This allowed scientists and engineers to start troubleshooting the probe and other operational factors to better understand and monitor the process via this simple three-step process.

5 Conclusion

As the pharmaceutical industry implements QbD principles towards process and product development and commercialization, it is critical that pharmaceutical companies have an efficient and effective approach towards knowledge management and process monitoring. The combination of data visualization and sophisticated statistical techniques, such as those discussed in this chapter, and advanced analytical tools will facilitate efficient process monitoring. This in turn will result in increased consistency of product quality as well as efficiency in manufacturing of pharmaceutical products and bring us closer to full implementation of QbD and realizing its benefits.

References

1. Awad EM, Ghaziri HM (2007) Understanding knowledge. Knowledge management, 1st edn. Pearson Education, India, pp 60–65
2. Bansal A, Hans J, Rathore AS (2011) Operation excellence: more data or smarter approach? BioPharm Int 24(6):36–41
3. Cinar A, Parulekar SJ, Undey C, Birol G (2003) Batch fermentation: modeling, monitoring and control. CRC, New York
4. Gunther JC, Conner JS, Seborg DE (2009) Process monitoring and quality variable prediction utilizing PLS in industrial fed-batch cell culture. J Process Control 19:914–921
5. Guidance for Industry, Process Validation: General Principles and Practices (2011) US Department of Health and Human Services, Food and Drug Administration, Revision 1

6. Hans J, Bansal A (2009) Biopharmaceutical information infrastructure 2.0. BioProcess Int 7(11):10–19
7. ICH Harmonised Tripartite Guideline: Q8(R1) Pharmaceutical Development (2008) http://www.ich.org/LOB/media/MEDIA4986.pdf
8. ICH Harmonised Tripartite Guideline: Q9 Quality Risk Management (2005) http://www.ich.org/LOB/media/MEDIA1957.pdf
9. ICH Harmonised Tripartite Guideline: Q10 Pharmaceutical Quality System (2008) http://www.ich.org/LOB/media/MEDIA3917.pdf
10. Kirdar AO, Connor JS, Baclaski J, Rathore AS (2007) Application of multivariate analysis toward biotech processes: case study of a cell-culture unit operation. Biotechnol Prog 23:61–67
11. Konold P, Woolfenden R II, Undey C, Rathore AS (2009) Monitoring of biopharmaceutical processes: present and future approaches. BioPharm Int 22(5):26–39
12. Kozlowski S, Swann P (2009) Considerations for biotechnology product quality by design. In: Rathore AS, Mhatre R (ed) Quality by design for biopharmaceuticals: perspectives and case studies. Wiley, New Jersey, pp 9–30
13. Martin EB, Morris AJ (2002) Enhanced bio-manufacturing through advanced multivariate statistical technologies. J Biotechnol 99:223–235
14. Nucci ER, Cruz AJG, Giordano RC (2010) Monitoring bioreactors using principal component analysis: production of penicillin G acylase as a case study. Bioprocess Biosyst Eng 33: 557–564. doi:10.1007/s00449-009-0377-y
15. PAT Guidance for Industry—A Framework for Innovative Pharmaceutical Development, Manufacturing and Quality Assurance (2004) US Department of Health and Human Services, Food and Drug Administration (FDA), Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), Office of Regulatory Affairs (ORA)
16. Part 211 Current Good Manufacturing Practice for Finished Pharmaceuticals (2008) Code of Federal Regulations, Title 21, vol 4, US Department of Health and Human Service, Food and Drug Administration (FDA)
17. Rathore AS (2009) A roadmap for implementation of quality by design (QbD) for biotechnology products. Trends Biotechnol 27:546–553
18. Rathore AS, Winkle H (2009) Quality by design for pharmaceuticals: regulatory perspective and approach. Nat Biotechnol 27:26–34
19. Rathore AS, Parr L, Dermawan S, Lawson K, Lu Y (2010) Large scale demonstration of process analytical technology (PAT) in bioprocessing: use of high performance liquid chromatography (HPLC) for making real time pooling decisions for process chromatography. Biotechnol Prog 26:448–457
20. Undey C, Tatara E, Cinar A (2003) Real-time batch process supervision by integrated knowledge-based systems and multivariate statistical methods. Eng Appl Artif Intell 16: 555–566
21. Undey C, Ertunc S, Cinar A (2003) Online batch/fed-batch process performance monitoring, quality prediction and variable contributions analysis for diagnosis. Ind Eng Chem Res 42(20):4645–4658
22. Undey C, Tatara E, Cinar A (2004) Intelligent real-time performance monitoring and quality prediction for batch/fed-batch cultivations. J Biotechnol 108(1):61–77
23. Ündey C, Ertunc S, Mistretta T, Looze B (2010) Applied advanced process analytics in biopharmaceutical manufacturing: Challenges and prospects in real-time monitoring and control. J Process Control 20:1009–1018
24. Yoo CK, Lee IB (2006) Nonlinear multivariate filtering and bioprocess monitoring for supervising nonlinear biological processes. Process Biochem 41:1854–1863

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The Choice of Suitable Online Analytical Techniques and Data Processing for Monitoring of Bioprocesses

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Abstract With increasing pressure from regulatory authorities on industry to develop processes embracing process analytical technology (PAT) initiatives, there is a growing demand to establish reliable tools and systems capable of meeting this need. With regard to monitoring and control of bioprocesses, this need translates to a search for robust instrumentation capable of monitoring the critical process parameters in real time. The application of such technologies at all stages of the process, from the initial R&D phase to process optimisation and production, enhances process understanding and paves the way for the development of control platforms. An examination of the PAT concept and selected tools (NIR, MIR, Raman, dielectric spectroscopy and calorimetry) are presented here. A description of each tool is given, with particular emphasis on the nature of the signal produced and how these relate to measurements of biomass, metabolites and product. A description of the signal processing that is necessary to gain meaningful results from the different tools is also given, together with online data reconciliation techniques based on mass and energy balances. Many techniques such as those based on vibrational spectroscopy are of particular interest, since they are capable of monitoring several critical process parameters which are typically controlled in a bioprocess. A window of application for each of the techniques, when used in the area of bioprocessing, is suggested based on their uses and inherent limitations.

Keywords Bioprocess control · Bioprocess monitoring · Calorimetry · Critical process parameters · Data reconciliation · PAT · Spectroscopy

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Contents

1 Introduction

The last decade has introduced a significant number of changes to the pharmaceutical and biopharmaceutical industries, not least in the areas of quality assurance and regulatory compliance. This new focus has largely been driven by the Food and Drug Administration (FDA). In 2002 the FDA announced a new initiative, "Pharmaceutical cGMPs for the 21st century", the purpose of which was to modernise the regulation of pharmaceutical quality. The initiative supports and promotes the use of risk-based and science-based approaches for regulatory decision making, throughout the entire lifecycle of a product [1]. After 2 years in development, the final report outlines the envisioned direction in which the pharmaceutical and biopharmaceutical industries should be moving, but also provides guidance on how to make the proposed changes and embrace the new concepts put forward.

Central to the implementation of this new system is the use of science and engineering knowledge to establish boundaries and evaluate processes. Previously, a manufacturing process was developed and quality control and assurance tests were then applied to ensure compliance. This new initiative aims to use knowledge to mitigate risk, by reducing process and product variability and applying continuous process improvement. Industry guidelines published by the FDA in May 2006, "Q8 Pharmaceutical Development", state, "quality cannot be tested into the products, it should be built in by design" [2]. Essentially this means that the rigorous testing of the past cannot improve product quality or enhance the process but rather quality should be pivotal throughout the lifecycle of a process and a key factor from the initial stages of development and process design. This introduces the concept of "quality by design" (QbD), whereby a "design space" is established, within which the product quality profile is defined, the critical quality attributes (CQAs) and critical process parameters (CPPs) are identified and the manufacturing process is controlled. Process changes that occur within the design space are acceptable, as the design space would have been subject to regulatory assessment and approval at the time the manufacturing process was filed. However

movement outside the design boundary is considered a change and as such would most likely require regulatory post-approval.

In order to establish a design space that will allow for maximum process flexibility while ensuring all CPPs and CQAs are identified and maintained, a large degree of process understanding is essential. Process analytical technology (PAT) is a "pillar/guiding principle" of the cGMP initiative [1]. The PAT framework published in September 2004 defines process understanding and highlights the tools required to achieve this standard of process knowledge:

> *A process is generally considered well understood when (1) all critical sources of variability are identified and explained; (2) variability is managed by the process; and, (3) product quality attributes can be accurately and reliably predicted over the design space established* [3].

PAT provides in-depth process understanding, but to implement PAT and operate under the principle of quality by design the process must be well understood. Many in the industry have applied these to processes to glean greater process knowledge. However, although PAT is a relatively new concept, it has evolved over the last decade. It has transitioned from being an analysis *in* the process, to supplement quality control, to being an analysis *of* the process [4]. As a result of PAT being embraced by industry, tools that are capable of real-time monitoring and control must be developed. Currently, few developed tools exist and even fewer have actually been implemented in a manufacturing environment.

This chapter explores the use of selected PAT tools which can be used in the context of M^3C in bioprocess applications and looks at the advantages and limitations of each. Calorimetry is examined in terms of its operating principle and signal processing methods. A description of the current state and potential future developments is provided along with a summary of its reported use as a PAT tool. The history of the development of dielectric spectroscopy for bioprocess purposes is then described within the scope of PAT, and a detailed overview of the different applications in the field of process engineering in the production of biological products is provided. Issues relating to correlation and data pre-processing techniques are also discussed as well as the potential industrial applications of dielectric spectroscopy. The final set of PAT analysers considered are those based on vibrational spectroscopy. The theory behind the use of MIR, NIR and Raman spectroscopy for bioprocessing applications is outlined, and the necessity of using multivariate data processing is explained. Reported uses of these techniques for bioprocess monitoring and control applications are summarised, and the current state of the different technologies are compared.

Finally, a synopsis of available control strategies for bioprocesses based on measurements from PAT tools and data analysis is given.

2 Techniques for Bioprocessing Monitoring

2.1 Heat-Flow Biocalorimetry

Bioprocesses are complex systems involving multiple ongoing biochemical reactions at both intra- and extra-cellular levels. According to classical biothermodynamics "All living cells involve heat exchange with their surroundings in order to sustain their cell metabolism". The heat generated during cell metabolism is due to dissipation of excessive internal (Gibb's) energy stored inside the living cell. The amount of heat generated by a living cell is determined by the metabolic activity of the cell itself. Each living cell sustains the balance between the anabolic and catabolic processes by regulating the amount of heat dissipation and, hence, persists as metabolically active under different process conditions [5]. As a result, the measurement of metabolic heat production should provide valuable information on the physiological activity of the organism and may be regarded as a 'metabolic variable' of any bioprocess system. The measurement of metabolic heat has been gaining attention in both industry and academia due to its non-specific, non-invasive and insensitive properties in relation to the process systems to which it is applied [6–8].

2.1.1 Working Principle and Operation

Reaction calorimeters have been especially designed for bioprocess monitoring. This section deals only with advances concerning bench-scale heat-flow biocalorimeters; related fields such as micro-calorimetry are not discussed here. Several reports are available which describe the principle of heat-flow biocalorimetry [9, 10], which is similar to a bench-scale fermenter but with additional calorimetric sensors for metabolic heat flow rate measurement.

2.1.2 Development of Biocalorimetry

'Biocalorimetry' is an old branch of science that dates back to the eighteenth century. It facilitates a quantitative interpretation of metabolic heat generated from living systems as a useful 'process signal' for monitoring purposes [11]. Since the start of the nineteenth century, temperature sensors with improved sensitivity and sophisticated measurement techniques have become available, and these have been deployed by several research groups for metabolic heat measurements, rendering 'biocalorimetry' popular among scientists and academics. Micro-calorimeters were primitive models of biocalorimeters used in the mid-nineteenth century to effectively monitor fermentation processes [12, 13]. Micro-calorimeters accomplished high-sensitivity measurements of heat flow signals; however the technical difficulties associated with the design, viz. pH control, mixing and oxygen supply, remained a hurdle for technical applications [7]. These technical challenges led to

the development of a calorimetric technique that could be readily integrated into a bench-scale bioreactor, known as 'heat-flow calorimetry' [11]. Ciba-Geigy AG (Basel, Switzerland) developed a new bench-scale calorimeter based on the heat flow calorimetric technique, commercialised by Mettler Instruments AG (Switzerland) as a 'reaction calorimeter, RC1' suitable for monitoring chemical reactions [14]. The advent of bench-scale reaction calorimetry in the 1980s paved the way for biocalorimetrists all over world to make advances in calorimetric science [15]. A high-resolution version of the reaction calorimeter suitable for monitoring biochemical reactions was developed by Marison et al. [16], and it caused a paradigm shift in biocalorimetric research and demonstrated the usefulness of heat-flow biocalorimeters in bioprocess monitoring. Further studies illustrated that heat-flow biocalorimeters are high-performing (bio)reactors suitable for all kinds of bioprocess applications, viz. cultivation of different cell lines, quantitative studies including process monitoring and control [17] and biothermodyanamic studies [5]. Due to their versatile nature, heat-flow biocalorimeters are widely employed nowadays for bioprocess monitoring and control applications. Moreover, the decreasing surface-to-volume ratio in large-scale bioreactors results in a good-quality heat flow signal. Hence huge scope exists for deploying heat-flow calorimetry in industrial-scale bioreactors in the near future. Though several research works employing bench-scale biocalorimeters have been reported, their real potential is yet to be realised in the industrial biotechnology sector.

2.1.3 High-Resolution Heat Flow Rate Signal

The successful application of calorimetric techniques to bioprocess monitoring is inherently related to the sensitivity of the instrument and the signal noise [16]. García-Payo et al. improved the resolution to 4–12 mW L^{-1}, and this is regarded as a significant milestone in heat-flow biocalorimetric research [18], paving the way for employing heat-flow calorimetry to monitor weakly exothermic reactions, viz. anaerobic and animal cell growth processes. However, long-term signal noise during calorimetric measurements is still an issue. Long-term noise disturbances in measured calorimetric signals are attributed to ambient temperature fluctuations and could be minimised by effectively thermostatting both the reactor housing and its head plate. Such signal noise becomes less important with large-scale reactors since the heat balances around the reactor would compensate for these.

2.1.4 Scope for Bench- and Large-Scale Biocalorimetry in General as a PAT Process Analyser

Direct measurement of the metabolic activity of organisms is desirable, and this may be achieved by quantitative measurement of metabolic activity via heat flow rate measurements. Table 1 chronologically summarises the significant progress achieved in biocalorimetry in real-time monitoring and control applications for a

Table 1 Bioprocess monitoring and control applications of heat-flow biocalorimetry

Bioprocess system	Calorimeter model	Growth metabolism	Sensitivity (mW L^{-1})	Key process parameters (CPPs)[a]	**Reference**
Cultivation in mixed substrate mode	BSC-81	Aerobic	≤100	Onset of diauxic growth	[21]
Batch cultivation of *E. coli*	BSC-81	Aerobic	≤100	Glucose limitation	[8]
Dual limitation of substrates	RC1	Aerobic respiration/ aerobic fermentative	100	Onset of uncoupled respiration	[19]
Controlled fed-batch cultivation	RC1	Aerobic respiration/ fermentative	100	Sustaining oxidative growth metabolism, high productivity using repeated fed-batch addition of limiting N-source	[27–29]
Anaerobic growth of methanogen and UASB sludge	BioRC1	Anaerobic	5–10	Cell growth monitoring, biomass adaptation, onset of aceticlastic methanogenesis	[23, 24, 30]
Investigation of biological activity of WWT system	BioRC1	Aerobic/anaerobic/ anoxic	5–10	Real-time biomass activity, toxicity tolerance and nitrification/ denitrification assessment	[10, 20]
Fed-batch cultivation of *Bacillus spharecius*	BioRC1	Aerobic	>10	Substrate consumption rate and feed control	[11]
Quantitative studies on micro-algal cultivation	BioRC1	Photoautotrophic	5–10	Real-time characterization of autotrophic, heterotrophic and mixotrophic growth phases & estimation of photosynthetic efficiency	[25, 26, 31]
Large-scale calorimetry of *Bacillus* sphaericus	300-L bioreactor	Aerobic	150–500	Monitoring and control of a *Bacillus spaericus* culture	[32]
Large-scale calorimetry	100,000-L bioreactor	Aerobic		Real-time monitoring of a *S. cerevisiae* culture by biocalorimetry at large-scale	[33]

(continued)

Table 1 (continued)

Bioprocess system	Calorimeter model	Growth metabolism	Sensitivity (mW L^{-1})	Key process parameters (CPPs)[a]	**Reference**
Bench-scale Calorimetry of *Halomonas elongata*	2-L bioreactor	Aerobic	50	Real-time monitoring of the critical process parameters by biocalorimetry	[34]
Feedback control	BioRC1	Aerobic	5–8	Specific growth rate	[35, 36]
Feedback control	20-L bioreactor	Aerobic		Specific growth rate control in *S. cerevisiae* and *E. coli* cultures to avoid production of overflow metabolites	[37, 38]

[a] Bioprocess parameter/anomalies monitored based on metabolic heat flow rate profile

variety of bioprocess systems. These findings show the versatility for investigation of almost all types of cell metabolism, viz. aerobic, fermentative, anaerobic, anoxic, photoautotrophic and mixotrophic. Changes in the measured heat flow rate profile were effectively used by several research groups to understand anomalies encountered in a bioprocess system such as diauxic growth, change in physiology of organism, dual substrate limitation and substrate and toxic inhibitions [19–21]. Furthermore, the estimation of heat yield coefficients could confirm the existence of these anomalies, e.g., values of heat yield due to cell growth ($Y_{Q/X}$) and oxy-calorific coefficient ($Y_{Q/O}$) could provide quantitative information on diauxic behaviour and metabolic shifts in an ongoing bioprocess [12]. The development of a high-sensitivity biocalorimetry [22] proved its ability to monitor weakly exothermic biochemical reactions encountered in anaerobic and WWT systems; For instance, Liu et al. first reported the existence of endothermic microbial growth by cultivation of the acetotrophic methanogen *Methanosarcina barkeri* [23]. Also, Daverio et al. successfully monitored acidogenic and methanogenic phases of anaerobic granular sludge originating from an up-flow anaerobic sludge blanket (UASB) digester [24]. Calorimetric investigations in micro-algal cultures, viz. *Chlorella vulgaris* and *Chlorella sorokiniana*, were carried out using an improved 'photobiocalorimeter' [25, 26]. The heat flow measurements were utilised to quantify the stored chemical energy (converted from incident light) inside algal biomass and to estimate photosynthetic efficiency.

Recent studies by the authors proved the robustness of a heat flow signal compared with process signals acquired in parallel to PAT process analysers such as dielectric spectroscopy and exhaust gas analysers [35]. Apart from monitoring, the measured heat flow rate signal can also serve as an input to control the bioprocess in order to improve the product yield, e.g. initiating limiting substrate feed during fed-batch culture and/or the induction phase. This is illustrated in Fig. 1, which represents a typical heat flow rate (power–time) profile during aerobic respiratory growth of *Kluyveromyces marxianus*. Since there is no fermentative by-product formation during respiratory metabolism, the majority of the heat generation is from the cell growth process. This phenomenon can be inferred from Fig. 1, since the measured heat flow rate signal clearly depicts distinct phases of cell growth. The logarithmic growth phase of *K. marxianus* corresponds to an exponential rise in the heat flow rate, while substrate (glucose) limitation leads to a dramatic fall in the heat profile at approximately 8 h after inoculation. This shift in heat profile was used as the signal to start the fed-batch operation in order to maintain the growth trajectory of *K. marxianus* in the exponential mode (Fig. 1). A further improvement has been achieved via feedback control employing heat flow rate measurements and a proposed real-time fed-batch control [35]. A simple estimator was developed for biomass and specific growth rate using heat flow rate measurements, and its reliability was investigated in a fed-batch process in real time [36]. The robustness of feedback control to maintain specific growth rate at a desired set value employing such estimators is shown in Fig. 2. It can be seen that the average tracking error between the controlled and the actual set value (0.21 h^{-1}) of the specific growth rate is 0.03 h^{-1} over a 5-h period. These results

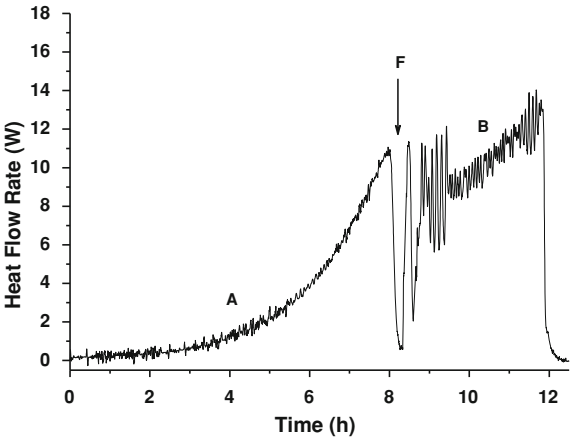

Fig. 1 Heat flow rate profile for pure aerobic respiratory growth of *K. marxianus* cultivated in glucose-limited mineral salt medium. Phases: A—exponential growth, B—fed-batch growth, and F—feed start

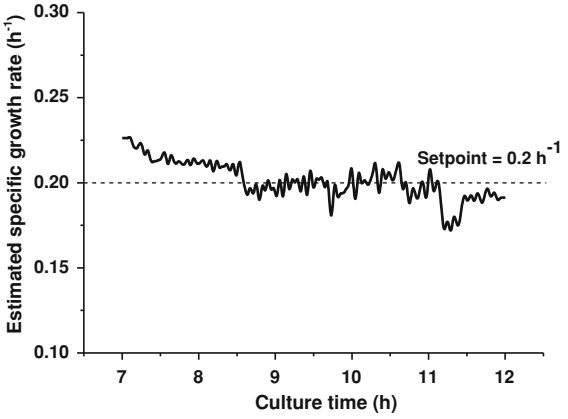

Fig. 2 Feedback control of specific growth rate of *K. marxianus* employing real-time heat flow rate measurements

are encouraging and suggest that heat flow calorimetry may be a potential PAT process analyser in a variety of bioprocess systems.

Very few reports have been published on the use of heat-flow calorimetry using mammalian bioprocess systems. Real-time monitoring and control of mammalian bioprocesses is gaining considerable attention in the biopharmaceutical industry to achieve high yields of monoclonal antibodies (mAbs) and other recombinant proteins. Mammalian cell growth processes are slow reactions and weakly exothermic compared with microbial anaerobic systems [16], and may require modifications to increase instrument sensitivity or to yield a high-resolution signal by minimizing the heat losses to the environment through insulation and ambient temperature control. Signal quality could also be improved by employing robust noise filtering techniques.

2.1.5 Custom Bioreactors—The Future Biocalorimeters?

Recently, conversion of industrial-scale bioreactors into biocalorimeters is attracting interest due to the ease of measurement, the non-invasive nature of the measurement and cost-effectiveness. The move from bench-scale to industrial-scale biocalorimeters results in a decrease in surface area-to-volume ratio. This facilitates heat measurements by reducing the heat transfer surface, resulting in better signal quality [33]. Voisard et al. first converted a pilot-scale (300-L) fermenter into a calorimeter and successfully monitored the growth of *B. sphaericus* [32]. An approach for conversion of bioreactors, irrespective of size, to biocalorimeters by integrating suitable calorimetric measurement principles has been proposed [34, 39]. However, a power compensation technique for heat flow rate measurements was employed, and this is not feasible in industrial-scale bioreactors, which are usually jacketed vessels in which the reactor temperature is controlled using cooling fluid circulating through the jacket. Moreover, the measured heat signal sensitivity was 50 mW L^{-1}, signal stability was of the order of 0.2 mW L^{-1} and the response time was in the range of 1–2 min. Technical-scale calorimetric monitoring demands a high-sensitivity heat flow rate signal (short-term noise <0.003 mW L^{-1}) and dynamic temperature sensors (time constant <10 s). Current progress in temperature sensor instrumentation and data acquisition (DAQ) tools may render the conversion of large-scale bioreactors into high-sensitivity biocalorimeters. Integration of a heat flow biocalorimeter into a PAT platform would provide the end-user with insight into metabolic changes encountered in an ongoing bioprocess and ensure a robust process control leading to high-titre product yield.

2.2 Dielectric Spectroscopy

Dielectric spectroscopy exploits the complex electrical properties of viable cells. Any such complex, passive, electrical system can be defined by two characteristics: capacitance measured in farads (F) and conductance measured in siemens (S). Dielectric spectroscopy can provide information on the total and viable cell volume, since only cells with intact membranes act like capacitors when placed in an electrical field. Obtaining information about the viable cell volume is important, since monitoring the growth of the organism of interest can be crucial to the process, for instance to determine the appropriate time for induction of recombinant protein production. In addition, some interesting products are growth related and may be indirectly monitored using dielectric spectroscopy. The measurement of the evolution of the viable cell volume may identify the specific product formation rate. In order to design control strategies to maintain a particular specific growth rate or act on the product formation rate, it is crucial to make in situ measurements of biomass or bioactivity. As highlighted in Chap. 1, such measurements should be in real time, a feature which is possible with dielectric spectroscopy.

2.2.1 History of the Development of the Technique

Dielectric spectroscopy is based on impedance, as highlighted in Chap. 1, and is used extensively in different fields, such as electronic engineering and chemistry. The first publication relating to biological applications reported the use of radio-frequency impendence to measure viable biomass [40]. Since its origin in the biotechnology area, the technique has gained in importance, particularly since the PAT initiative, as shown in a recent review [41].

2.2.2 Dielectric Spectroscopy in the Scope of the PAT Initiative

Process analysers are an important part of the PAT initiative [42], and they should provide real-time process-related information, if possible of multivariate nature, through non-destructive, non-invasive measurements. Currently, commercially available dielectric spectrometers are built to withstand cleaning–in-place (CIP) and sterilisation-in-place (SIP) while allowing in situ monitoring to provide real-time information through high-frequency measurements. Most available devices are highly customized and include a wide range of approved filtering and data pre-processing techniques. The instruments are versatile and applicable to all types of cells. On the other hand, capacitance measurements show dependence on temperature, pressure, mixing rate, aeration rate, reactor volume, probe position and proximity to metal components. However, the signal is reasonably stable if all the above-mentioned parameters are kept as constant as possible [43]. The validity and reliability of the gathered process information is highly dependent on correlation to off-line measurements, as discussed later. Despite the advantages of dielectric spectroscopy as a process analyser, its application is concentrated in the academic field rather than in industry, with the exception of brewing [44–48].

The evolution of the application of dielectric spectroscopy over time, the importance of data processing and the range of cell densities measured are summarized in Tables 2 and 3. “Application” refers to the cell type and cell line used, as well as to the culture conditions and experimental set-up. “Settings” summarizes, as far as the information available, the frequency settings used, the ranges scanned and the interval for acquiring data applied.

Dielectric spectroscopy finds its application in fields such as molecular biology, monitoring of transfection efficiency [72, 73], protein folding [74] and food technology [75]. Alternative but related methods include dielectrophoresis [60, 76] and electrochemical impedance spectroscopy [77].

Early work in dielectric spectroscopy was mostly concerned with improving the instrumentation and the mathematical translation of the signal [45, 47–50, 78] as well as exploration of the dielectric properties of cells [79, 80]. A series of papers was dedicated to exploring the frequency dependence of the capacitance measurements and the appropriate techniques to extract meaningful information from the obtained data [44, 45, 81, 82]. Several types of corrections for changes in the conductivity of the medium were proposed, and the most appropriate ones are now

Table 2 Summary of applications of dielectric spectroscopy to microbial cell cultures, with details of the device and settings used, the data processing applied and the cell density range measured

Device	Application	Settings and data pre-processing	Cell density	**References**
Four-terminal dielectric spectrometer, Aber biomass monitor	*S. cerevisiae*	Low-pass filter with time constant of 1 s, measurements at 0.4 MHz		[49–51]
Aber instrument 214 A	Baker's yeast	25 frequencies from 0.2 to 10 MHz; ANN compared with PLS and PCA	0–70 g/L	[44]
Aber biomass monitor 386SX IBM PC with DT2911-PGH 12-bit A/D I/O board in combination with PERMSTAT.exe, an in-house built QUICKBasicv4.5 program	Baker's yeast	0.4 MHz and 9.5 MHz were used during the fermentation; offline, each sample was scanned at 12 frequencies from 0.2 mHz to 9.6 MHz; various non-linear times series	–	[45]
BM 214-M	Baker's yeast	Study of data treatment and filtering (Butterworth etc.)	12–25 g/L	[46]
Three-electrode Aber instrument probes BM 214 A	Lysed baker's yeast	0.4 and 10 MHz	–	[47, 48]
Aber BM 214A	*Candida utilis*, *V. paradoxus* DSM 4065	0.6 and 10 MHz; moving-point average	0–2 g/L	[52, 53]
Aber BM 214	*Streptomyces claviguleris*	Dual frequency 0.1–1.0 MHz and approximately 10 MHz, low-pass filter to 1 s and low-pass range, low and high frequencies chosen as 0.4 and 10 MHz, data acquisition at 0.2 per min	0–30 g/L	[54]
Aber 214 M	*Streptomyces genus*	Dual frequency 0.1–1.0 MHZ and 9.6 MHz, low range, low-pass filter to 1 s, excitation frequency 0.4 MHz, data acquisition at 0.2 per min, working volume of the reactor of 200 L; Raw conductance measurements averaged over 25 points	–	[55]

(continued)

Table 2 (continued)

Device	Application	Settings and data pre-processing	Cell density	**References**
Aber 214 M	*E. coli* HMS174 (DE3) expressing viral autoprotease fusion protein	Dual frequency at 0.5 MHz, high range, low-pass filter at 1 s; Raw data with Savitzky–Golay over 25 data points	0–40 g/L	[56]
BM220 Aber instruments, 1.12 MHz and background compensation at 15.65 MHz	*E. coli* JM 105pUC19-GFP, *E. coli* BL21 star (DE3) p6xHis-GFP, *E. coli* K12DH5alpha pTRCHIS T-Saphire	1.12 MHz and background compensation at 15.65 MHz	70 g/L	[57]
Aber BM 210	*A. adeninivorans* LS3	25 logarithmically increasing frequencies from 0.1 to 19.49 MHz; Combination of moving-point average and Savitzky–Golay, Cole–Cole with Leuvenberg–Marquart	0–20 g/L	[58, 59]
Aber 220	*S. cerevisiae*, *S. cerevisiae* T65	Frequency spectra from 100 kHz to 500 MHz acquired; Multifrequency mode 0.1–10 MHz	0–120 g/L	[60, 61]
Aber BM 210	*S. cerevisiae*, *K. marxianus*	For each run, 25 frequencies from 0.1 to 20 MHz were scanned every 15 s; 81-point (20 min) moving point average and Savitzky–Golay smoothening on top, linear prediction model	0–10 g/L	[62]
Aber BM 210	*S. cerevisiae*, *K. marxianus*, *C. utilis*, *P. pastoris*	Dual-frequency measurements at 500 kHz and 10 MHz, low-pass filter at 60 s, data acquisition every 20 s; Moving-point average over routine interval of 2 min, linear prediction model	0–8 g/L	[63, 64]
Fogale biosystem	*S. cerevisiae*	0.3–10 MHz; Cole–Cole with constrained Leuvenberg–Marquart	–	[65]

Table 3 Summary of applications of dielectric spectroscopy to monitor growth and physiology of animal and plant cell cultures, with details of the device and settings used, the data processing applied and the cell density range measured

Device	Application	Settings and data pre-processing	Cell density	**Reference**
–	Erythrocytes	–	–	[40]
Aber Bugmeter	*Catharanthus roseus*, *Nicotiana tabacum*, *Cinchona robust* (plant cells)	Measurements at 0.4 MHz	Up to 44 g/L	[66]
Aber viable cell monitor	CHO 320 producing interferon-γ	Raw signal smoothening by moving-point average	Detection limit: 3.5×10^5 cells cm^{-3}	[67]
Aber BM 214 A	Mouse/mouse hybridoma expressing mAB EGF	Single frequency, 0.6 MHz, frequency chosen as "best compromise" between sensitivity and extensive independence from changes in conductivity of the medium in the given system, high range and low-pass filter at 1 s	Up to 14×10^5 cells/mL	[68]
Aber BM 214 M	CHO SSF3, immobilized and freely suspended	Calibration by differential method	Up to 1.6×10^6 cells/mL	[69]
Aber BM 214 M	CHO SSF3, immobilized and freely suspended	0.6 MHz single-frequency mode with low-pass filter at 5 s	Up to 7×10^6 cells/mL measurement range	[70]
Aber BM 214 M	CHO SSF3	Operated at "high range" with a maximal conductivity of 36 mS/cm, low-pass filter with time constant of 5 s, frequency scanning; Moving-point average over 5 points	5×10^6 cells/mL	[71]

built into the most recent developed devices. Once the technique had reached a sufficient level of reliability, the vast majority of processes involving commercially relevant cell types (animal cells [71, 83], plant cells [66] and industrially exploited microbes) were monitored with the aim of drawing correlations [61] and developing prediction models [62]. The method became increasingly popular for monitoring process singularities or particular events such as storage of intracellular compounds [58], virus production within infected animal cells [84] or bacteriophage production [85]. A number of authors have reviewed the application of dielectric spectroscopy to cell monitoring [41, 86–89].

Table 4 Reference techniques used for correlating dielectric spectroscopy signals

Technique	References
Cell counts and staining methods	[61, 70, 71]
Cell diameter, size	[56, 58, 59, 73]
Colony-forming units (CFU)	[61]
Cross-sectional area	[58, 59]
Dry cell weight (DCW)	[43, 54, 57, 61, 66, 90]
DNA	[54]
NTP	[68]
Packed (mycelium) volume	[57, 61, 66]
OD/turbidity	[43, 57]
Viscosity	[54, 55]

2.2.3 Reference Methods for Use in Dielectric Spectroscopic Correlations

The quest for a reference standard for the correlation of the capacitance reading to relevant biological information is still ongoing, as highlighted in Chap. 1. Regardless of the nature of the cells considered, the capacitance of a lipid membrane is on average 0.5–1 μF per cm^2 of membrane area [45]. The capacitance measurement still needs to be transformed into relevant information depending on the aim of the study. Research groups have described several correlation methods over the past two decades (Table 4). Xiong et al. [61] compared the most common techniques, namely optical density at 600 nm, dry cell weight (DCW), packed mycelial volume and colony-forming units (CFU), and obtained linear correlations in the range 3.1×10^6 cell/mL to 9.2×10^9 cells/mL for *Saccharomyces cerevisiae* with all techniques. Cell counting methods, especially when associated with Trypan blue staining to differentiate between viable and non-viable cells, are commonly used to correlate capacitance measurements to cell number or cell viability for animal cells. However, if crystal violet is used for nuclei counting, deviations from linearity can be observed if cells tend to be multi-nucleated under specific culture conditions [70]. Neves et al. [54] observed linear correlations of capacitance with DNA, packed mycelial volume and CO_2 production during the exponential growth phase, while correlations between DCW and capacitance measurements tended to be linear during exponential, transition and stationary phases. Correlations tend to be linear as long as no metabolic, physiological or morphological changes occur within the culture [61]. Indeed, as mentioned previously, dielectric spectroscopy is not only a tool to measure viable cell volume, but also to detect changes during biotechnological processes [73, 84]. Maskow et al. discussed the reasons and implication of non-linear correlations between biomass and capacitance correlations in the scope of bioprocess monitoring and control [59].

2.2.4 Data Pre-Treatment and Enhancement

Finding and exploiting an appropriate correlation method is not the only challenge to be tackled when implementing dielectric spectroscopy for monitoring a process. Signal noise and lack of robustness are the principal hurdles that need to be overcome. The former issue has been extensively addressed by implementing appropriate filtering techniques [44, 45, 49, 50, 81, 87]. Noise resulting from agitation can be filtered out using a low-pass filter with time constant of 1 s [49, 50] Most manufacturers of dielectric spectrometers have integrated appropriate filters into the provided software. However, there is still a need to correct for changes in conditions over time. Predictive modelling [46] as well as data reconciliation [63] can improve the reliability of the measurements.

2.2.5 Range of Measurement and Limits of Application

Dielectric spectroscopy gives real-time information about cell volume and viable cell number and, depending on the application, even insights into changes in physiology or morphology of cells. Commercially available devices have different ranges of validity and linearity of measurements and also find different applications. Real-time culture monitoring in a 96-well plate is possible using the xCELLigence system and is applied to mammalian cell cultures at micro-scale [91]. Aber Instruments as well as the more recent Fogal range of devices find their application in monitoring cultures of all types of organisms, but mostly at laboratory scale. Aber devices are very commonly used in brewing industry, where there are even well-established control tools. Devices from Hewlett Packard [43, 90, 92–94] and Edgerton et al. [81], as well as some micro-devices [80, 95, 96] only play a marginal role in the field of bioprocess monitoring by dielectric spectroscopy. The most commonly encountered devices have validity ranges from 2×10^5 cell/mL to a maximum of 10^9 cell/mL, corresponding to approximately 1–200 g/L of dry cell weight for yeast cells. When working with Gram-positive organisms, it should be kept in mind that the α-dispersion, not relevant when working with animal cells or Gram-negative microbes, plays an important role [45, 81, 97]. Cultures involving highly conductive medium cannot be monitored by traditional dielectric spectrometers since these have an upper conductivity limit of 100 mS/cm [61]. The sensitivity of the method, especially at low cell concentration, is limited [98].

2.2.6 Potential of Industrial Application: Perspectives and Limitations

Monitoring and process understanding is one part of PAT; process control is quite another. Dielectric spectroscopy is a potentially important tool to gain better process understanding and to monitor one of the most vital process parameters in biotechnology. Applications in the area of bioprocess control are less common.

A combination of capacitance and heat-flow measurements has been suggested and simulated by Guan et al. [67] and later applied to control the bacterial conversion of toxic substrates into polyhydroxyalkanoates [53]. Noll et al. [68] correlated capacitance to glutamine consumption rate and based a successful feeding strategy on this particular correlation. Dielectric spectroscopy measurements were used to trigger automated cell harvest [57], and Dabros et al. [63] used biomass measured by dielectric spectroscopy as the main control variable. Justice et al. [41] reviewed the different control applications of dielectric spectroscopy at laboratory scale. Dielectric spectroscopy is not yet widely applied in industrial production processes for biological, and this is probably due to requirements in terms of data pre-processing, filtering or predictive modelling. On the other hand, in the brewing industry, the technique is routinely used both for monitoring and control. Interesting fields of application are automatic pitching rate control or automatic yeast recovery control to minimize biowaste [97]. Applications in pharmaceutical processes have been reported by Eli Lilly (*Pichia pastoris* cultures for recombinant protein production [99]) and by Novo Nordisk (processes involving the culture of BHK cells [100]). The need to find appropriate correlation methods and develop a suitable technique to reduce noise may outweigh the advantages that dielectric spectroscopy has in some cases.

2.3 Vibrational Spectroscopy

Infrared spectroscopy is concerned with the region of the electromagnetic spectrum between the visible and microwave regions. This region, the infrared region, is further broken down into the near-infrared region (12,500–4,000 cm^{-1} or 800–2,500 nm), mid-infrared region (4,000–400 cm^{-1} or 2,500–25,000 nm) and far-infrared region (<400 cm^{-1} or >25,000 nm). Energy in the mid-infrared region has lower frequency and so lower energy than that of the near-infrared region. Mid-infrared spectroscopy looks at the change in energy when the molecule is radiated and moves from the ground state to the next excited energy level. As a result, absorption bands within the mid-infrared region can be attributed to specific molecules or functional groups within the molecule; hence MIR spectroscopy is sometimes referred to as a "fingerprinting" technique. The higher energy of the near-infrared region results in the radiated molecule moving from the ground state to excited energy levels above the fundamental absorption state, therefore NIR is focussed on the combination bands and overtones.

Spectra are most commonly presented in terms of intensity or absorbance versus wavenumber v. The absorbance and concentration can be calculated using the Beer–Lambert law (Eq. 1).

$$A_v = -\log_{10}\left(\frac{I_v}{I_{v,0}}\right) = \varepsilon bc, \tag{1}$$

where A_v is the absorbance at wavenumber v (no units), I_v is the intensity of light emitted from the sample at wavenumber v (arbitrary units), $I_{v,\,0}$ is the intensity of light emitted from the background at wavenumber v (arbitrary units), ε is the molar absorption coefficient (M^{-1} cm^{-1}), b is the path length (cm), and c is the concentration (M).

Raman spectroscopy is a complementary technique to infrared spectroscopy and is based on the scattering of light [101, 102].

To transform spectral data into meaningful results, it is necessary to develop a calibration model which relates the spectra to a process parameter e.g. concentration values of a substrate. Chemometric techniques are exploited to extract the relevant data (see Chap. 7).

2.3.1 Development of MIR Spectroscopy

Infrared radiation was discovered in 1800 by Sir William Herschel, and following this, the first mid-infrared spectrometer was constructed by Melloni in 1833. The first half of the twentieth century saw little development in FT-IR spectroscopy, and its potential as an analytical tool remained largely untapped until the late 1950s and early 1960s. Since the commercial debut of the FT-IR system in the 1970s, the technology has been embraced by manufacturing industries and research communities alike. Instruments have been adapted and improved to meet the specific needs of the end user.

MIR immersion probes have been available since the late 1980s. At the early stages of development MIR, optic fibres suffered from high material absorption and scattering and poor mechanical and chemical stability, therefore "fixed" arm probes with parallel light pipes using internal reflection spectroscopy were found to be more suitable. However, when placed in a process environment, this design is far from ideal. These probes need to be precisely aligned and are highly sensitive to vibrations in the surrounding area, which can result in alignment changes and hence spectral differences [103–105]. There have been major advances in the development of fibre-optic materials over the last 10 years, and these improvements have had far-reaching consequences. In the case of MIR instrumentation, this has resulted in flexible, more robust immersion probes which address many of the problems encountered with the rigid conduit probes. However, regardless of probe type, process disturbances will regularly impact the spectra collected, and these disturbances need to be accounted for when developing multivariate calibration models. The short path length of MIR, when compared with that of NIR, means that from a sampling perspective MIR does not penetrate as far into the material and may not be as representative of the sample as NIR would be; however, in the presence of particulate matter the shorter path length of MIR reduces light scattering, which is commonly experienced when NIR is used in a similar situation [106].

2.3.2 Development of NIR Spectroscopy

The development of NIR spectroscopy as a quality and process control tool is largely due to the availability of efficient chemometric techniques and varying spectrometer configurations [107].

Process environments vary considerably, and selecting the most appropriate sampling interface is of paramount importance. A large degree of process understanding is vital prior to choosing the sampling interface. The physical, chemical and optical nature of the process stream should be known in order to determine if the results will be significantly impacted by light scattering. This is particularly important where NIR is concerned, as the strong light source and the weak absorbance allow infrared radiation to penetrate further into the sample, allowing particulates present in a suspension or slurry to cause light scattering. Other aspects such as the potential of the process fluid to foul the probe or sample system should also be evaluated [107]. Typically NIR sampling systems for in situ process monitoring are in the form of one of the following two types: extractive sampling systems, or immersion probe sampling. A number of variations of each of the above two types exist. Invariably, it is the process conditions which dictate the system of choice.

2.3.3 Development of Raman Spectroscopy

During the 1920s, scattering effect theory was investigated by a number of researchers, including the physicist C. V. Raman, who in 1928 was the first to experimentally demonstrate the Raman effect in liquids [108]. A modified FT-IR instrument was also used to collect Raman spectra, and the use of such instruments is now known as FT-Raman spectroscopy. Dispersive Raman spectroscopy and FT-Raman spectroscopy each have their own specific advantages, and both continue to be used for different applications [109–111].

The basic components of any Raman spectrometer are the excitation source, the spectrometer and the detector. Significant advances in all three areas have led to the possibility of using Raman spectroscopy as a PAT tool.

2.3.4 Interpretation of Spectral Data Using Chemometrics

It is almost impossible to discuss the use of spectral data without a brief discussion on the concept of chemometrics, as the various chemometric techniques maximise the information available from spectroscopic instruments. Chemometrics can be defined as the chemical discipline that uses mathematical, statistical and other methods employing formal logic (a) to design or select optimal measurement procedures and experiments, and (b) to provide maximum relevant chemical information by analysing chemical data [112] (see also Chap. 7). When applied to spectra collected online during a bioprocess by MIR, NIR or Raman spectrometers,

it is the second function of chemometrics that is of most interest. Process data from a spectrometer are analysed in a multivariate rather than a univariate way; i.e. for each sample, the responses at multiple wavenumbers are taken into account. If the spectrum of a sample was recorded at three wavenumbers using any spectroscopic technique, a simple two-dimensional plot of response versus wavenumber could be used to visualise the data. The same data can also represented by a single point in three dimensions, where each dimension corresponds to a wavenumber.

An individual spectrum recorded on a spectrometer can have hundreds of data points, and a single component can have a response in multiple places within the one region, making the data highly correlated [113]. Rather than representing the spectral data in two-dimensional space, chemometric techniques use multi-dimensional space or hyperspace to represent the same spectrum by a single point. As there is usually much redundant information in spectra due to variables being highly correlated, data do not need to be represented in space with as many dimensions as the original data points. The spectral data containing hundreds of data points can be fully characterised in as few as 20 dimensions [114]. Chemometric or multivariate calibration techniques allow the concentration of a given analyte to be related to spectral features. They are also useful for distinguishing real chemical information from instrument noise [113].

Pre-Treatments

Prior to analysing spectral data, a mathematical pre-treatment may be necessary. Common pre-treatments include mean centring, mean normalisation and using the first or second derivative of the spectra [114]. Leverage is a measure of how extreme a data point is compared with the majority. A data point with high leverage will have a high influence on any model created. Mean normalisation is an adjustment to a data set that equalizes the magnitude of each sample. When the spectra have been normalized, qualitative information that distinguishes one sample from another is retained but information that would separate two samples of identical composition but different concentration is removed. A standard normal variate (SNV) pre-treatment is one which centres and scales individual spectra. The effect of this pre-treatment is that on the vertical scale each spectrum is centred on zero and varies roughly from -2 to $+2$. This effectively removes the multiplicative interferences of scatter and particle size in spectral data [115].

The first derivate of a spectrum is the slope of the curve at every point. It has peaks where the original has maximum slope and crosses zero where there was a peak in the original spectrum. As the slope is not affected by additive baseline offsets in the spectrum, calculating the first derivative is an effective method of removing baseline effects. The second derivative is the slope of the first derivative. It has peaks in roughly the same places as the original spectrum, but these peaks are in the inverted direction. Calculating the second derivative of a spectrum will remove additive baseline effects as well as multiplicative baseline effects

[116, 117]. Small spectral differences are enhanced and overlapping peaks are separated by the use of derivative pre-treatments.

As a measured spectrum is not a continuous mathematic curve, but rather a series of equally spaced points, traditional derivative calculation performed by using the difference in values between two adjacent points has the effect of reducing the signal-to-noise ratio in the data. It is therefore necessary to include some form of smoothing in the calculation. One method of calculating the derivate of the spectra is to use the algorithm described by Savitzky and Golay [118]. This works by taking a narrow window centred at the wavelength of interest, and fitting a low-order polynomial to the data points in this window using least squares. The calculated polynomial is a continuous curve of the form $y = a + bx + cx^2 \ldots$, where x is the wavelength and y is the spectral response. The first and second derivatives of this fitted curve are then used as estimates of the derivatives of the underlying spectrum.

The choice of pre-treatment can depend on the type of spectra being analysed; e.g. Raman and NIR will often have derivative pre-treatments applied [119–121]. A multi-component mixture or a sample collected online which may be subject to instrument drift will also be pre-treated with a procedure such as SNV or derivatives. Many other pre-treatments are possible, and the nature of the application will dictate the most suitable one or indeed combination to choose.

Quantitative Analysis

Interpretation of spectra can be a challenge, as many different components can have a response in similar regions of the electromagnetic spectrum. This becomes an issue when the aim is to identify and quantify individual components in a mixture. The first step in developing a calibration model is to do a simple feasibility study such as that described in the ASTM international standards [122] for each component of interest. The procedure described involves the collection of spectra from 30–50 samples incorporating the expected variations in particle size, sample presentation and process conditions which are expected during analysis. If the results of this simple study are favourable as judged by error values from cross-validation methods and the required precision was obtained, the study can be expanded to see if multi-component mixtures can be adequately modelled.

To make a good calibration model, a suitable experimental design must be employed. The samples used for developing the model are known as the training or calibration set and should ideally comprise several uniformly distributed concentrations for each component of interest. The factors in an experimental design for a multi-component mixture are the individual components, and these factors should be mutually independent or orthogonal; i.e. the correlation coefficient between each pair of factors should be zero [123]. There has been some discussion in the literature on the importance of using uncorrelated samples in the development of chemometric models for online metabolite monitoring [124–126]. As the

performance of any model is directly affected by the training set used in its development, the training set should fulfil certain criteria. It should:

- Contain all expected components
- Span the concentration ranges of interest
- Span the conditions of interest
- Contain mutually independent samples.

The calibration should also be validated using a set of samples (validation set) which is independent of the training set. Strategies on how to determine an experimental design which will achieve these aims can be found elsewhere [123, 127, 128].

Partial Least-Squares Regression

An often-used chemometric calibration technique for bioprocessing applications is partial least-squares regression (PLS). This is a multivariate statistical technique developed from classical least-squares and inverse least-squares regression used in economic forecasting and later in chemical applications [127] (see Chap. 7).

2.3.5 PAT Applications of Vibrational Spectroscopy in Bioprocessing

The applications or potential applications of vibrational spectroscopy in bioprocessing are largely dependent on the sampling interfaces available. A number of instruments exist, and sample interfaces vary from sample cavities using cuvettes or vials to immersion probes. Where real-time data are required for monitoring and control purposes, the type of available instruments is very much reduced, as all offline techniques are eliminated. Bioprocess applications to date have used either flow cells, where the sample of interest is passed through a measuring chamber, or immersion probes, where a probe is inserted into a reactor and the sample is scanned in situ by transflectance, transmission or reflectance methods. The development of high-quality fibre optics and autoclavable probes has increased the capabilities of these techniques. The most common applications in bioprocessing are analyte, metabolite and biomass monitoring, with monitoring systems in some cases further developed to enable process control.

MIR Applications

MIR lags behind its infrared counterpart, NIR, when it comes to applications in bioprocessing. Despite the fact that MIR can detect and quantify components in aqueous solutions at significantly lower levels than NIR [121], MIR is less extensively used. Only in the last decade has MIR been considered a potentially useful tool for bioprocess monitoring. Work to date has mainly focussed on

detection of substrates and metabolites in yeast and bacterial cultures, but it has also been applied to suspended and immobilised animal cell cultures [129]. Most methods use synthetic samples or samples taken from cell cultures to build multivariate models capable of predicting changing concentration values.

The most common component modelled is glucose. This is the predominant substrate in cell culturing and, so, is of most interest from a detection and monitoring point of view [130–132]. Other substrates detected using online MIR techniques include fructose, lactose, galactose, ammonia and methyl oleate [106, 133, 134]. Accuracy values vary between studies, with standard prediction errors ranging from 0.26 to 0.86 g/L for glucose. Subtle differences exist between the various techniques developed. The sample presentation method is of some importance for this application, as many cell cultures require aeration, resulting in gas bubbles forming on the probe tip. Automated flow systems can help mitigate this problem, while a recessed geometry of the probe tip can facilitate the formation of pockets on the crystal surface [121]. In addition to the sampling interface, the models employed are specific to each individual set-up. Although multivariate chemometric modelling is used to develop these models, each model is unique.

This technique has also been applied to determine the rate of product formation. Cell culture products that have been successfully detected using MIR include ethanol, lactic acid and glucuronic acid [131, 132, 135].

Online MIR measurements have been used not just to detect or monitor cell culture substrates and metabolites, but also to control cultures. Kornmann et al. used *Gluconacetobactor xylinus* to develop a control strategy based on the depletion of two substrates, fructose and ethanol [136]. Real-time spectroscopic scans were collected every 5 min, concentrations were sent to an adaptive control algorithm, and fructose and ethanol were fed to the culture in controlled volumes. Schenk et al. showed that a similar system could be used to control methanol feeding to *Pichia pastoris* cultures [137].

NIR Applications

NIR spectroscopy can provide online information on substrate, biomass, product and metabolite concentrations [138]. This information can be further used to control and optimise cell cultures. Extensive work has been carried out in this area to date. NIR has been used to monitor concentration changes in yeast, bacterial and even mammalian cell cultures. Crowley et al. used NIR to monitor the main substrates, glycerol and methanol, as well as biomass, in a *Pichia pastoris* culture [139], Petersen et al. used NIR to predict the changing concentrations of glucose, ammonium and biomass in a *Streptomyces coelicolor* culture [126], while Rodrigues et al. developed an NIR model to monitor clavulanic acid, the product of a fed-batch process with *S. clavuligerus* [140].

The technique has also been applied to monitoring of mammalian cell cultures. Four key analytes of a CHO-K1 mammalian cell culture, i.e. glucose, lactate, glutamine and ammonia, were monitored by Arnold et al. [141], and this work was

further developed by Roychoudhury et al. [142], who used a multiplexed calibration technique.

As with MIR, NIR predictive models have also been applied to control systems in order to allow fed-batch cultures to react in "real time". As early as 1994 Vaccari et al. proposed the use of NIR to control the glucose feed in the production of lactic acid by *Lactobacillus casei* [143]. Many others have developed control strategies for various yeast and microbial cultures [144, 145].

Raman Applications

The reported use of Raman spectroscopy for monitoring bioprocesses in situ and in real time is limited, and this is most likely due to the need for low-frequency lasers to avoid fluorescence, which can have heating effects due to the long exposure times necessary for such lasers. Most reported studies describe use of Raman spectroscopy to monitor yeast cultures. One of the earliest applications of in situ Raman spectroscopy was monitoring the production of ethanol in yeast fermentations [146]. In this study the concentrations of fructose and glucose were also measured. Shaw et al. used a dispersive Raman instrument to monitor the change in substrate and metabolite concentrations as well as product formation in yeast fermentation and found it necessary to include a by-pass filter to remove cells as they were causing interference to the photon scattering process [147]. The production of carotenoids in *Phaffia rhodozyma* cultures has also been monitored by dispersive Raman spectroscopy [148]. Bacterial cultures with monitoring of glucose, acetate, formate, lactate and phenylalanine by in situ measurements have also being reported [149]. In a more recent study, Raman spectra were collected in situ in a mammalian cell bioreactor. As well as monitoring substrates and metabolites, the spectra were correlated to total cell density and viable cell density, showing that it may be possible for Raman spectroscopy to distinguish between live and dead cells [119]. While these studies all demonstrate the potential of Raman spectroscopy as a monitoring tool, it has yet to be proved capable of control in industrial bioprocesses.

Although separate techniques, both MIR and NIR have similar applications in bioprocessing; both have been used for monitoring and control purposes. Raman spectroscopy has been used to monitor bioprocesses, but to a lesser degree than the other vibrational spectroscopies. The manner in which these techniques are exploited is similar. In all cases, multivariate chemometric models are developed based on synthetic, semi-synthetic or actual samples from a cell culture. Typically, these models are then validated and applied to a culture online. These techniques all have their benefits and limitations, but to date NIR has been the subject of more investigation and as a result is more developed in terms of applications in bioprocessing. However, the potential of MIR and Raman should not be underestimated or overshadowed.

3 Conclusions

Choosing a suitable online analytical technique and data processing method for bioprocess applications is essential if reliable monitoring and control are to be achieved. Each of the process analysers described here has the potential to be used for online measurement, but it is only through proper understanding of their specific advantages and limitations that they can be applied to monitor the appropriate process variables. The relationship between the measureable parameters and critical process parameters needs to be recognised in order to develop calibrations for the critical process parameters of interest, and knowledge of the likely signal interferences will allow the employment of data treatments which can minimise or even eliminate their effects. Advanced data processing methods such as data reconciliation and artificial neural networks can also enhance the accuracy of the measured variables by using inputs from a number of online sensors. The combination of suitable analytical techniques and data processing methods should provide an increase in bioprocess knowledge, which will in turn allow the process to be tightly controlled and operate within a previously established design space.

References

1. U.S. FDA (2004) Pharmaceutical cGMPs for the 21st century—A risk-based approach—final report
2. U.S. FDA (2006) Guidance for industry, Q8 pharmaceutical development
3. U.S. FDA (2004) Guidance for industry PAT—a framework for innovative pharmaceutical development, manufacturing, and quality assurance
4. Baughmann E (2005) Process analytical chemistry: introduction and historical. In: Bakeev KA (ed) Process analytical technology: spectroscopic tools and implementation strategies for chemical and pharmaceutical industries. Blackwell, Oxford
5. von Stockar U, Maskow T, Liu J, Marison IW, Patiño R (2006) Thermodynamics of microbial growth and metabolism: an analysis of the current situation. J Biotechnol 121:517–533
6. Winkelmann M, Hüttl R, Wolf G (2004) Application of batch-calorimetry for the investigation of microbial activity. Thermochimica Acta 415:75–82
7. von Stockar U, Marison IW (1991) Large-scale calorimetry and biotechnology. Thermochimica Acta 193:215–242
8. Birou B, Marison IW, Stockar UV (1987) Calorimetric investigation of aerobic fermentations. Biotechnol Bioeng 30:650–660
9. Buttiglieri G, Bouju H, Malpei F, Ficara E, Canziani R (2010) Microcalorimetry: a tool to investigate aerobic, anoxic and anaerobic autotrophic and heterotrophic biodegradation. Biochem Eng J 52:25–32
10. Voisard D, von Stockar U, Marison IW (2002) Quantitative calorimetric investigation of fed-batch cultures of *Bacillus sphaericus* 1593 M. Thermochimica Acta 394:99–111
11. von Stockar U, Marison I (1989) The use of calorimetry in biotechnology, bioprocesses and engineering. Springer, Berlin/Heidelberg, 40:93–136
12. Redl B, Tiefenbrunner F (1981) Determination of hydrolytic activities in wastewater systems by microcalorimetry. Water Res 15:87–90
13. Wadsö I (1986) Bio-calorimetry. Trends Biotechnol 4:45–51

14. Grob B, Riesen R (1987) Reaction calorimetry for the development of chemical reactions. Thermochimica Acta 114:83–90
15. Marison IW, von Stockar U (1985) A novel bench-scale calorimeter for biological process development work. Thermochimica Acta 85:496
16. Marison I, Liu JS, Ampuero S, Von Stockar U, Schenker B (1998) Biological reaction calorimetry: development of high sensitivity bio-calorimeters. Thermochimica Acta 309:157–173
17. Zentgraf B (1991) Bench-scale calorimetry in biotechnology Thermochimica Acta 193:243–251
18. García-Payo MC, Ampuero S, Liu JS, Marison IW, von Stockar U (2002) The development and characterization of a high resolution bio-reaction calorimeter for weakly exothermic cultures. Thermochimica Acta 391:25–39
19. von Stockar U, Larsson C, Marison IW, Cooney MJ (1995) Calorimetry of dual limitations in yeast cultures. Thermochimica Acta 250:247–258
20. Aulenta F, Bassani C, Ligthart J, Majone M, Tilche A (2002) Calorimetry: a tool for assessing microbial activity under aerobic and anoxic conditions. Water Res 36:1297–1305
21. Birou B (1986) Etude De La Chaleur Dégagée Par Des Cultures Microbiennes Dans Un Fermenteur De Laboratoire. E.P.F.L., Lausanne
22. Marison I, Linder M, Schenker B (1998) High-sensitive heat-flow calorimetry. Thermochimica Acta 310:43-46
23. Liu J, Marison IW, von Stockar U (2001) Microbial growth by a net heat up-take: a calorimetric and thermodynamic study on acetotrophic methanogenesis by *Methanosarcina barkeri*. Biotechnol Bioeng 75:170–180
24. Daverio E, Spanjers H, Bassani C, Ligthart J, Nieman H (2003) Calorimetric investigation of anaerobic digestion: biomass adaptation and temperature effect. Biotechnol Bioeng 82:499–505
25. Janssen M, Patiño R, von Stockar U (2005) Application of bench-scale biocalorimetry to photoautotrophic cultures. Thermochimica Acta 435:18–27
26. Janssen M, Wijffels R, von Stockar U (2007) Biocalorimetric monitoring of photoautotrophic batch cultures. Thermochimica Acta 458:54–64
27. Randolph TW, Marison IW, Martens DE, VonStockar U (1990) Calorumetric control of fed-batch fermentations. Biotechnol Bioeng 36:678–684
28. von Stockar U, Duboc P, Menoud L, Marison IW (1997) On-line calorimetry as a technique for process monitoring and control in biotechnology. Thermochimica Acta 300:225–236
29. Voisard D, Claivaz C, Menoud L, Marison IW, von Stockar U (1998) Use of reaction calorimetry to monitor and control microbial cultures producing industrially relevant secondary metabolites. Thermochimica Acta 309:87–96
30. Liu J, Marison I, von Stockar U (1999) Anaerobic calorimetry of the growth of *Lactobacillus helveticus* using a highly sensitive bio-RCl. Biochimica et Biophysica Acta 56:1191–1195
31. Patiño R, Janssen M, von Stockar U (2007) A study of the growth for the microalga *Chlorella vulgaris* by photo-bio-calorimetry and other on-line and off-line techniques. Biotechnol Bioeng 96:757–767
32. Voisard D, Pugeaud P, Kumar AR, Jenny K, Jayaraman K, Marison IW, von Stockar U (2002) Development of a large-scale biocalorimeter to monitor and control bioprocesses. Biotechnol Bioeng 80:125–138
33. Türker M (2004) Development of biocalorimetry as a technique for process monitoring and control in technical scale fermentations. Thermochimica Acta 419:73–81
34. Schubert T, Breuer U, Harms H, Maskow T (2007) Calorimetric bioprocess monitoring by small modifications to a standard bench-scale bioreactor. J Biotechnol 130:24–31
35. Sivaprakasam S, Schuler M, Hama A, Hughes K, Marison I (2011) Biocalorimetry as a process analytical technology process analyser; robust in-line monitoring and control of aerobic fed-batch cultures of crabtree-negative yeast cells. J Therm Anal Calorim 104:75–85

36. Schuler M, Sivaprakasam S, Freeland B, Hama A, Hughes K, Marison I Investigation of the potential of biocalorimetry as a process analytical technology (PAT) tool for monitoring and control of Crabtree-negative yeast cultures. Appl Microbiol Biotechnol :1–10
37. Biener R, Steinkämper A, Hofmann J (2010) Calorimetric control for high cell density cultivation of a recombinant Escherichia coli strain. J Biotechnol 146:45–53
38. Biener R, Steinkämper A, Horn T (2012) Calorimetric control of the specific growth rate during fed-batch cultures of Saccharomyces cerevisiae. J Biotechnol 160:195–201
39. van Kleeff BHA, Kuenen JG, Honderd G, Heijnen JJ (1998) Using heat-flow measurements for the feed control of a fed batch fermentation of *Saccharomyces cerevisiae*. Thermochimica Acta 309:175–180
40. Kirkpatrick DS, McGinness JE, Moorhead WD, Corry PM, Proctor PH (1978) High-frequency dielectric spectroscopy of concentrated membrane suspensions. Biophys J 24:243–245
41. Justice C, Brix A, Freimark D, Kraume M, Pfromm P, Eichenmueller B, Czermak P (2011) Process control in cell culture technology using dielectric spectroscopy. Biotechnol Adv 29:391–401
42. FDA (2004) Guidance for industry PAT—a framework for innovative pharmaceutical development, Manufacturing and Quality Assurance
43. Soley A, Lecina M, Gamez X, Cairo JJ, Riu P, Rosell X, Bragos R, Godia F (2005) On-line monitoring of yeast cell growth by impedance spectroscopy. J Biotechnol 118:398–405
44. Nicholson DJ, Kell DB, Davey CL (1996) Deconvolution of the dielectric spectra of microbial cell suspensions using multivariate calibration and artificial neural networks. Bioelectrochem Bioenerget 39:185–193
45. Davey HM, Davey CL, Woodward AM, Edmonds AN, Lee AW, Kell DB (1996) Oscillatory, stochastic and chaotic growth rate fluctuations in permittistatically controlled yeast cultures. BioSystems 39:43–61
46. Claes JE, Van Impe JF (1999) On-line estimation of the specific growth rate based on viable biomass measurements: experimental validation. Bioprocess Biosystems Eng 21:389–395
47. Davey CL, Kell DB (1998) The influence of electrode polarisation on dielectric spectra, with special reference to capacitive biomass measurements: (II) Reduction in the contribution of electrode polarisation to dielectric spectra using a two-frequency method. Bioelectrochem Bioenerg 46:105–114
48. Davey CL, Kell DB (1998) The influence of electrode polarisation on dielectric spectra, with special reference to capacitive biomass measurements—I. Quantifying the effects on electrode polarisation of factors likely to occur during fermentations. Bioelectrochem Bioenerg 46:91–103
49. Davey CL (1993) The theory of the β-dielectric dispersion and its use in the estimation of cellular biomass. Aber instruments handbook pp. 38
50. Davey CL, Davey HM, Kell DB, Todd RW (1993) Introduction to the dielectric estimation of cellular biomass in real-time, with special emphasis on measurements at high-volume fractions. Anal Chim Acta 279:155–161
51. Davey CL, GH Markx, Kell DB (1993) On the dielectric method of monitoring cellular viability. Pure Appl Chem 65:1921–1926
52. November EJ, Van Impe JF (2000) Evaluation of on-line viable biomass measurements during fermentations of *Candida utilis*. Bioprocess Biosystems Eng 23:473–477
53. Maskow T, Olomolaiye D, Breuer U, Kemp R (2004) Flow calorimetry and dielectric spectroscopy to control the bacterial conversion of toxic substrates into polyhydroxyalcanoates. Biotechnol Bioeng 85:547–552
54. Neves AA, Pereira DA, Vieira LM, Menezes JC (2000) Real time monitoring biomass concentration in Streptomyces clavuligerus cultivations with industrial media using a capacitance probe. J Biotechnol 84:45–52
55. Ferreira AP, Vieira LM, Cardoso JP, Menezes JC (2005) Evaluation of a new annular capacitance probe for biomass monitoring in industrial pilot-scale fermentations. J Biotechnol 116:403–409

56. Clementschitsch F, Jürgen K, Florentina P, Karl B (2005) Sensor combination and chemometric modelling for improved process monitoring in recombinant *E. coli* fed-batch cultivations. J Biotechnol 120:183–196
57. Kaiser C, Pototzki T, Ellert A, Luttmann R (2008) Applications of PAT-process analytical technology in recombinant protein processes with *Escherichia coli*. Eng Life Sci 8:132–138
58. Maskow T, Röllich A, Fetzer I, Ackermann J, Harms H (2008) On-line monitoring of lipid storage in yeasts using impedance spectroscopy. J Biotechnol 135:64–70
59. Maskow T, Röllich A, Fetzer I, Yao J, Harms H (2008) Observation of non-linear biomass–capacitance correlations: Reasons and implications for bioprocess control. Biosens Bioelectron 24:123–128
60. Patel PM, Bhat A, Markx GH (2008) A comparative study of cell death using electrical capacitance measurements and dielectrophoresis. Enzyme Microb Technol 43:523–530
61. Xiong Z, Guo M, Guo Y, Chu J, Zhuang Y, Zhang S (2008) Real-time viable-cell mass monitoring in high-cell-density fed-batch glutathione fermentation by *Saccharomyces cerevisiae* T65 in industrial complex medium. J Biosci Bioeng 105:409–413
62. Dabros M, Dennewald D, Currie D, Lee M, Todd R, Marison I, von Stockar U (2009) Cole–cole, linear and multivariate modeling of capacitance data for on-line monitoring of biomass. Bioprocess Biosystems Eng 32:161–173
63. Dabros M, Amrhein M, Bonvin D, Marison IW, von Stockar U (2009) Data reconciliation of concentration estimates from mid-infrared and dielectric spectral measurements for improved on-line monitoring of bioprocesses. Biotechnol Prog 25:578–588
64. Dabros M, Schuler M, Marison I (2010) Simple control of specific growth rate in biotechnological fed-batch processes based on enhanced online measurements of biomass. Bioprocess Biosystems Eng 33:1109–1118
65. Tibayrenc P, Preziosi-Belloy L, Ghommidh C (2011) On-line monitoring of dielectrical properties of yeast cells during a stress-model alcoholic fermentation. Process Biochem 46:193–201
66. Markx GH, Davey CL, Kell DB (1991) To what extent is the magnitude of the Cole–Cole-alpha of the beta-dielectric dispersion of cell-suspensions explicable in terms of the cell-size distribution. Bioelectrochem Bioenerg 25:195–211
67. Guan Y, Evans PM, Kemp RB (1998) Specific heat flow rate: an on-line monitor and potential control variable of specific metabolic rate in animal cell culture that combines microcalorimetry with dielectric spectroscopy. Biotechnol Bioeng 58:464–477
68. Noll T, Biselli M (1998) Dielectric spectroscopy in the cultivation of suspended and immobilized hybridoma cells. J Biotechnol 63:187–198
69. Ducommun P, Kadouri A, von Stockar U, Marison IW (2002) On-line determination of animal cell concentration in two industrial high-density culture processes by dielectric spectroscopy. Biotechnol Bioeng 77:316–323
70. Ducommun P, Bolzonella I, Rhiel M, Pugeaud P, von Stockar U, Marison IW (2001) On-line determination of animal cell concentration. Biotechnol Bioeng 72:515–522
71. Cannizzaro C, Gugerli R, Marison I, von Stockar U (2003) On-line biomass monitoring of CHO perfusion culture with scanning dielectric spectroscopy. Biotechnol Bioeng 84:597–610
72. Negrete A, Esteban G, Kotin R (2007) Process optimization of large-scale production of recombinant adeno-associated vectors using dielectric spectroscopy. Appl Microbiol Biotechnol 76:761–772
73. Ansorge S, Esteban G, Schmid G (2007) On-line monitoring of infected Sf-9 insect cell cultures by scanning permittivity measurements and comparison with off-line biovolume measurements. Cytotechnology 55:115–124
74. Bonincontro A, Risuleo G (2003) Dielectric spectroscopy as a probe for the investigation of conformational properties of proteins. Spectrochim Acta A 59:2677–2684
75. Castro-Giráldez M, Fito PJ, Rosa MD, Fito P (2011) Application of microwaves dielectric spectroscopy for controlling osmotic dehydration of kiwifruit (*Actinidia deliciosa* cv Hayward). Innovative Food Sci Emer Tech 12:623–627

76. Pohl HA, Kaler K, Pollock K (1981) The continuous positive and negative dielectrophoresis of microorganisms. J Biol Phys 9:67–86
77. Kim T, Kang J, Lee J, Yoon J (2011) Influence of attached bacteria and biofilm on double-layer capacitance during biofilm monitoring by electrochemical impedance spectroscopy. Water Res 45:4615–4622
78. Davey CL, Markx GH, Kell DB (1993) On the dielectric method of monitoring cellular viability. Pure Appl Chem 65:1921–1926
79. Gastrock G, Lemke K, Metze J (2001) Sampling and monitoring in bioprocessing using microtechniques. Rev Mol Biotechnol 82:123–135
80. Krommenhoek EE, Gardeniers JGE, Bomer JG, Van den Berg A, Li X, Ottens M, van der Wielen LAM, van Dedem GWK, Van Leeuwen M, van Gulik WM, Heijnen JJ (2006) Monitoring of yeast cell concentration using a micromachined impedance sensor. Sensor Actuat B-Chem 115:384–389
81. Ciureanu M, Levadoux W, Goldstein S (1997) Electrical impedance studies on a culture of a newly discovered strain of streptomyces. Enzyme Microb Technol 21:441–449
82. Asami K, Yonezawa T (1996) Dielectric behavior of wild-type yeast and vacuole-deficient mutant over a frequency range of 10 kHz to 10 GHz. Biophys J 71:2192–2200
83. Ducommun P, Ruffieux P, Furter M, Marison I, von Stockar U (2000) A new method for on-line measurement of the volumetric oxygen uptake rate in membrane aerated animal cell cultures. J Biotechnol 78:139–147
84. Ansorge S, Lanthier S, Transfiguracion J, Henry O, Kamen A (2011) Monitoring lentiviral vector production kinetics using online permittivity measurements. Biochem Eng J 54:16–25
85. Maskow T, Kiesel B, Schubert T, Yong Z, Harms H, Yao J (2010) Calorimetric real time monitoring of lambda prophage induction. J Virol Methods 168:126–132
86. Markx GH, Davey CL (1999) The dielectric properties of biological cells at radiofrequencies: applications in biotechnology. Enzyme Microb Technol 25:161–171
87. Asami K (2002) Characterization of biological cells by dielectric spectroscopy. J Non-Cryst Solids 305:268–277
88. Carvell J, Poppleton J, Dowd J (2006) Measurements and control of viable cell density in cGMP manufacturing processes. Bioprocess J 5:58–63
89. Kiviharju K, Salonen K, Moilanen U, Eerikäinen T (2008) Biomass measurement online: the performance of in situ measurements and software sensors. Ind Microbiol Biotechnol 35:657–665
90. Krairak S, Yamamura K, Nakajima M, Shimizu H, Shioya S (1999) On-line monitoring of fungal cell concentration by dielectric spectroscopy. J Biotechnol 69:115–123
91. Roche Diagnostics GmbH (2009) The xCELLigence system—new horizons in cellular technology.http://www.roche-applied-science.com/sis/xcelligence/xce_docs/xCELL-SystBrosch_NL2_LR.pdf
92. Asami K, Gheorghiu E, Yonezawa T (1999) Real-time monitoring of yeast cell division by dielectric spectroscopy. Biophys J 76:3345–3348
93. Hofmann MC, Ellersiek D, Kensy F, Büchs J, Mokwa W, Schnakenberg U (2005) Galvanic decoupled sensor for monitoring biomass concentration during fermentation processes. Sensors Actuators B Chem 111–112:370–375
94. Hofmann MC, Kensy F, Buechs J, Mokwa W, Schnakenberg U (2005) Transponder-based sensor for monitoring electrical properties of biological cell solutions. J Biosci Bioeng 100:172–177
95. Kim Y, Park J, Jung H (2009) An impedimetric biosensor for real-time monitoring of bacterial growth in a microbial fermentor. Sensors Actuators B Chem 138:270–277
96. Hofmann MC, Funke M, Buechs J, Mokwa W, Schnakenberg U (2010) Development of a four electrode sensor array for impedance spectroscopy in high content screenings of fermentation processes. Sensors Actuators B Chem 147:93–99

97. Carvell, J. and K. Turner (2003) New applications and methods utilizing radio-frequency impedance measurements for improving yeast management. Master Brewers Assoc Am 40:30–38
98. Vojinovic V, Cabral JMS, Fonseca LP (2006) Real-time bioprocess monitoring. Part I In Situ Sens 114:1083–1091
99. O'Reilly BT, Hilton MD (2006) Improved fed-batch through maintenance of specific productivity by indexing the glucose feed rate to capacitance-measured biomass in *Pichia pastoris*, BIOT 443: Upstream Processing: microbial fermentation process development. Advances in Process Engineering, San Francisco
100. Carvell J, Dowd J (2006) On-line measurements and control of viable cell density in cell culture manufacturing processes using radio-frequency impedance. Cytotechnology 50:35–48
101. Günzler H, Gremlich H (2002) Spectroscopy in near- and far-infrared as well as related methods. In: Anonymous IR spectroscopy: an introduction. Wilcy-VCH, Weinheim, p 309
102. Brown JM (1998) Molecular spectroscopy. Oxford University Press, New York
103. Schenk J, Dabros M, Marison IW, von Stockar U (2005) Simple and quick in situ calibration of a FTIR instrument to control fed-batch fermentations of *Pichia pastoris*. J Biotechnol 118:S37–S37
104. Schenk J, Marison IW, von Stockar U (2007) Simplified Fourier-transform mid-infrared spectroscopy calibration based on a spectra library for the on-line monitoring of bioprocesses. Analytica Chimica Acta 591:132–140
105. Schenk J, Viscasillas C, Marison IW, von Stockar U (2008) On-line monitoring of nine different batch cultures of *E. coli* by mid-infrared spectroscopy, using a single spectra library for calibration. J Biotechnol 134:93–102
106. Gabriele R (2005) Near-infrared spectroscopy and imaging: basic principles and pharmaceutical applications. Adv Drug Deliv Rev 57:1109–1143
107. Simpson MB (2005) Near-infrared spectroscopy for process analytical chemistry: theory, technology and implementation. In: Bakeev KA (ed) Process analytical technology: spectroscopic tools and implementation for the chemical and pharmaceutical industries. Blackwell, Oxford, p 39
108. Singh R (2002) C. V. Raman and the discovery of the Raman effect. Phys Perspect 4:399–420
109. Das RS, Agrawal YK Raman spectroscopy: recent advancements, techniques and applications. Vib Spectrosc 57:163–176
110. Wartewig S, Nuebert RHH (2005) Pharmaceutical applications of mid-IR and Raman spectroscopy. Adv Drug Deliver Rev 57:1144–1170
111. Chase B (1994) A new generation of Raman instrumentation. Appl Spectrosc 48:14A–19A
112. Massart DL, Vandeginste BGM, Deming BM, Michotte Y, Kaufman L (1988) Chemometrics: a textbook. data handling in science and technology. Elsevier, Amsterdam
113. Brown, S. D., (2001) A Short Primer on Chemometrics for Spectroscopists. Educational Article. http://www.spectroscopynow.com/details/education/sepspec10349education/A-Short-Primer-on-Chemometrics-for-Spectroscopists.html. Accessed 16 Nov 2012
114. Kramer R (1998) Chemometric techniques for quantitative analysis. Dekker, New York, pp 216
115. Barnes RJ, Dhanoa MS, Lister SJ (1989) Standard normal variate transformation and de-trending of near-infrared diffuse reflectance spectra. Appl Spectrosc 43:772–777
116. Naes T, Isaksson T, Fearn T, Davies T (2002) A user-friendly guide to multivariate calibration and classification. NIR, Chichester, pp 344
117. Gabrielsson J, Jonsson H, Airiau C, Schmidt B, Escott R, Trygg J (2006) OPLS methodology for analysis of pre-processing effects on spectroscopic data. Chemom Intellig Lab Syst 84:153–158
118. Savitzky A, Golay MJE (1964) Smoothing and differentiation of data by simplified least squares procedures. Anal Chem 36:1627–1639

119. Abu-Absi NR, Kenty BM, Cuellar ME, Borys MC, Sakhamuri S, Strachan DJ, Hausladen MC, Li ZJ (2011) Real time monitoring of multiple parameters in mammalian cell culture bioreactors using an in-line Raman spectroscopy probe. Biotechnol Bioeng 108:1215–1221
120. Henriques J, Buziol S, Stocker E, Voogd A, Menezes J (2010) Monitoring mammalian cell cultivations for monoclonal antibody production using near-infrared spectroscopy. Adv Biochem Eng Biotechnol 116:73–97
121. Landgrebe D, Haake C, Höpfner T, Beutel S, Hitzmann B, Scheper T, Rhiel M, Reardon K (2010) On-line infrared spectroscopy for bioprocess monitoring. Appl Microbiol Biot 88:11–22
122. ASTM International (2012) Standard Practices for Infrared Multivariate Quantitative Analysis, ASTM Standard 1655–05. doi:10.1520/E1655-05R12, West Conshohocken, PA
123. Brereton RG (1997) Multilevel multifactor designs for multivariate analysis. Analyst 122:1521–1529
124. Petiot E, Bernard-Moulin P, Magadoux T, Gény C, Pinton H, Marc A (2010) In situ quantification of microcarrier animal cell cultures using near-infrared spectroscopy. Process Biochem 45:1427–1431
125. Rhiel MH, Amrhein M, Marison IW, von Stockar U (2002) The influence of correlated calibration samples on the prediction performance of multivariate models based on mid-infrared spectra of animal cell cultures. Anal Chem 74:5227–5236
126. Petersen N, Ödman P, Padrell AEC, Stocks S, Lantz AE, Gernaey KV (2010) In situ near infrared spectroscopy for analyte-specific monitoring of glucose and ammonium in streptomyces coelicolor fermentations. Biotechnol Prog 26:263–271
127. Brereton RG (2007) Applied chemometrics for scientists. Wiley, Chichester
128. Munoz JA, Brereton RG (1998) Partial factorial designs for multivariate calibration: extension to seven levels and comparison of strategy. Chemometrics Intell Lab Syst 43:89–105
129. Rhiel M, Ducommun P, Bolzonella I, Marison I, von Stockar U (2002) Real-time in situ monitoring of freely suspended and immobilized cell cultures based on mid-infrared spectroscopic measurements. Biotechnol Bioeng 77:174–185
130. Doak DL, Phillips JA (1999) In situ monitoring of an *Escherichia coli* fermentation using a diamond composition ATR probe and mid-infrared spectroscopy. Biotechnol Prog 15:529–539
131. Franco VG, Perín JC, Mantovani VE, Goicoechea HC (2006) Monitoring substrate and products in a bioprocess with FTIR spectroscopy coupled to artificial neural networks enhanced with a genetic-algorithm-based method for wavelength selection. Talanta 68:1005–1012
132. Mazarevica G, Diewok J, Baena JR, Rosenberg E, Lendl B (2004) On-line fermentation monitoring by mid-infrared spectroscopy. Appl Spectrosc 58:804–810
133. Roychoudhury P, Harvey LM, McNeil B (2006) At-line monitoring of ammonium, glucose, methyl oleate and biomass in a complex antibiotic fermentation process using attenuated total reflectance-mid-infrared (ATR-MIR) spectroscopy. Analytica Chimica Acta 561:218–224
134. Dabros M, Amrhein M, Bonvin D, Marison IW, von Stockar U (2007) Data reconciliation of mid-infrared and dielectric spectral measurements for improved on-line monitoring of bioprocesses. Biotechnol Prog 25(2):578–588
135. Fayolle P, Picque D, Corrieu G (2000) On-line monitoring of fermentation processes by a new remote dispersive middle-infrared spectrometer. Food Control 11:291–296
136. Kornmann H, Valentinotti S, Duboc P, Marison I, von Stockar U (2004) Monitoring and control of Gluconacetobacter xylinus fed-batch cultures using in situ mid-IR spectroscopy. J Biotechnol 113:231–245
137. Schenk J, Marison IW, von Stockar U (2007) A simple method to monitor and control methanol feeding of *Pichia pastoris* fermentations using mid-IR spectroscopy. J Biotechnol 128:344–353

138. Cervera AE, Petersen N, Lantz AE, Larsen A, Gernaey KV (2009) Application of near-infrared spectroscopy for monitoring and control of cell culture and fermentation. Biotechnol Progr 25:1561–1581
139. Crowley J, Arnold SA, Wood N, Harvey LM, McNeil B (2005) Monitoring a high cell density recombinant *Pichia pastoris* fed-batch bioprocess using transmission and reflectance near infrared spectroscopy. Enzyme Microb Tech 36:621–628
140. Rodrigues L, Vieira L, Cardoso J, Menezes J (2008) The use of NIR as a multi-parametric in situ monitoring technique in filamentous fermentation systems. Talanta 75:1356
141. Arnold SA, Crowley J, Woods N, Harvey LM, McNeill B (2003) In-situ near infrared spectroscopy to monitor key analytes in mammalian cell cultivation. Biotechnol Bioeng 84:13–19
142. Roychoudhury P, O'Kennedy R, McNeil B, Harvey LM (2007) Multiplexing fibre optic near infrared (NIR) spectroscopy as an emerging technology to monitor industrial bioprocesses. Anal Chim Acta 590:110–117
143. Vaccari G, Dosi E, Campi AL, Mantovani G, Gonzalez-Vara y RA, Matteuzzi D (1994) A near-infrarod spectroscopy technique for the control of fermentation processes: an application to lactic acid fermentation. Biotechnol Bioeng 43:913–917
144. González-Vara y RA, Vaccari G, Dosi E, Trilli A, Rossi M, Matteuzzi D (2000) Enhanced production of L-(+)-lactic acid in chemostat by *Lactobacillus casei* DSM 20011 using ion-exchange resins and cross-flow filtration in a fully automated pilot plant controlled via NIR. Biotechnol Bioeng 67:147–156
145. Cimander C, Mandenius C (2002) Online monitoring of a bioprocess based on a multi-analyser system and multivariate statistical process modelling. J Chem Technol Biotechnol 77:1157–1168
146. Gomy C, Jouan M, Dao NQ (1988) Methode d'analyse quantitative par spectrometrie Raman-laser associee aux fibres optiques pour le suivi d'une fermentation alcoolique. Anal Chim Acta 215:211–221
147. Shaw AD, Kaderbhai N, Jones A, Woodward AM, Goodacre R, Rowland JJ, Kell DB (1999) Noninvasive, on-line monitoring of the biotransformation by yeast of glucose to ethanol using dispersive Raman spectroscopy and chemometrics. Appl Spectrosc 53:1419–1428
148. Cannizzaro C, Rhiel M, Marison I, von Stockar U (2003) On-line monitoring of *Phaffia rhodozyma* fed-batch process with in situ dispersive Raman spectroscopy. Biotechnol Bioeng 83:668–680
149. Lee HLT, Boccazzi P, Gorret N, Ram RJ, Sinskey AJ (2004) In situ bioprocess monitoring of *Escherichia coli* bioreactions using Raman spectroscopy. Vib Spectrosc 35:131–137

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