Part One

Overview of State-of-the-Art Technologies and Challenges

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Continuous Bioprocess Development: Methods for Control and Characterization of the Biological System

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1.1 Proposed Advantages of Continuous Bioprocessing

1.1.1 Introduction

The change from batch to continuous processing has led to the intensification of processes in a number of industries, including steel casting, automobile and other devices, petrochemicals, food, and pharmaceuticals. Advantages include, aside from a significant increase in volumetric productivity, reduced equipment size, steady-state operation, low cycle times, streamed process flows, and reduced capital cost.

In bioengineering, continuous processing is the standard in wastewater treatment, composting, and some bioenergy processes such as biogas and bioethanol fermentations. In contrast, most production processes run as batch type operations or more specifically fed-batch processes, which is the major production technology today.

Konstantinov and Cooney provide a definition of a continuous process as "A unit operation is continuous if it is capable of processing a continuous flow input for prolonged periods of time. A continuous unit operation has minimal internal hold volume. The output can be continuous or discretized in small packets produced in a cyclic manner." [1]. They also differentiate between full continuous processes with no or minimal hold volume in the process line or hybrid processes that contain both batch and continuous process operations.

Obviously, the push in continuous manufacturing technologies was initiated by the BioPAT initiative of the Food and Drug Administration (FDA) in 2002 and the published guidance to PAT in 2004 [2], which initially aimed at a better understanding of the connections between product quality and process conditions. This lead to the need to develop quality by design (QbD), that is, the implementation of process analytical tools over the whole developmental pipeline from early product screening over the process development in the laboratory scale and during scale up. The needs for a better understanding of the impact of process parameters on the critical quality attributes (CQA) of the respective product also increased the interest in the development and implementation of novel sensors and analytical

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tools. As a consequence, this better understanding of processes resulted in further process intensification and provided the instrumental basis to approach challenges in relation to continuous operation.

Aside the FDA initiative, there are several drivers for the increasing interest in continuous processing, not only in the pharmaceutical industry but also in the industrial (white) biotech industry. On one side, we see an increasing demand and thus also increasing production scale for industrial bioproducts (enzymes, small molecules, and bioenergy market) with a need for reduced costs for the products and increased competition. Considering that production scales are steadily growing and that a scale reduction close to factor 10 would be possible by continuous processing, plant sizes and the efficiency of bioprocesses could be increased significantly. On the other side, the opportunity of the selection of new biocatalysts and its implementation in the chemical synthesis for integrated chemoenzymatic processes (i.e., processes which combine chemical and enzymatic reactions) have to be competitive with the existing chemical processes and need to be integrated into the chemical production schemes. Here, continuous processes offer clear advantages.

In biopharma for recombinant proteins, antibodies, highly complex proteins, recombinant enzymes and blood factors, the efficiency of the cell factories, and production systems have dramatically increased during the last decade. Opportunities for high cell density processes with a higher volumetric product yield and quality, as well as the changing situation in view of the intellectual properties by the termination of many patents for important drugs with novel commercial opportunities for new biosimilars and biobetters are a strong driver in increasing the competition especially from emerging markets. In parallel, there is an increasing demand for establishing local production sites for defined regional markets, rather than having single production sites. Strict cost calculations as a developmental driver demand for smaller and effective, but also flexible production plants. This directs interest to evaluate continuous bioprocessing opportunities to minimize investments for production facilities, and thinking about parallelization rather than larger scales. Parallelization would also be an advantage in processes with longer plant cycle times [3] as, for example, cell culture-based products. A nice example that shows the opportunities in significantly decreasing operational and capital expenses by changing from conventional bioprocessing to continuous bioprocessing in the case of production on monoclonal antibodies (mAB) and other non-mAB processes is shown by Walther *et al.* [4].

However, despite the obvious opportunities of continuous processes there are many challenges to solve, mainly the demand for fast realization and risk minimization. Currently, it seems to be easier to transfer a batch process into production than to start a new, longer, and more expensive development of a continuous process even though it is expected to be more efficient.

These scenarios show that there is a big need in strategic methods concerning the development of continuous process strategies for either new products or to derive a continuous process from existing batch type processes. As early-phase product development can practically be only performed as batch processes, a key question in product development is how we can transfer a batch strategy to a continuous operation in a large process.

Specific challenges for continuous operations in the biotech industry compared to other industries are (i) the inherent nature of a natural whole cell biocatalyst, that is, a prokaryotic or eukaryotic cell, for steady evolution of its genome. (ii) Biotechnological processes generally need much higher amounts of catalyst compared to chemical industry. This biocatalyst has to be maintained by feeding. Thereby, the feed is mostly the same substance as the original substrate for the biochemical reaction and thus it competes with the yield. As a consequence, diffusion and mass transfer in the reactor have not only an effect on the efficiency of the process but also are critical for long-term operation and maintenance of the product quality.

Especially in view of the evolutionary adaptation, it is obvious that in contrast to batch procedures, continuous operation cannot be set up by trial and error but needs a fundamental basic understanding of the process in kinetic terms to allow a control of the process. This is a fundamental paradigm change in bioprocessing industries, where most operations included only a limited application of mathematical models. Also, available sensors and ways for process control are traditionally very limited.

Although current examples of so-called continuous cell culture processes in the pharma area are only semicontinuous if compared to, for example, biogas or wastewater bioprocessing, the expansion of cell cultivation processes from days to months, for example, by the perfusion technology, goes into the direction of continuous bioprocessing. However, also for these continuous bioprocess operations a big amount of labor and time is needed to establish a new process as a continuous production system. Similar as in traditional biotech, the development is currently mainly based on wet-lab experiments rather than on a systematic developmental approach. This raises the question, whether this is the only way that has proven to be successful or whether a paradigm change in the application of technologies is needed to advance the field of continuous bioprocessing. In this chapter we will discuss the methods and data which are needed to develop a continuous operation with a focus on modeling approaches.

1.2 Special Challenges for Continuous Bioprocesses

1.2.1 The Biological System in Continuous Biomanufacturing

In process engineering, there are different reactor designs typically used depending on the characteristics of the reaction system. It should be pointed, that continuous stirred tank reactors (CSTR) are known to have some disadvantages over plug flow reactors (PFR), which are typically used in the chemical industry and batch and fed-batch type of cultures – the most important being a lower concentration of product due to a constant dilution. In chemical processes, typically PFRs are preferred when high yields are required, and batches are chosen when low volumes of different product are to be produced. In addition, considering process development, continuous processes require higher investments and times at small scales. Hence, even in the chemical industry many processes with similar characteristics to protein synthesis run in PFR or batch. Still there are

many process setups that allow a significant reduction in footprint, time, and costs by using continuous processes and these should be considered in bioengineering.

Now let us focus on the processes that should run continuously in bioengineering. Continuous bioprocesses are characterized by a continuous addition of substrates and simultaneous harvest, thus that the bioreactor volume stays approximately constant. If the product is released from the cell and accumulates in the culture broth, recycling by filtration units or centrifuges can be applied and this separation can be supported by immobilization of the biocatalyst. Alternatively, the bioprocess can be performed in solid-state fermentation processes where the medium runs through a static matrix where the biocatalyst is immobilized.

The biological system, which is the core component of a biotechnological production process, has many specific features that favor continuous production on one side, but also cause the specific challenges that restrict continuous processing in the biotech industries so far.

In difference to chemistry, where continuous operation became a standard, and where the change from batch to continuous operation has contributed to a significant drop in reactor sizes and investment costs and lead to modularization of production plants, bioprocesses are rather different. To understand the special challenges of continuous bioprocessing, it is important to analyze these differences between chemical and biocatalytic processes, both of which are different routes for the same outcome – the production of a chemical molecule.

A chemical reaction is characterized by the high yield of the reaction from a substrate into a product using small amounts of catalysts (order of μg/l or mg/l). In contrast, in a bioprocess the catalyst is the (micro)-organism, which has to be produced from the substrate in a very high concentration (in industry mostly 50–100 g/l). This is mostly done in the first phase of a process (called growth phase). The product is not produced until the second phase, called production phase, which may be equal in length or even shorter than the growth phase. Interestingly, the product reaches low yields if compared to single reaction chemical processes. Highest concentrations of bioproducts are in the order between 100 and 200 g/l, or up to the order of 10–100 g/l for more complex molecules, and for many processes the yield is even lower. Generally, the yield of the product from glucose is significantly lower than 0.1 g/g since most of the glucose goes in the biomass, that is, the production of the biocatalyst. If one assumes that after biomass production the cells could be used to produce the product over unlimited time (considering that substrate required for the own maintenance is less than 10% of the amount needed for the growth phase), a significant increase of the product yield per substrate in a bioprocess would be possible. Furthermore, if turn-around times are avoided, such as harvesting of the reactor, cleaning, and preparation of the new batch including preparation of the starter cultures, time costs would also be reduced further increasing process efficiency.

1.2.2 Inherent Changes in the Microbial System – Problem of Evolution

In contrast to continuous processes in chemical engineering, bioprocesses include a perpetual evolution process, that is, a genetic change of the biocatalyst.

The rate of mutations has been extensively discussed and investigated, mainly in the context of how a continuous culture can be used for strain evolution.

However, it needs to be stated that the problem of evolution is inherent to any bioprocess. In a bioprocess the operation always starts with a very low number of cells that are multiplied during inoculum preparation and scale-up from flasks to the final size bioreactor. For a large scale bioreactor running at a scale of $10\,m^3$ (which is small in view of most common microbial bioprocesses) and a final cell number of 1.2×10^{18} for a bacterial process (i.e., 1.2×10^{11} cells/ml),[1] this makes approximately 60 generations from a selected one-cell clone and even approximately 27 generations from an inoculum stock. If the calculations concerning the accumulation of mutations as performed by Ref. [5] were assumed to be 1×10^{-3} nucleotide substitutions per genome in each generation for *Escherichia coli* [6] and 4×10^{-3} nucleotide substitutions per genome per generation in *Saccharomyces cerevisiae* [7], and would be adopted to the considered large-scale cultivation case, when assuming a homogenic frozen stock culture (which is not the case), there would be at the end of the cultivation a probability of approximately 30 mutations per each position of the nucleotide sequence of *E. coli* and 0.07 mutations per position for the yeast *S. cerevisiae*. This is an incredible diversity, which is rarely applied in molecular evolution experiments and still not exploited. These considerations may even underestimate the possible mutation frequency, as discussed in detail for chemostat environments by Ferency [8]. Therefore, it is a great challenge in continuous cultivation to direct evolution in order to guarantee continuous production of a constant product with equal quality despite the steady evolution process. While this is greatly ignored in industrial batch processes, this evolution and selection of the fittest is a critical point in continuous processing.

What can be learned from a large number of evolution experiments, especially from the extensive work of Lensky *et al*. [9], is that characteristic changes occur stepwise, that is, suddenly. Also, most importantly, fitness gain is constant with steady improvements without an upper end even in very long experiments over (tens of) thousands of generations. Experiences with natural evolution and steady fitness gain with a selection pressure for important cellular parameters, like affinity constants and maximum specific rates as described in the model below, and steady change of them by mutations [5] are a clear advantage if the continuous culture is aimed at degrading a compound which serves as a key substrate, like in degradation of wastes. However, the same process is a challenge for control in production of biomolecules where it is not possible to set a selection pressure toward the product.

1.2.3 Lack of Process Information

Historically, the low reproducibility, observability, controllability, and understanding of the biotechnological processes has driven large-scale production to an approach based on: (i) as fast as possible after inoculation, (ii) as fast as possible after induction, (iii) as fragmented as possible to avoid mixing of failed charges with high purity product. For these reasons, the fed-batch technology has become the most widely

1 Assuming $1\,g/l = 2 \times 10^{12}$ cells/l, final dry cell weight of 60 g/l.

applied standard upstream production method in large scale. Consequently, all other processes taking place before (pretreatment and medium preparation) and after (down-stream operations) were developed to meet the requirements of discrete production plants. Despite this success of the fed-batch technology, it is remarking that continuous bioprocesses have been early established [10] in parallel to the development of the fed-batch technology by Hayduck [11].

Today's advances in technology are paving the way for continuous systems with improvements in PAT, higher automation of the production, advanced control strategies, methods to direct and apply the naturally occurring evolution process to higher productivity, and most importantly regulations that promote continuous processes. This may clearly offer significant advantages by solving important challenges as are higher overall productivity, lower risk of infection, smaller reaction vessels and plants.

1.2.3.1 Models-Based Process Development and Control for Continuous Processes

In bioprocess engineering, models are used for process design, monitoring, control, and optimization [12–16]. Bioprocess complexity and restrictions have driven design and control to demand accurate and robust models [17], triggering a rapid development over the last years [18]. Advanced sensor techniques and fast computer processors enable the creation of very complex models processing enormous amounts of information [15,19]. Models are not only used to describe the behavior of living organisms but also essential to map complex systems into smaller dimension and also to obtain indirect measurements and observe non-observable events when applied as software sensors [20], for example.

The new regulations of the PAT initiative of the FDA and EMA show the importance that modeling applied to process monitoring and control is gaining in the pharmaceutical and in general in biotechnological processes [21].

Biological processes are characterized by [22–25]

- the complexity of the biological processes taking place in the bioreactor
- lack of reproducibility
- monitoring of unstable intracellular compounds at very low concentrations
- extremely fast and sensible reaction to environmental changes (offline measurements are inaccurate)
- mutations
- insufficient sensor technology
- expensive and inaccurate sensors
- highly invasive
- difficult to calibrate
- large time delays and low frequency of observations

1.2.3.2 Engineering Approach to Complex Systems

In chemical engineering, the implementation of different methods to deal with large complex systems has a long history. Engineers have developed methods like hierarchical modeling, model reusability, model inheritance, and so on. An extensive discussion of these methods and their application for the simulation

of chemical plants is presented by Barton [12]. In biological systems, the modularization of separated instances of the system is not always possible. In traditional process engineering, a pump can be modeled in a modular form and then added to the flow sheet of the plant and reused as many times as needed [26]. Contrary to this, biological systems tend to show different behavior under *in vitro* conditions compared to their *in vivo* state [27]. Still, some approaches intend an analysis and modeling of biological systems with methods taken from engineering [28,29]. Kitano [30,31] emphasizes that the only possibility to understand living organisms is to consider the system as a whole. Identifying genes and proteins is only the first step, whereas real understanding can only be achieved by uncovering the structure and dynamics of the system. Kitano states the following four key properties [30]:

- *System structure*
  System structure identification refers to understanding both the topological relationship of the network components and the parameters for each relation.
- *System dynamics*
  System behavior analysis suggests the application of standardized techniques such as sensitivity, stability, stiffness, and bifurcation.
- *The control method*
  System control is concerned with establishing methods to control the state of biological systems.
- *The design method*
  System design is the effort to establish new technologies to design biological systems aimed at specific goals, for example, organ cloning techniques.

For this reason, two things are necessary in order to control a biological system and comply with the strict food and pharmaceutical regulations, namely, to observe or at least deduce the state of critical quantities and to predict to some extent the behavior of the system. The first issue is tackled using state and parameter estimation methods [32–36] in an effort to infer the conditions of the process using the information that is available. Second, the evolution of the system over time as well as its response to the control actions, characterized by nonlinear dynamics, can be predicted using mathematical models.

1.2.4 Limited Control Strategies

1.2.4.1 Traditional Control Strategies for Continuous Cultures

As the chemostat works at a preset dilution rate without any feedback control, it cannot stabilize in a process that runs close to the maximum specific growth rate. Although the chemostat is the most used continuous culture technique in research, it is rarely applied in industry. In this context other processes with a feedback control loop have developed, such as the turbidostat and the pH auxostat.

The *turbidostat* (see Ref. [37] for an excellent early review) is a continuous process where the feed rate is controlled by the online measurement of the turbidity, mostly in the outlet stream. By maximizing the flow rate at a high biomass concentration, the turbidostat can operate the process at μ_{max} and, at the same time, avoids outwashing of the biomass. Therefore, it is generally applied in

processes where the flow rate should be maximized, but while maintaining the cells in the system, for example, in processes which aim the degradation of toxic compounds in wastewater treatment, or directly for the production of biomass (single cell protein), or other growth-related molecules. The turbidostat has been also a powerful tool for the selection of faster growing strains, that is, in natural selection, due to its permanent adaptation of the flow rate [38–40].

The necessity of the turbidostat to have a continuous measurement of the turbidity limits its applications to systems with no biofilm formation at the walls. Therefore, the *pH auxostat (pH stat)*, which uses the pH as a state control variable, is more robust, but is also more sophisticated in terms of the design of the medium. In the pH-auxostat the flow rate is set by the pH controller through the feeding of fresh medium to keep the pH constant. Thus, the pH auxostat can be used only for processes where biomass growth is closely correlated to changes on the pH [41]. Early pH auxostats [42,43] were typically applied for microbial processes with acidifying products, for example, in the dairy fermentation [44] or similar anaerobic processes. Such processes needed special pretreatment of the fed medium that had to be preadjusted for a certain pH and thus the biomass concentration in the reactor depended on the difference between the pH difference between the feed solution and the fermenter broth as well as on the buffer capacity of the medium, and normally not high cell densities were obtained [42].

The theoretical solution of the performance of a pH auxostat with two inlet flows, medium and a pH controlling agent, by Larsson *et al*. [45] made the pH auxostat more easy to handle and applicable as a tool also for aerobic processes. The kinetic model contains an extra function that calculates the hydrogen ions in the added base, and considers that the added new medium has the same pH as the control point for the pH in the bioreactor. The process is controlled by the definition of the inlet flow ratio of the two inlet streams, which can be easily set. This inlet flow ratio is a parameter that provides a reliable tool for process optimization. This principle was adapted to processes with an ammonia (NH_4^+) feed, for which the H^+ concentration is in good relation with the cellular uptake of NH_3 and the yield coefficient for hydrogen ions on substrate used is constant, such as for *S. cerevisiae* [41].

A third principle for control of a continuous bioprocess, namely, the nutristat, aiming at maintaining the substrate concentration at a certain level, has found less application. If online methods for the determination of the substrate are available, the nutristat is well suited to run a process at higher growth rates which are lower than μ_{max}. This is a clear advantage over the pH- or turbidostat. However, an interesting study by Rice and Hempfling [46] shows that even a pH stat can run stable at different concentrations of the growth limiting substrate, that is, specific growth rates, by variation of the substrate concentration in the feed solution or by changing the buffering capacity of the feed solution. The nutristat is clearly useful for the degradation of waste compounds, as shown, for example, by Refs [47,48]. When using glucose as substrate, the nutristat can be applied [48], however due to the low K_S value and high specific substrate uptake rate, a proper control below μ_{max} is becoming challenging, especially at high cell densities.

A special solution for culture processes with production of secreted products, such as monoclonal antibodies, at low or even zero growth rates is cell

recycling. The theory of continuous cultures with cell recycling was already developed and experimentally proven in Ref. [49] based on the idea of continuous culture with cell recycling [50]. While in these processes the theory of a chemostat (see above) is valid with the cell recycling term (which can be from 0 to 1), most production processes rely on higher growth rates, as the metabolic activities at low growth rates are low and the energy supply is going to the maintenance mainly. However, the cell recycling (or cell retention, retentiostat, perfusion culture) is a practical method to increase degradation capabilities, for example, in waste treatment or maintaining organisms for which even μ_{max} is very low.

The method of perfusion culture is widely and very successfully applied in mammalian cell culture and similar processes with a low growth rate, where, for example, the production of monoclonal antibodies can be stably maintained for longer times (see Chapter 7 of this volume). Cell retention in these systems today is mainly achieved either by immobilization of the cells, by filtration, for example, use of alternating tangential flow filtration, or by centrifugation [51,52].

1.3 Changes Required to Integrate Continuous Processes in Biotech

1.3.1 A Better Physiological Understanding of the Organisms and Their Responses on the Reactor Environment

1.3.1.1 Model Complexity

The biggest challenge for modeling is to develop a general and systematic approach to find the simplest manner to describe complex systems aiming at the strictly required accuracy. The meaning of model simplification becomes more important with the increasing complexity of bioprocesses analyzed in research. The complexity of biological processes makes it very difficult to fully describe cultivations using a computer model. To name one example consider the phenotype of a microbial cell determined by >30 million macromolecules, >1000 species of small organic molecules fine-tuned in composition and number to the comprehensive set of its environmental factors [53].

In order to achieve a robust and efficient continuous process, a close monitoring of the system and a tight control are required. Due to the complexity of the microbial behavior, standard feedback control methods are not adequate and more advanced methods are required. Process control has a long tradition in development of model-based techniques, for monitoring (e.g., softsensors, observers, moving horizon estimators) and control (e.g., model predictive control, adaptive control). These methods relay on a mathematical model that fulfils some specifications as are follows:

- Ability to accurately describe the dynamic behavior of the system
- Identifiability
- Tractability

This obliges a close interaction between control experts and bioengineers in order to develop control systems that assure a secure process that will fulfill quality regulation with high reliability.

1.3.1.2 Models

A model is a poor mathematical representation of a physical system. Lack of accurate knowledge of the process to be modeled, insufficient measurement techniques, and extensive computation time hinder an exact representation of the phenomena to be described [54]. Nevertheless, models are widely used in science and their contribution to a better understanding of engineering processes and their proper design, optimization and control is out of question. Computer aided tools using model-based methods allow optimal design and operation of plants, reducing energy consumption, hazard, and environmental impact, while allowing better monitoring and control [12].

From this it can be deduced that the best model to describe a certain process is not necessarily the most accurate, but the one that describes only the relevant aspects of the system so as to get a good description with minimal effort [55].

Modeling includes a wide number of tools [56] as are principal component analysis (PCA) or partial least squares (PLS) [57], nonlinear models like neural networks [58] and also multivariate statistics [57]. Roughly said, these methods search for data correlation to reduce the dimension of the data set [59]. Also more advanced methods in knowledge discovery of data (KDD) like data mining [60] have been developed for treatment of large data sets and are applied in bioinformatics. Still, generally speaking, first principle modeling (white box) is the preferred approach to describe a complex system when mechanistic understanding (mass balances, thermodynamics, kinetics, etc.) is at hand [25,61–63]. These methods study the data characteristics to find new relations between variables and create black-box type models that describe it. By these means it is possible to look through high dimensional data and detect the most important characteristics of the system [64–66].

Contrary to black box models, mechanistic models are based on physical knowledge of the system to be described. In engineering, for example, rigorous modeling includes mass and energy balances, detailed reaction pathways, and so on. Models are the core of computer aided process engineering (CAPE) [67] and computer aided biology (CAB). The quality of every work on simulation, optimization, design, and model-based control, depends on the characteristic of the model. In engineering, models are not only used to describe the behavior of systems but also essential to map complex systems into smaller dimension more comprehensible to humans. Finally, they also serve to obtain indirect measurements of states or parameters of interest with software sensors [20], for example. Software sensors substitute measurements, which are not possible due to physical limitations, with models which predict the behavior of the nonmeasurable variable based on indirect measurements. Whenever a state of the system is to be determined, observer can be applied [35,36] or the Kalman filter [33] with its variations [34].

In system biology, various methods exist aiming at an adequate description of the dynamics of living organisms studying their gene regulatory networks [68].

Still, differential equation systems settle the standard modeling method in engineering. Systems of ordinary differential equations (ODE) have been widely applied for the description of gene regulatory networks. Usually, the system comprises rate equations of the form

$$\frac{dx_i}{dt} = f_i(x, u), \tag{1.1}$$

where x is the vector of concentration of proteins, mRNAs, or other molecules, u the vector of inputs, and f_i is a nonlinear function. Also, time delays can be added if necessary. Typical types of equations used are Monod type, switching, Heaviside, and logoid functions among others. An important advantage of nonlinear ODEs is the possibility to describe multiple steady-states and oscillations in the system [69]. Besides the requirement of testing the global convergence of the optimal solution, the bottleneck is still the state information of the parameter set creating identifiability problems. Nevertheless, many successful applications have been published showing the possibilities of ODEs to describe gene regulatory networks [70].

Although today gene regulatory network models are not applicable in industrial scales, it can be expected that systematic conversion of complex gene regulatory network models in simple tractable models will be possible in near future. Nevertheless, model complexity is closely related to instability, over parameterization, parameter correlation, and low parameter identifiability [71]. The effort required to develop and fit a model has to be justified by its application. It is useless to apply computational fluid dynamics (CFD) to the simulation of a 1 l reactor knowing that the concentration gradients can be neglected. On the other hand, simulating a reaction in a tank with 10 000 l without considering mass transfer limitations may yield in results far from reality. Summarizing, the key dynamics of a system need to be identified, isolated, and analyzed before any model is built. Currently, limitations are mainly due to the scarcity of measurement possibilities but also to the insufficiency of adequate mathematical tools.

1.3.2 Model-Based Process Monitoring

A key task in process control is to monitor the critical states and parameters of the process in order to secure proper operating conditions and desired quality even under perturbations and model mismatch [72,73]. Unfortunately, bioprocesses are characterized by their low information content caused by low concentrations, complex media, and the lack of noninvasive online sensors that can measure intracellular concentrations [74]. To overcome these problems, model-based methods to infer the conditions of the process can be applied. There is a long list of methods and applications for online state estimation [32], state observers including the classical Kalman filters [33] with their variations [34] and nonlinear observers [35,36]. Some authors use the expression software sensor or softsensor [75,76] in account of the fact that a "software" or computer-based calculation of a nonmeasurable variable which is not always a state variable, like the respiration coefficient, provides more information than the initial variable that can be directly measured.

1.3.3 Implementation of Model Predictive Control

1.3.3.1 Model-Based Control

Advances in computer capacity, sensor technology, and a better understanding of the biological system are giving place to very successful applications of advanced control strategies in continuous processes [77,78]. In the case of continuous processes, different approaches have been developed and successfully applied. Some representative examples are classical approaches to various control strategies [79–84] and state feedback control strategies [85,86]. Additionally, efforts to exploit the data generated using neural networks [64,87,88] without the need of a thorough understanding of the system have been shown. In an effort to simplify the control strategy, fuzzy logic controls [89] have also been used in bioreactors. Furthermore, advanced methods using model predictive controllers [64–66], and even based on population models [22] can be found in literature. An interesting approach is to overcome the limitations of the existing models by performing recursive estimation of its parameter estimates. By these means, models can be used also in processes that change over time. Some proves of the potential of these methods are the use of adaptive control techniques [90–92]. Finally, investigation of complex formulation for optimal control show the controllability potential of biological systems if simplified mathematical descriptions succeed to predict the dynamics of the process [93,94].

In general, the theory of monitoring and control is used under the assumption that the system to be described is time invariant and properly described by the model. Nevertheless, there exists the possibility to adapt the observer to changing system behavior by estimating the model parameters together with the states in order to adapt the model to changes in the system. This is called adaptive control [95] and proved to be very effective for many applications including bioreactors [90]. Adaptive control can be used to overcome structural deficiencies of the model as well as uncertainty in the parameters. Still an important drawback is the need of increasing information in order to find accurate estimates of both, the parameters and the states. Methods for moving horizon estimation can be used to increase the robustness of the parameter estimates if sufficient computer capacity is at hand [96]. These methods are especially relevant in biotechnology application since changes in the system (e.g., between cultivations, mutants, over time) can be observed by small variations in the parameters without the need of a change in the model structure.

1.4 Role of Iterative Process Development to Push Continuous Processes in Biotech

1.4.1 Methods for Development of Continuous Processes

In general, bioprocess development suffers from significantly longer times and costs compared to other industries [97,98]. Additionally, development of continuous processes follows different strategies than batch processes. While it is advantageous to implement the cultivation strategy at the screening or product

development stage, it is difficult to implement continuous strategies in the early phases. Thus, the successful development of continuous bioprocesses depends to a bigger part than batch bioprocess development on a comprehensive understanding of the biological system. While batch processes have been traditionally developed with trial-and-error approaches, this is not possible for continuous processes so that the implementation of robust process control strategies is an important basis.

A key parameter for any bioprocess is the specific product formation rate q_p. This rate has a close connection to the specific growth rate, but this correlation is different for different products. In many cases there is no linear dependency, but q_p has an optimum below μ_{max}. Thus, to run a process with a high productivity, it is a major task in continuous bioprocess development to find this correlation between μ and q_p.

Traditionally, this is performed in chemostats, which is a long-lasting process, as the steady state must be established for each dilution rate, which takes about four to seven reactor exchanges. While generally in scientific investigations with chemostats more steady states are established in a series from an initial batch process, such consecutive long-time cultivation can lead to the selection of mutants and thus has to be performed with good controls, for example, by returning to the original dilution rate in the end of an experiment.

While for process development systems for the parallel performance of continuous bioreactors would be very interesting, the setup of such systems is a technical challenge. Parallel chemostats can minimize the problem of evolutionary selection by running experiments with different dilution rates in parallel. However, parallel experiments benefit from miniaturization, especially if big liquid volumes are handled like in the case of chemostats. Although miniaturization has made a big progress in discontinuous cultivation technologies, it is difficult to achieve in the milliliter and submilliliter scale when well defined and controlled dilution rates must be guaranteed over long time intervals.

In the past a number of approaches have been realized for parallel continuous mini and microbioreactors. Balagadde *et al.* [99] developed a microchip-based circulating loop bioreactor with a segment-wise sterilization option to avoid biofilm formation. They demonstrated this reactor for cultivations with *E. coli*. The authors observed oscillations in the cell number. No other online parameters were measured. The feed control, steady states, and so on, were not characterized. The system was developed to investigate evolution, but probably it would not be applicable in its current form for process development.

Nanchen *et al.* [100] used a 10-ml parallel continuous culture system in 17 ml Hungate tubes for 13C labeling and metabolic flux analysis. Aeration with 2 vvm, was used to also mix the system, feeding was done by a peristaltic pump, pH was measured in outlet stream and DO by a microelectrode. The system was characterized with steady states obtained over five volume changes and compared against stirred tank reactor cultivations. The system is very simple to establish and is also very valuable for parameter estimation for continuous process development. A shaken system that can be easily parallelized was developed by Akgün *et al.* [101]. The authors developed a continuous bioreactor based on shake flasks

with a controlled feed, an overflow outlet channel at the side of the flask, and a top-phase aeration. While this system was extensively characterized in connection to filling volume constancy and different technical parameters and applied for a continuous culture of *S. cerevisiae*, the system has possibly so far not been applied for other studies. In view of real microbioreactors a modification to a commercial 48 bioreactor system (2 mag, Munich, Germany) was recently published by Schmideder *et al*. [102] showing first promising results for the feasibility of transferring this system to a continuous bioreactor. However, in this case so far only eight of the bioreactors were connected and the determination of key parameters, such as the K_s value had a relatively high error. While continuous cultivation until seven volume exchanges was possible, the presented data do not allow deep-going evaluation about the quality of the steady states, which even has been a problem in larger continuous cultivations.

A faster and elegant method for obtaining the necessary cellular parameters and characteristics with a lower effort compared to parallel chemostats is the A stat technology [103]. With this technology, it is possible to scan the whole growth rate space of an organism in a single experiment [104], either by continuously increasing (Acelerostat) or by decreasing (Decelerostat) the dilution rate in a way that the culture always is in a steady state. However, the technology even has wider use by applying this concept also to the continuous change of other parameters than the feed rate of the limiting substrate; therefore, the term changestat was introduced [104]. This technology of the Gradiostat was applied by various authors, but only Vilu *et al*. developed the scientific basis for it and showed the strength in view of data collection over the whole growth range (see Chapter 9 of this volume).

1.4.1.1 Alternative: Fed-Batch as a System to Simulate Quasi Steady-State Conditions

The strength of the A-stat technology, screening the whole growth space in a single experiment, starting with either a high or a low specific growth rate, is principally also possible by the application of the fed-batch technology. Here, in a similar way as in the A-stat, one can realize a feeding rate, which leads to a continuous gradual change of the specific growth rate of the system. However, in difference to a real continuous culture technology, the fed-batch is more easily to apply in high throughput approaches, as standard (micro)-reactors can be used. Simply instead of having an outgoing steam, one can live with the typical volume change of a fed-batch, which is dependent on the substrate concentration in the feed solution and the feed rate.

Although the controlled feeding to miniaturized cultures is still a challenge, first solutions are available which provide an interesting technological basis, such as 48 microplate base real feeding systems with integrated bottom channels and a hydrogel filling the capillaries [105], or even the integration of micropumps [106]. Alternatively, and more easy to use, would be systems with internal release of the substrate either from silicon as the feed-bead technology [107] or the Feed plate® (PS Biotech, Aachen, Germany). While these systems rely simply on the diffusion of the substrate into the medium, the EnBase® technology [108] (for a comprehensive review see Ref. [109]), which is based on a biocatalytic release of glucose

from a polymer of glucose, allows easily varying the feed rate by the amount of the added biocatalyst. Thus, it is easy with this technology to screen for a wide range of growth rates in microplates or parallel shake flasks [108,110]. Recently, this technology was applied with the aim of finding an optimal specific growth rate for the specific production rate of a secreted heterologous enzyme in the yeast *S. cerevisiae*. In comparison to the A-stat technology the same optima was found, but in a very much shorter time [111].

As it was discussed above, the big challenge for the future is, to combine model-based and experimental approaches for continuous bioprocess development. As models in this direction must provide knowledge on the system, mechanistic models, rather than black-box approaches are needed. Therefore, it is necessary to develop design of experiment (DoE) approaches which allow the fast estimation of parameters of nonlinear models. Traditionally, in other disciplines this has been done with online optimal experimental designs (OED). In a recent study, we have applied this approach also to identify the parameters of a dynamic model for *E. coli* cultivations [112]. The strength of the application of the fed-batch approach with a model-based DoE with a sequential reoptimization of the model by a sliding windows approach succeeded in identifying the model parameters in a single parallel experiment of one day, which shows how process development can benefit from model-based and automation approaches.

1.4.2 Mimicking Industrial Scale Conditions in the Lab: Continuous-Like Experiments

1.4.2.1 A Simple Model for Continuous Processes

The challenges of continuous processes and comparison against batch or fed-batch can be better understood using a simplified description of its dynamic system. The continuous process is one of three main cultivation technologies together with batch and fed-batch. Other extensions such as sequencing batch and fed-batch also exist but will be covered later. The main difference between these three setups is the inflow and outflow streams. We can use a simple generalized dynamic model to describe the reactor in Eqs (1.2–1.5). The reader is referred to Refs [25,113] for a more detailed description of the system of equations.

The simplest system of a continuous process is the chemostat, which is characterized by a continuous flow of the incoming medium at a rate that limits one nutrient component in the bioreactor. As the rates for inflow and outflow are equal, the volume in a chemostat is constant. Through the limiting component, the specific growth rate can be controlled simply by the pump speed.

The experimental system and kinetic basis of the chemostat were developed in the groundbreaking papers by Novick and Szillard [114] and Monod [115] and further theoretically refined by Powell [116]. The chemostat applies the concept of the metabolic control, which has been earlier established by the fed-batch method, to continuous processing. By this these authors have set the basis for the wider application of the chemostat providing a detailed procedure for control, long before this was done for the fed-batch by Pirt and Kurowski [49].

A number of assumptions are necessary, the most important being: (i) species and conditions that are not described by the model (temperature, pH, trace elements, etc.) are constant, (ii) ideal mixing, (iii) monoculture. The behavior of the process can be approximately described by the following set of equations:

$$\frac{\mathrm{d}S_j}{\mathrm{d}t} = \frac{F_{\mathrm{in}}}{V}\left(S_{j,\mathrm{in}} - S_j\right) - q_{S_j}X, \tag{1.2}$$

$$\frac{\mathrm{d}X}{\mathrm{d}t} = \frac{F_{\mathrm{in}}}{V}(X_{\mathrm{in}} - X) - \frac{F_{\mathrm{out}}}{V}(\delta X - X) + \mu X, \tag{1.3}$$

$$\frac{\mathrm{d}O_{\mathrm{d}}}{\mathrm{d}t} = \mathrm{Kla}\left(O_{\mathrm{d}}^{*} - O_{\mathrm{d}}\right) - q_{\mathrm{O}}XH, \tag{1.4}$$

$$\frac{\mathrm{d}V}{\mathrm{d}t} = F_{\mathrm{in}} - F_{\mathrm{out}}, \tag{1.5}$$

with S_j being the $j = 1 \ldots N$ soluble components (substrate or product) concentrations in the medium in (g/l) ($q_{S_j} > 0$ for substrate uptake and $q_{S_j} < 0$ product secretion), X the cell dry weight of the organisms in (g/l), O_{d} the oxygen dissolved in the medium in (%) of saturation, V the volume of medium in the reactor in (l), and F a flow stream of the reactor in (l/h). The subindexes in and out represent the inlet and outlet streams, respectively, q_{S} and q_{O} the uptake rates of soluble components and oxygen, respectively, in (g/(g l)), Kla is the oxygen diffusion constant, O_{d}^{*} the saturation concentration of oxygen in the medium in (%), H the Henry related coefficient, and δ the cell retention (−) by membrane or perfusion systems (1 for no retention and 0 for complete recirculation).

This simple model, allows us to analyze the basic characteristics and differences of the discontinuous and continuous cultivation processes. In batch $F_{\mathrm{in}} = F_{\mathrm{out}} = 0$, in fed-batch $F_{\mathrm{in}} > 0; F_{\mathrm{out}} = 0$, and in continuous $F = F_{\mathrm{in}} = F_{\mathrm{out}} > 0$ with $D = F/V$ being the dilution rate.

1.4.2.2 Continuous-Like Fed-Batch Cultivations

It is worth stressing out that, in systems with no recirculation ($\delta = 1$), F_{out} enters only in the volume Eq. (1.5), so that equilibrium in all other states can be reached also in the fed-batch setup considering off course an infinitely large vessel.

In a continuous process, the growth rate can be easily obtained by solving Eqs (1.2) and (1.3) at steady state and considering that concentration of biomass or products in the inlet are equal to zero.

In a continuous process, the growth rate can be obtained by reformulating Eq. (1.3) to

$$0 = \frac{\mathrm{d}X}{\mathrm{d}t} = \left(-\frac{F}{V}\delta + \mu\right)X, \tag{1.6}$$

and further solving it to

$$\mu = \frac{F}{V}\delta = D\delta,$$

for the biomass with S_1 being the substrate and $q_{S_1} < 0$ being the specific substrate uptake rate

$$\begin{aligned} 0 &= \frac{\mathrm{d}S_1}{\mathrm{d}t} = \frac{F_{\text{in}}}{V}\left(S_{1,\text{in}} - S_1\right) + q_{S_1}X = D\left(S_{1,\text{in}} - S_1\right) + q_{S_1}X, \\ X &= -\frac{D\left(S_{1,\text{in}} - S_1\right)}{q_{S_1}}, \quad \text{considering} \left(S_{1,\text{in}} - S_1\right) \approx S_{1,\text{in}}, \\ X &= -\frac{DS_{1,\text{in}}}{q_{S_1}}, \end{aligned} \tag{1.7}$$

now if we consider $q_{S_1} = -\mu / Y_{X/S}$, and $\mu = D\delta$ we obtain

$$X = \frac{S_{1,\text{in}} Y_{X/S}}{\delta}, \tag{1.8}$$

and for the product S_2 or P, with $q_p > 0$ being the production rate

$$\begin{aligned} 0 &= \frac{\mathrm{d}P}{\mathrm{d}t} = -\frac{F_{\text{in}}}{V}P + q_P X = -DP + q_P X, \\ q_P &= D\frac{P}{X}, \text{ or } P = \frac{q_P X}{D}. \end{aligned} \tag{1.9}$$

Figure 1.1 depicts the growth rate of an *E. coli* cultivation with regard to different levels of constant feeding. Even at large feeding differences, the change in biomass concentration drives the process to a similar growth rate.

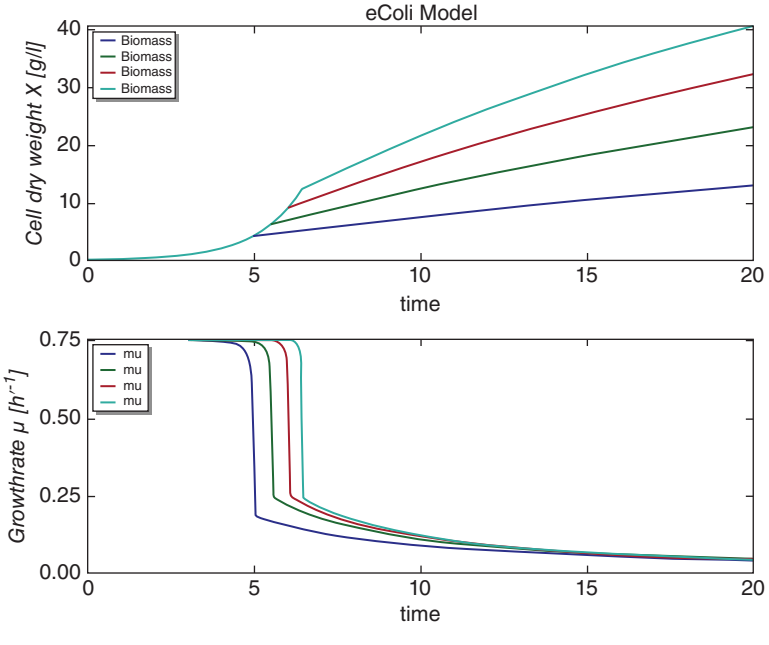

Figure 1.1 Effect of constant feeding profiles in biomass and growth rate.

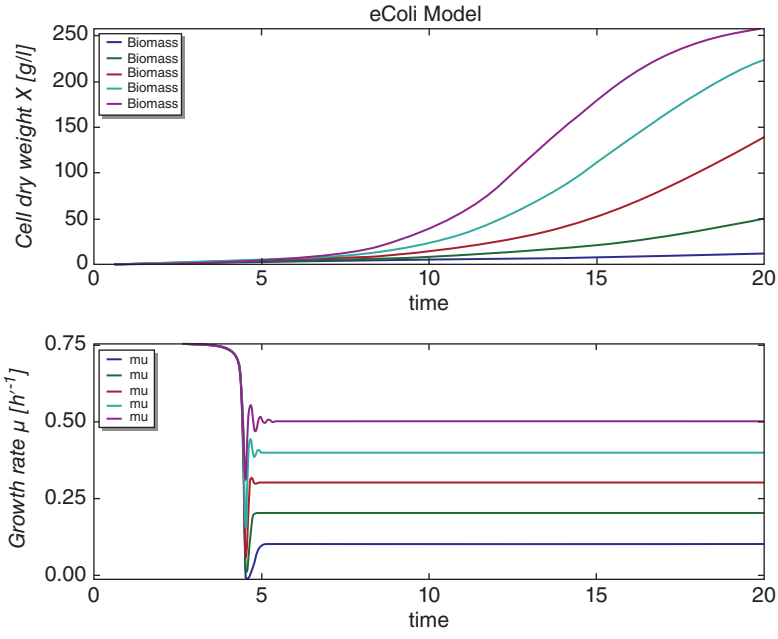

Figure 1.2 Effect of exponential feeding profiles in biomass and growth rate on the behavior of a fed-batch process.

If we apply an exponential feeding as in Figure 1.2, the dilution rate remains constant. By this, the growth rate, which is directly related to D, can be held constant so as to investigate the effect of continuous cultures in the organisms.

These exponential feeding experiments recreate conditions very similar to continuous cultures, reducing drastically material costs (experimental setup and volumes) and experimental time.

1.4.3 Fast and Parallel Experimental Approaches with High Information Content

1.4.3.1 Computer-Aided Operation of Robotic Facilities

One of the most important differences of continuous processes compared to batch or even fed-batch is its level of sophistication hence control sophistication required. The actions that can be taken to operate a batch process are limited so that control can be simple. But continuous processes require a complex control strategy to assure stability and product quality. In other words, the acceptance and profitability of continuous processes strongly depends on the progress in bioprocess monitoring, understanding, and control.

But before a model can be used for monitoring, control, and optimization purposes, it has to be build and validated with experimental data. This is not a trivial task since, as mentioned before, the reproducibility and scalability of processes is especially difficult in biological systems. The experimental efforts related are extremely high so that model building is usually left aside since scale-up and process development are carried out under high time pressure. Nowadays,

advances in robotics, miniturazion, and data handling are being used to create high-throughput (HT) facilities able to perform thousands of experiments in parallel automatically. With this, new opportunities arise for a better process understanding and model building. Together with scale down techniques it is possible to create many experiments at "process like conditions" in order to rapidly fit model parameters and test the response of the system in a larger operation space.

1.4.3.2 Model Building and Experimental Validation

In biotechnology, model building is necessarily coupled to a reiterative experimental validation. Regardless of its level of complexity, models have to be constantly fitted against real observations to adjust its parameters to changes caused by variations in the environment or in the microorganism itself. On the other hand, such robotic systems require a respective control strategy, posing new challenges to experimental design and control. Process automation from product development to production requires a horizontal transfer of information. In near future, a miniaturized scale down robotic facility will be connected to the plant and run in parallel creating a miniaturized twin of the large-scale process. By this, the mathematical model traditionally used in MPC will be substituted by a more accurate description of the system with a faster response time due to the dimension difference.

Regarding the experimental planning for model validation, the efficiency in the design of multiple parallel experiments can be increased by using existing methods for data analysis and design of experiments [117–120] in order to allow an automatic evaluation of experimental results as well as the design and run of following experiments. If we managed to build a proper model to describe a process, we first need to fit the model to real data. This model will contain a set of unknown parameters that can be varied to fit the outputs of the model against observations of the real process. The aim is to find the experimental setups such that the statistical uncertainty of estimates of the unknown model parameters is minimized [121,122]. Nevertheless, there are some problems again related with the complexity and nonlinear dynamics of biological processes. First, the experiments carried out in the screening phase should emulate real process conditions [123] and generate high quality data so that systems beyond simple plates, as are mini-bioreactors [124–129] are needed. But more important is that, even for continuous processes, we have to go beyond "endpoint" or "steady-state" experiments. The dynamics of the process are essential to predict its evolution over time and the proper control strategies and for these we need dynamic experiments or at least different steady states. Because of the size and possibility of modern HT facilities, the number of factors that can be varied is very large including "actions" (pipetting, mixing, incubating, measuring, etc.) and "resources" (1-, 8-, or 96-channel pipette, shaker, photometer, flow cytometer, reaction vessels, plates, etc.). For these reasons, computer aided tools for optimal experimental design (OED) [121,130–132] are needed to maximize the efficiency of automatized laboratories. Achievements in online applications, allowing the use of the data generated to redesign the running experiment also are being developed [130,133–137] and applied in real case studies with bacterial cultivations [138], solving a number of complications as are the complexity of the biological system, the control of the experimental facilities, the low information

content and long delays of the measurements, the scheduling of all actions considering resource availability, and a robust and cheap computation of the optimization.

Generally, the main factors that affect the identifiability of a model are: (i) the structure of the model, (ii) the quality of the measurements (frequency, accuracy, etc.), and (iii) the design of the experiment (inputs, conditions, etc.). OED thus, by realization of the computed experimental conditions, the information content of the measurements is maximized and the parameters are determined most accurately.

There are still important challenges that need to be solved before OED methods for optimal design and operation of robotic liquid handling stations can be reliably applied for bioprocess development. Some of the most important are the design of a robust optimization program that can assure convergence to a global solution in a limited time, the addition of nonlinear path constraints to define a more accurate search, the computation of the error propagation caused by model uncertainties, efficient methods for the solution of the scheduling problem considering all resources.

1.5 Conclusions

Continuous bioprocessing which is a standard in some bioprocesses, such as for example, waste-water treatment and biogas production, is still at its infancy in pharmaceutical production. While long-term continuous experiments are limited in view of labor and also would only provide limited knowledge in connection to the randomly occurring mutations, deeper going knowledge is needed and quality has to be implemented in the process. Therefore, modeling and advanced process control approaches form a solid basis. Especially, mechanistic and hybrid models can provide here important information on the system and the process. For their application, it has to be considered that due to variations in the process the parameters of the model will be not constant over longer time intervals but have to be regularly adjusted automatically. While such continuous optimization strategies are the state of the art in various engineering disciplines they are new in the area of bioprocessing, but can provide a significant benefit. In this context it is an advantage that various complex cellular models exist, which however by typical methods of model reduction should be adapted in a way to make the parameters identifiable. If successful, such approaches can than be a solid basis for continuous bioprocessing.

References

1 Konstantinov, K.B. and Cooney, C.L. (2015) White paper on continuous bioprocessing May 20–21, 2014 continuous manufacturing symposium. *J. Pharm. Sci.*, **104**, 813–820.

2 FDA (2004) Guidance for industry PAT – A framework for innovative pharmaceutical development, manufacturing, and quality assurance.

3 Han, C., Nelson, P., and Tsai, A. (2010) Process development's impact on Cost of Goods Manufactured (COGM). *BioProcess Int.*, **8**, 48–55.
4 Walther, J., Godawat, R., Hwang, C., Abe, Y., Sinclair, A., and Konstantinov, K. (2015) The business impact of an integrated continuous biomanufacturing platform for recombinant protein production. *J. Biotechnol.*, **213**, 3–12.
5 Gresham, D. and Hong, J. (2015) The functional basis of adaptive evolution in chemostats. *FEMS Microbiol. Rev.*, **39**, 2–16.
6 Lee, H., Popodi, E., Tang, H.X., and Foster, P.L. (2012) Rate and molecular spectrum of spontaneous mutations in the bacterium *Escherichia Coli* as determined by whole-genome sequencing. *Proc. Natl. Acad. Sci. USA*, **109**, E2774–E2783.
7 Lynch, M., Sung, W., Morris, K., Coffey, N., Landry, C.R., Dopman, E.B., Dickinson, W.J., Okamoto, K., Kulkarni, S., Hartl, D.L., and Thomas, W.K. (2008) A genome-wide view of the spectrum of spontaneous mutations in yeast. *Proc. Natl. Acad. Sci. USA*, **105**, 9272–9277.
8 Ferenci, T. (2008) Bacterial physiology, regulation and mutational adaptation in a chemostat environment. *Adv. Microb. Physiol.*, **53**, 169–229.
9 Lenski, R.E., Wiser, M.J., Ribeck, N., Blount, Z.D., Nahum, J.R., Morris, J.J., Zaman, L., Turner, C.B., Wade, B.D., Maddamsetti, R. *et al.* (2015) Sustained fitness gains and variability in fitness trajectories in the long-term evolution experiment with *Escherichia coli*. *Proc. Biol. Sci.*, **282**, 20152292.
10 Hayduck, F. (1923) *Low-Alcohol Yeast Process*, US Patent 1449107, Fleischmann Company.
11 Hayduck, F. (1915) Verfahren der Hefefabrikation ohne oder mit nur geringer Alkohlolerzeugung. German Patent 300662.
12 Barton, P.I., (1992) The modelling and simulation of combined discrete/continuous processes. Ph.D. dissertation. University of London.
13 Alvarez, M., Stocks, S., and Jørgensen, S. (2009) Bioprocess modelling for learning model predictive control (L-MPC), in *Computational Intelligence Techniques for Bioprocess Modelling, Supervision and Control* (eds M. C. Nicoletti and L.C. Jain), Springer, Berlin, pp. 237–280.
14 Lübbert, A. and Simutis, R. (1994) Using measurement data in bioprocess modelling and control. *Trends Biotechnol.*, **12**, 304–311.
15 Nicoletti, M., Jain, L., and Giordano, R. (2009) Computational intelligence techniques as tools for bioprocess modelling, optimization, supervision and control in *Computational Intelligence Techniques for Bioprocess Modelling, Supervision and Control*, (eds M.C. Nicoletti and L.C. Jain), Springer, Berlin, pp. 1–23.
16 Tabrizi, H.O., Amoabediny, G., Moshiri, B., Haji Abbas, M.P., Pouran, B., Imenipour, E., Rashedi, H., and Büchs, J. (2011) Novel dynamic model for aerated shaking bioreactors. *Biotechnol. Appl. Biochem.*, **58**, 128–137.
17 Chhatre, S., Farid, S.S., Coffman, J., Bird, P., Newcombe, A.R., and Titchener-Hooker, N.J. (2011) How implementation of quality by design and advances in biochemical engineering are enabling efficient bioprocess development and manufacture. *J. Chem. Technol. Biotechnol*, **86**, 1125–1129.
18 Preisig, H.A. (2010) Constructing and maintaining proper process models. *Comput. Chem. Eng.*, **34**, 1543–1555.

19 Li, D., Ivanchenko, O., Sindhwani, N., Lueshen, E., and Linninger, A.A. (2010) Optimal catheter placement for chemotherapy. *Comp. Aid. Chem. Eng.*, **28**, 223–228.

20 Dochain, D. (2003) State and parameter estimation in chemical and biochemical processes: a tutorial. *J. Process Control.*, **13**, 801–818.

21 fda.gov (2011) Guidance for Industry Process Validation: General Principles and Practice.

22 Henson, M.A. (2003) Dynamic modeling and control of yeast cell populations in continuous biochemical reactors. *Comput. Chem. Eng.*, **27**, 1185–1199.

23 Lei, F. and Jørgensen, S.B. (2001) Dynamics and nonlinear phenomena in continuous cultivations of *Saccharomyces cerevisiae*. Technical University of Denmark, Department of Chemical and Biochemical Engineering.

24 Lei, F., Olsson, L., and Jørgensen, S.B. (2004) Dynamic effects related to steady-state multiplicity in continuous *Saccharomyces cerevisiae* cultivations. *Biotechnol. Bioeng.*, **88**, 838–848.

25 Dochain, D. (2008) Bioprocess control. ISTE London.

26 Hady, L. and Wozny, G. (2010) Computer-aided web-based application to modular plant design. *Comp. Aid. Chem. Eng.*, **28**, 685–690.

27 Bailey, J.E. (1998) Mathematical modeling and analysis in biochemical engineering: past accomplishments and future opportunities. *Biotechnol. Progr.*, **14**, 8–20.

28 Kremling, A., Fischer, S., Gadkar, K., Doyle, F.J., Sauter, T., Bullinger, E., Allgöwer, F., and Gilles, E.D. (2004) A benchmark for methods in reverse engineering and model discrimination: problem formulation and solutions. *Genome Res.*, **14**, 1773–1785.

29 Csete, M.E. and Doyle, J.C. (2002) Reverse engineering of biological complexity. *Supramol. Sci.*, **295**, 1664–1669.

30 Kitano, H. (2002) Systems biology: a brief overview. *Supramol. Sci.*, **295**, 1662–1664.

31 Kitano, H. (2002) Computational systems biology. *Nature*, **420**, 206–210.

32 Kravaris, C., Hahn, J., and Chu, Y. (2013) Advances and selected recent developments in state and parameter estimation. *Comput. Chem. Eng.*, **51**, 111–123.

33 Welch, G. and Bishop, G. (1995) An introduction to the Kalman filter.

34 Daum, F. (2005) Nonlinear filters: beyond the Kalman filter. *IEEE Aerosp. Electron. Syst. Mag.*, **20**, 57–69.

35 Soroush, M. (1997) Nonlinear state-observer design with application to reactors. *Chem. Eng. Sci.*, **52**, 387–404.

36 Boulkroune, B., Darouach, M., Zasadzinski, M., Gillé, S., and Fiorelli, D. (2009) A nonlinear observer design for an activated sludge wastewater treatment process. *J. Process Control*, **19**, 1558–1565.

37 Watson, T.G. (1972) Present status and future prospects of turbidostat. *J. Appl. Chem. Biotechnol.*, **22**, 229–243.

38 Evdokimov, E.V. and Pecherkin, M.P. (1992) Directed autoselection of bacteria cultured in a turbidostat. *Microbiology*, **61**, 460–466.

39 Bennett, W.N. (1988) Assessment of selenium toxicity in algae using turbidostat culture. *Water Res.*, **22**, 939–942.

40 Aarnio, T.H., Suihko, M.L., and Kauppinen, V.S. (1991) Isolation of acetic acid-tolerant bakers-yeast variants in a turbidostat. *Appl. Biochem. Biotechnol.*, **27**, 55–63.

41 Pham, H.T.B., Larsson, G., and Enfors, S.O. (1999) Modelling of aerobic growth of *Saccharomyces cerevisiae* in a pH-auxostat. *Bioprocess. Eng.*, **20**, 537–544.

42 Martin, G.A. and Hempfling, W.P. (1976) A method for the regulation of microbial population density during continuous culture at high growth rates. *Arch. Microbiol.*, **107**, 41–47.

43 Büttner, R., Uebel, B., Genz, I.L., and Köhler, M. (1985) Researches with a substrate-limited Ph-auxostat .1. Bistability of growth under potassium-limited conditions. *J. Basic Microbiol.*, **25**, 227–232.

44 Pettersson, H.E. (1975) Growth of a mixed species lactic starter in a continuous "pH-Stat" fermentor. *Adv. Appl. Microbiol.*, **29**, 437–443.

45 Larsson, G., Enfors, S.O., and Pham, H. (1990) The ph-auxostat as a tool for studying microbial dynamics in continuous fermentation. *Biotechnol. Bioeng.*, **36**, 224–232.

46 Rice, C.W. and Hempfling, W.P. (1985) Nutrient-limited continuous culture in the phauxostat. *Biotechnol. Bioeng.*, **27**, 187–191.

47 Müller, R.H. and Babel, W. (2004) Delftia acidovorans MC1 resists high herbicide concentrations–a study of nutristat growth on (RS)-2-(2,4-dichlorophenoxy)propionate and 2,4-dichlorophenoxyacetate. *Biosci. Biotechnol. Biochem.*, **68**, 622–630.

48 Kleman, G.L., Chalmers, J.J., Luli, G.W., and Strohl, W.R. (1991) Glucose-stat, a glucose-controlled continuous culture. *Appl. Environ. Microbiol.*, **57**, 918–923.

49 Pirt, S.J. and Kurowski, W.M. (1970) An extension of the theory of the chemostat with feedback of organisms. Its experimental realization with a yeast culture. *J. Gen. Microbiol.*, **63**, 357–366.

50 Herbert, D. (1961) A theoretical analysis of continuous culture systems, in *Continuous Culture*, vol. 12, Society of Chemistry and Industry, London, pp. 21–53.

51 Zhang, Y., Stobbe, P., Silvander, C.O., and Chotteau, V. (2015) Very high cell density perfusion of CHO cells anchored in a non-woven matrix-based bioreactor. *J. Biotechnol.*, **213**, 28–41.

52 Kempken, R., Preissmann, A., and Berthold, W. (1995) Clarification of animal cell cultures on a large scale by continuous centrifugation. *J. Ind. Microbiol.*, **14**, 52–57.

53 Neidhardt, F.C., Ingraham, J.L., and Schaechter, M. (1990) *Physiology of the Bacterial Cell: A Molecular Approach*, Sinauer Associates.

54 Box, G.E.P. (1999) Statistics as a catalyst to learning by scientific method part II-a discussion. *J. Qual. Technol.*, **31**, 16–29.

55 Raduly, B., Capodaglio, A.G., and Vaccari, D.A. (2004) Simplification of wastewater treatment plant models using empirical modelling techniques. *Young Res.*, **2004**, 51.

56 Nomikos, P. and MacGregor, J.F. (1994) Monitoring batch processes using multiway principal component analysis. *AIChE J.*, **40**, 1361–1375.

57 AlGhazzawi, A. and Lennox, B. (2009) Model predictive control monitoring using multivariate statistics. *J. Process Control*, **19**, 314–327.

58 Kern, P., Wolf, C., Bongards, M., Oyetoyan, T.D., and McLoone, S. (2011) Self-organizing map based operating regime estimation for state based control of wastewater treatment plants. 2011 International Conference of IEEE on Soft Computing and Pattern Recognition (SoCPaR), pp. 390–395.

59 Georgescu, R., Berger, C.R., Willett, P., Azam, M., and Ghoshal, S. (2010) Comparison of data reduction techniques based on the performance of SVM-type classifiers, 2010 IEEE Aerospace Conference, pp. 1–9.

60 Witten, I.H. and Frank, E. (2005) *Data Mining: Practical Machine Learning Tools and Techniques*, 2nd edn, Morgan Kaufmann, San Francisco.

61 Barton, P.I. and Lee, C.K. (2002) Modeling, simulation, sensitivity analysis, and optimization of hybrid systems. *ACM Trans. Model. Comput. Simul. (TOMACS)*, **12**, 256–289.

62 Rippin, D.W.T. (1993) Batch process systems engineering: a retrospective and prospective review. *Comput. Chem. Eng.*, **17**, 1–13.

63 Lima, F.V. and Rawlings, J.B. (2011) Nonlinear stochastic modeling to improve state estimation in process monitoring and control. *AIChE J.*, **57**, 996–1007.

64 Ławryńczuk, M. (2008) Modelling and nonlinear predictive control of a yeast fermentation biochemical reactor using neural networks. *Chem. Eng. J.*, **145**, 290–307.

65 Zhu, G.-Y., Zamamiri, A., Henson, M.A., and Hjortsø, M.A. (2000) Model predictive control of continuous yeast bioreactors using cell population balance models. *Chem. Eng. Sci.*, **55**, 6155–6167.

66 Ramaswamy, S., Cutright, T., and Qammar, H. (2005) Control of a continuous bioreactor using model predictive control. *Process Biochem.*, **40**, 2763–2770.

67 Bayer, B. and Marquardt, W. (2004) Towards integrated information models for data and documents. *Comput. Chem. Eng.*, **28**, 1249–1266.

68 Spieth, C., Hassis, N., and Streichert, F. (2006) *Comparing Mathematical Models on the Problem of Network Inference*, ACM, pp. 279–286.

69 Heinrich, R. and Schuster, S. (1996) *The Regulation of Cellular Systems*, Springer, US.

70 Vilela, M., Chou, I.C., Vinga, S., Vasconcelos, A., Voit, E., and Almeida, J. (2008) Parameter optimization in S-system models. *BMC Syst. Biol.*, **2**, 35.

71 Stewart, W.E., Shon, Y., and Box, G.E.P. (1998) Discrimination and goodness of fit of multiresponse mechanistic models. *AIChE J.*, **44**, 1404–1412.

72 Harms, P., Kostov, Y., and Rao, G. (2002) Bioprocess monitoring. *Curr. Opin. Biotechnol.*, **13**, 124–127.

73 Schügerl, K. (2001) Progress in monitoring, modeling and control of bioprocesses during the last 20 years. *J. Biotechnol.*, **85**, 149–173.

74 Biechele, P., Busse, C., Solle, D., Scheper, T., and Reardon, K. (2015) Sensor systems for bioprocess monitoring. *Eng. Life Sci.*, **15**, 469–488.

75 de Assis, A.J. and Maciel Filho, R. (2000) Soft sensors development for on-line bioreactor state estimation. *Comput. Chem. Eng.*, **24**, 1099–1103.

76 Sundström, H. and Enfors, S.-O. (2008) Software sensors for fermentation processes. *Bioprocess Biosyst. Eng.*, **31**, 145–152.

77 Agrawal, P. and Lim, H.C. (1984) *Analyses of Various Control Schemes for Continuous Bioreactors*, Springer.

78 Henson, M.A. and Seborg, D.E. (1992) Nonlinear control strategies for continuous fermenters. *Chem. Eng. Sci.*, **47**, 821–835.

79 Andersen, M.Y., Pedersen, N.H., Brabrand, H., Hallager, L., and Jørgensen, S.B. (1997) Regulation of a continuous yeast bioreactor near the critical dilution rate using a productostat. *J. Biotechnol.*, **54**, 1–14.

80 Hu, X., Li, Z., and Xiang, X. (2013) Feedback control for a turbidostat model with ratio-dependent growth rate. *J. Appl. Math. Inform.*, **31**, 385–398.

81 Konstantinov, K.B., Tsai, Ys., Moles, D., and Matanguihan, R. (1996) Control of long-term perfusion chinese hamster ovary cell culture by glucose auxostat. *Biotechnol. Progr.*, **12**, 100–109.

82 Sowers, K.R., Nelson, M.J., and Ferry, J.G. (1984) Growth of acetotrophic, methane-producing bacteria in a pH auxostat. *Curr. Microbiol.*, **11**, 227–229.

83 Ullman, G., Wallden, M., Marklund, E.G., Mahmutovic, A., Razinkov, I., and Elf, J. (2013) High-throughput gene expression analysis at the level of single proteins using a microfluidic turbidostat and automated cell tracking. *Philos. Trans. R. Soc. B*, **368**, 20120025.

84 Yao, Y., Li, Z., and Liu, Z. (2015) Hopf bifurcation analysis of a turbidostat model with discrete delay. *Appl. Math. Comput.*, **262**, 267–281.

85 Olivieri, G., Russo, M.E., Maffettone, P.L., Mancusi, E., Marzocchella, A., and Salatino, P. (2013) Nonlinear analysis of substrate-inhibited continuous cultures operated with feedback control on dissolved oxygen. *Ind. Eng. Chem. Res.*, **52**, 13422–13431.

86 Tian, Y., Sun, K.B., Chen, L.S., and Kasperski, A. (2010) Studies on the dynamics of a continuous bioprocess with impulsive state feedback control. *Chem. Eng. J.*, **157**, 558–567.

87 Nagy, Z.K. (2007) Model based control of a yeast fermentation bioreactor using optimally designed artificial neural networks. *Chem. Eng. J.*, **127**, 95–109.

88 Sankpal, N.V., Cheema, J.J.S., Tambe, S.S., and Kulkarni, B.D. (2001) An artificial intelligence tool for bioprocess monitoring: application to continuous production of gluconic acid by immobilized *Aspergillus niger*. *Biotechnol. Lett.*, **23**, 911–916.

89 Kishimoto, M., Beluso, M., Omasa, T., Katakura, Y., Fukuda, H., and Suga, K-i. (2002) Construction of a fuzzy control system for a bioreactor using biomass support particles. *J. Mol. Catal. B Enzym.*, **17**, 207–213.

90 Bastin, G:. (2013) *On-Line Estimation and Adaptive Control of Bioreactors*, Elsevier.

91 Gresham, D. and Hong, J. (2015) The functional basis of adaptive evolution in chemostats. *FEMS Microbiol. Rev.*, **39**, 2–16.

92 Raissi, T., Ramdani, N., and Candau, Y. (2005) Bounded error moving horizon state estimator for non-linear continuous-time systems: application to a bioprocess system. *J. Process Control*, **15**, 537–545.

93 Alvarez-Vázquez, L.J. and Fernández, F.J. (2010) Optimal control of a bioreactor. *Appl. Math. Comput.*, **216**, 2559–2575.

94 Dondo, R.G. and Marqués, D. (2001) Optimal control of a batch bioreactor: a study on the use of an imperfect model. *Process Biochem.*, **37**, 379–385.

95 Åström, K.J. and Wittenmark, B. (2013) *Adaptive Control*, Courier Corporation.

96 Zavala, V.M., Laird, C.D., and Biegler, L.T. (2008) A fast moving horizon estimation algorithm based on nonlinear programming sensitivity. *J. Process Control*, **18**, 876–884.

97 Neubauer, P., Cruz, N., Glauche, F., Junne, S., Knepper, A., and Raven, M. (2013) Consistent development of bioprocesses from microliter cultures to the industrial scale. *Eng. Life Sci.*, **13**, 224–238.

98 Formenti, L.R., Nørregaard, A., Bolic, A., Hernandez, D.Q., Hagemann, T., Heins, A.L., Larsson, H., Mears, L., Mauricio-Iglesias, M., and Krühne, U. (2014) Challenges in industrial fermentation technology research. *Biotechnol. J.*, **9**, 727–738.

99 Balagadde, F.K., You, L., Hansen, C.L., Arnold, F.H., and Quake, S.R. (2005) Long-term monitoring of bacteria undergoing programmed population control in a microchemostat. *Supramol. Sci.*, **309**, 137–140.

100 Nanchen, A., Schicker, A., and Sauer, U. (2006) Nonlinear dependency of intracellular fluxes on growth rate in miniaturized continuous cultures of *Escherichia coli*. *Appl. Environ. Microbiol.*, **72**, 1164–1172.

101 Akgun, A., Maier, B., Preis, D., Roth, B., Klingelhofer, R., and Büchs, J. (2004) A novel parallel shaken bioreactor system for continuous operation. *Biotechnol. Progr.*, **20**, 1718–1724.

102 Schmideder, A., Severin, T.S., Cremer, J.H., and Weuster-Botz, D. (2015) A novel milliliter-scale chemostat system for parallel cultivation of microorganisms in stirred-tank bioreactors. *J. Biotechnol.*, **210**, 19–24.

103 Paalme, T., Elken, R., Kahru, A., Vanatalu, K., and Vilu, R. (1997) The growth rate control in *Escherichia coli* at near to maximum growth rates: the A-stat approach. *Antonie Van Leeuwenhoek*, **71**, 217–230.

104 Adamberg, K., Valgepea, K., and Vilu, R. (2015) Advanced continuous cultivation methods for systems microbiology. *Microbiology*, **161**, 1707–1719.

105 Wilming, A., Bahr, C., Kamerke, C., and Buchs, J. (2014) Fed-batch operation in special microtiter plates: a new method for screening under production conditions. *J. Ind. Microbiol. Biotechnol.*, **41**, 513–525.

106 Funke, M., Buchenauer, A., Schnakenberg, U., Mokwa, W., Diederichs, S., Mertens, A., Müller, C., Kensy, F., and Büchs, J. (2010) Microfluidic biolector-microfluidic bioprocess control in microtiter plates. *Biotechnol. Bioeng.*, **107**, 497–505.

107 Jeude, M., Dittrich, B., Niederschulte, H., Anderlei, T., Knocke, C., Klee, D., and Büchs, J. (2006) Fed-batch mode in shake flasks by slow-release technique. *Biotechnol. Bioeng.*, **95**, 433–445.

108 Panula-Perälä, J., Siurkus, J., Vasala, A., Wilmanowski, R., Casteleijn, M.G., and Neubauer, P. (2008) Enzyme controlled glucose auto-delivery for high cell density cultivations in microplates and shake flasks. *Microb. Cell Fact.*, **7**, 31.

109 Krause, M., Neubauer, A., and Neubauer, P. (2016) Scalable enzymatic delivery systems for controlled nutrient supply improve recombinant protein production. *Microb. Cell Fact.*, 15:110.

110 Siurkus, J., Panula-Perala, J., Horn, U., Kraft, M., Rimseliene, R., and Neubauer, P. (2010) Novel approach of high cell density recombinant bioprocess development: optimisation and scale-up from microliter to pilot scales while

maintaining the fed-batch cultivation mode of *E. coli* cultures. *Microb. Cell Fact.*, **9**, 35.

111 Glauche, F., Genth, R., Kiesewetter, G., Glazyrina, J., Bockisch, A., Cuda, F., Goellig, D., Raab, A., Lang, C., and Neubauer, P. (2017) New approach to determine the dependency between the specific rates for growth and product formation in miniaturized parallel robot-based fed-batch cultivations. *Eng. Life Sci.*, in press.

112 Cruz Bournazou, M.N., Barz, T., Nickel, D., Glauche, F., Knepper, A., and Neubauer, P., (2017) Online Optimal Experimental Re-Design in Robotic Parallel Fed-Batch Cultivation Facilities for Validation of Macro-Kinetic Growth Models at the Example of E. coli. *Biotechnol Bioengin*, **114**, 610–619.

113 Enfors, S.O. (2016) Fermentation Process Engineering. p. 99.

114 Novick, A. and Szilard, L. (1950) Description of the chemostat. *Supramol. Sci.*, **112**, 715–716.

115 Monod, J.L. (1950) La technique de culture continue. Théorie et applications. *Annales de l'Institut Pasteur*, **79**, 390–410.

116 Powell, E.O. (1965) Theory of the chemostat. *Lab. Pract.*, **14**, 1145–1149 passim.

117 Macarrón, R. and Hertzberg, R.P. (2011) Design and implementation of high throughput screening assays. *Mol. Biotechnol.*, **47**, 270–285.

118 Tai, M., Ly, A., Leung, I., and Nayar, G. (2015) Efficient high-throughput biological process characterization: definitive screening design with the Ambr250 bioreactor system. *Biotechnol. Prog*, **31**, 1388–1395.

119 Lutz, M.W., Menius, J.A., Choi, T.D., Laskody, R.G., Domanico, P.L., Goetz, A.S., and Saussy, D.L. (1996) Experimental design for high-throughput screening. *Drug Discov. Today*, **1**, 277–286.

120 Haaland, P.D. (1989) *Experimental Design in Biotechnology*, CRC Press.

121 Körkel, S., Kostina, E., Bock, H.G., and Schlöder, J.P. (2004) Numerical methods for optimal control problems in design of robust optimal experiments for nonlinear dynamic processes. *Optim. Method Softw.*, **19**, 327–338.

122 Korkel, D.M.S. (2002) Numerische Methoden fuer Optimale Versuchsplanungsprobleme bei nichtlinearen DAE-Modellen. Ruprecht-Karls-Universitaet Heidelberg.

123 Neubauer, P. and Junne, S. (2010) Scale-down simulators for metabolic analysis of large-scale bioprocesses. *Curr. Opin. Biotechnol.*, **21**, 114–121.

124 Hedrén, M., Ballagi, A., Mörtsell, L., Rajkai, G., Stenmark, P., Sturesson, C., and Nordlund, P. (2006) GRETA, a new multifermenter system for structural genomics and process optimization. *Acta Crystallogr. D*, **62**, 1227–1231.

125 Gill, N.K., Appleton, M., Baganz, F., and Lye, G.J. (2008) Design and characterisation of a miniature stirred bioreactor system for parallel microbial fermentations. *Biochem. Eng. J.*, **39**, 164–176.

126 Lamping, S.R., Zhang, H., Allen, B., and Shamlou, P.A. (2003) Design of a prototype miniature bioreactor for high throughput automated bioprocessing. *Chem. Eng. Sci.*, **58**, 747–758.

127 Zhang, Z., Perozziello, G., Boccazzi, P., Sinskey, A., Geschke, O., and Jensen, K. (2007) Microbioreactors for bioprocess development. *J. Assoc. Lab. Autom.*, **12**, 143–151.

128 Knorr, B., Schlieker, H., Hohmann, H.-P., and Weuster-Botz, D. (2007) Scale-down and parallel operation of the riboflavin production process with *Bacillus subtilis*. *Biochem. Eng. J.*, **33**, 263–274.

129 Huber, R., Ritter, D., Hering, T., Hillmer, A.-K., Kensy, F., Mueller, C., Wang, L., and Buechs, J. (2009) Robo-Lector: a novel platform for automated high-throughput cultivations in microtiter plates with high information content. *Microb. Cell Fact.*, **8**, doi: 10.1186/1475-2859-8-42.

130 Barz, T., López Cárdenas, D.C., Arellano-Garcia, H., and Wozny, G. (2013) Experimental evaluation of an approach to online redesign of experiments for parameter determination. *AIChE J*, **59**, 1981–1995..

131 Cruz Bournazou, M.N., Junne, S., Neubauer, P., Barz, T., Arellano-Garcia, H., and Kravaris, C. (2014) An approach to mechanistic event recognition applied on monitoring organic matter depletion in SBRs. *AIChE J.*, **60**, 3460–3472.

132 Franceschini, G. and Macchietto, S. (2007) Model-based design of experiments for parameter precision: state of the art. *Chem. Eng. Sci.*, **63**, 4846–4872.

133 Galvanin, F., Barolo, M., and Bezzo, F. (2009) Online model-based redesign of experiments for parameter estimation in dynamic systems. *Ind. Eng. Chem. Res.*, **48**, 4415–4427.

134 Galvanin, F., Barolo, M., Pannocchia, G., and Bezzo, F. (2012) Online model-based redesign of experiments with erratic models: a disturbance estimation approach. *Comput. Chem. Eng.*, **42**, 138–151.

135 Huang, B. (2010) Receding horizon experiment design with application in SOFC parameter estimation. 9th International Symposium on Dynamics and Control of Process Systems (DYCOPS 2010), July 5–7, Leuven, Belgium.

136 Stigter, J., Vries, D., and Keesman, K. (2006) On adaptive optimal input design: a bioreactor case study. *AIChE J.*, **52**, 3290–3296.

137 Zhu, Y. and Huang, B. (2011) Constrained receding-horizon experiment design and parameter estimation in the presence of poor initial conditions. *AIChE J.*, **57**, 2808–2820.

138 Cruz-Bournazou, M.N., Barz, T., Nickel, D., and Neubauer, P. (2014) Sliding-window optimal experimental re-design in parallel microbioreactors. *Chem. Ing. Tech.*, **86**, 1379–1380.