Advanced monitoring & control in animal cell cultivation processes for recombinant protein production

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Dipl.-Ing. Mathias Aehle
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Gutachter

1. Prof. Dr. rer. nat. habil. Andreas Lübbert
2. Prof. Dr. techn. Ing. Christoph Herwig

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Contents

Chapter 1

Introduction

ABSTRACT

Current animal cell culture processes are characterized by both large cell numbers and high protein titers and are cultivated in bioreactors with volumes up to 20 m³. Despite these impressive achievements during the last years, less focus was directed towards the design of cultivation strategies aiming at robust and highly reproducible processes. With the process analytical technology (PAT) initiative of the U.S. Food and Drug Administration (FDA) these aspects are today addressed from a regulatory perspective. In this regard, PAT supports innovation and efficiency for manufacturing by developing process understanding to ensure defined product quality and performance. In the present PhD thesis, new methods for animal cell processes are presented that provide innovative solutions for these requirements. In the experimental studies presented herein, a CHO cell line expressing the therapeutic recombinant protein EPO was used. In this perspective, methods for on-line state estimation, as well as the design of robust operational procedures and their automatic control were developed. In addition, the industrial applicability of these operational procedures was taken into consideration during their development.

INTRODUCTION

Currently licensed biopharmaceuticals using cell culture technology cover 50 % of today's worldwide marketed recombinant drugs. Up to 2009, 39 % originated from mammalian and 11.2 % from hybridoma cell manufacturing processes (Ferrer-Miralles et al. 2009). The field of therapeutical indications of these biologics being so diverse, they make up a considerably large amount of pharmaceutical companies' development pipelines and are sure to guarantee blockbuster marketed drugs. Treatment of cancer, endocrine, infectious, autoimmune and blood diseases are currently some of the most prominent medicinal applications.

From a scientific perspective, mammalian cell culture processes have evolved from an almost artistically abstract visionary approach to a widely renowned engineering discipline. This approach was driven by the continuous increase in demand for new therapeutic products and the escalating consumption rate of biopharmaceuticals world-wide (up to several 100 kg/a). Indeed, the use of chemically defined medium free of animal derived components, as well as the development of continuous cell lines, the switch from attached to suspension cell mode, the possibility to selectively chose and cultivate high-productive cell clones and the ever evolving technical process improvements have all made it economical feasible for commercial production in up to 20.000 L bioreactors (Wurm 2004, Ozturk 2006).

It is noteworthy to mention that the therapeutic efficacy of the proteins synthesized by mammalian cells is primarily dependent on their post-translational modifications, e.g. glycosylation, sialylation. Indeed, it was shown that these modifications influence the *in vivo* half-life, solubility, pharmacokinetics and immunogenicity of the final drug product (Andersen and Goochee 1994, Werner 2007). Compared to the template driven protein biosynthesis, glycosylation consists of a complex system of several enzymatic reactions. Thus, environmental factors like culture pH and osmolarity, dissolved oxygen and carbon dioxide concentration, temperature, nutrient concentrations and the entire process strategy play a crucial role in achieving the "right" glycosylation patterns (Harcum 2006). It is of paramount importance to understand the impact of these specific patterns which cannot be generalized among cell lines and their resulting recombinant protein products. Therefore, the post-translational modifications as quality attribute should be very tightly under control in order to achieve a high degree of homogeneity and to guarantee pre-defined acceptance criteria (Hossler 2009).

Over the last decades, good manufacturing practices (GMP) for the production of active pharmaceutical ingredients (APIs) have been established by companies together with the health authorities like the Food and Drug Administration (FDA) or the European Medicine Agency (EMA). Nevertheless, health authorities have recognized an increased number of deviations with regards to drug safety and efficacy during manufacturing campaigns when compared to previous registered data from clinical studies. This was shown to originate from the general procedures used for evaluating the final product quality that are based on extensive tests and analytics for product release which are only conducted after the manufacturing process. Such methodology prevents efficient manufacturing and bears a high risk of rejecting the product (Dünnebier and Tups 2007). In addition, as manufacturers spend several hundred million dollars for drug development and time-consuming clinical studies, less financial effort is put into the development of efficient manufacturing processes. To avoid additional costs and to guarantee agency

requirements, manufactures fix their processes in standard operational procedures (SOPs) at an early stage. But any further changes after approval require communication of all changes to health authorities for review and authorization. This process involves the preparation of costly and time-consuming variation submission files and the acceptance phase can vary from a few months to a couple of years, which could therefore lead to delays in production (Hinz 2006). This rigid regulatory system can be discouraging and companies hesitate to implement novel or innovative methods.

As a consequence, the FDA launched its process analytical technology (PAT) guidance in 2004 to encourage manufacturers to implement scientific principles and tools supporting innovation and efficiency together with a strategy to overcome the regulatory burden (FDA 2004). The contents of the PAT guidance have been adopted by EMA and other health authorities as well, resulting in a global impact for the pharmaceutical industry. In its framework, it is clearly stated that processes should be well understood to persistently ensure final product quality during manufacturing. It is important to note that process understanding should be based on mechanistic know-how. In addition, mechanistic process understanding facilitates continuous process improvements, one of the main requirements mentioned in this framework. The higher the level of process understanding, the better the identification of critical sources leading to process variability. Hence, manufacturers can predict in a more scientifically rationale and reliable manner the process related impacts on product quality attributes. Process understanding can be acquired through the implementation of process analyzers, preferably on/in-line sensors for real-time monitoring, which have to be robust, reliable and easy to use. Sensor(s) implementation should not only aim at using the information for simple monitoring, but should also be used to control critical attributes in real-time.

Technologies as potential PAT applications for bioprocesses have been existing for several decades. Examples of these technologies and their PAT status are summarized in literature (Junker et al. 2006, Rathore et al. 2010). What is evident from screening the applications is that there is a clear reduced number in the field of mammalian cell compared to microbial cell bioprocesses. Teixeira et al. (2009) evaluate the advances of on-line monitoring and control with a focus on mammalian cell cultures. From that, most of the advances have been made in the direction of monitoring of key process variables. However, control of these process variables is still insufficient to actively prevent process variability. There is a clear need for linking on-line information with control purposes which should be easy to implement and validate.

Developments in academia and sensor technology provide many possibilities to fulfill the aspects of PAT. But more effort has to be provided in the level of automation as this is currently low compared to other industries, for instance chemical industry. In addition, mining of data is crucial for extracting process information to identify critical process parameters and steps giving potential for process improvements (Charaniya et al. 2008). At the end, manufacturers of commercial products have to decide what of the currently available or upcoming technologies are feasible with respect to cost/benefit in their respective manufacturing environment.

In the work presented here, approaches for the upstream part of mammalian cell culture processes with respect to the PAT initiative *quality cannot be tested into products; it should be built-in by design* are provided. Essential points are the development of a robust operational design by process know-how and the extraction of easily accessible on-line state variables and their

inherent information. To guarantee a high degree of batch-to-batch reproducibility, different control strategies are applied that are not only evaluated on process performance but also on the quality of a recombinant therapeutic protein. To be attractive for industrial applicants and their demands, on-line monitoring and all feedback control strategies are employed fully automated and are thus proved to be highly reliable.

PROCESS MONITORING

State-of-the-art processes for manufacturing of APIs for commercial release are typically monitored for off-line and on-line variables. Common off-line variables like biomass, nutrient and metabolite concentrations are measured for In-Process Control (IPC). In animal cell cultures, these off-line IPC variables are normally measured once or twice a day. Derived quantities like the specific growth rate, substrate consumption and metabolite production rate can only be calculated when a sample is taken. Hence, the informative output of such off-line state variables is rather low during cultivation. Typical on-line measurements are mostly monitored to keep the environmental conditions under control, like temperature, dissolved oxygen and culture pH. As these variables are controlled at a pre-defined setpoint, their inherent information is low. Nevertheless, information about the state of the culture can be extracted when their control variables are taken into account. For example, the stirrer speed or airflow rate, to keep the pO_2 constant, and the base consumption for pH-control are very valuable data. But this necessitates an adequate process strategy together with reliable probes and a good controller tuning to guarantee high signal-to-noise ratios of the signals.

A very powerful analytical tool for aerobic cultivations is the measurement of the volume fractions of oxygen and carbon dioxide in the vent line of the bioreactors. Off-gas analyzers have been used as standard equipment for microbial systems since several decades but less in mammalian cell cultures for different reasons, e.g. low reaction rates, low biomass concentrations, use of medium buffers and rather complicated measurement devices like process mass spectrometers. The advantage of O_2 and CO_2 volume fraction measurements is the calculation of the corresponding oxygen uptake rate (OUR) and carbon dioxide production rate (CPR) that are quantitatively linked to cell growth. In addition, those sensors are commonly mounted after the sterile barrier and thus bear no additional risk for contamination.

For useful OUR and CPR values the same arguments regarding stable probes and proper controller tuning count here as well. The following equations show the general context.

$$\mathrm{OUR} = \mathrm{OTR} - \frac{\mathrm{dpO_2}}{\mathrm{dt}} \qquad (1)$$

As can be seen from Eq.1, the OUR can only be accurate calculated with a low noise if the pO_2 is well controlled or shows no significant fluctuations over a certain time interval.

For the CPR determination, the situation is a bit more complex due to the carbon dioxide solubility in water with respect to water-like medium. Here, the sensitivity of the CPR signal to

changes on the dissolved CO_2 depending on the pH and its control quality becomes evident from Eqs. 2 and 3 (Sperandio and Paul 1997).

$$CPR = \frac{dpCO_2}{dt} + CTR + CRR \tag{2}$$

$$CRR = k_1 \cdot CO_2 + k_2 \cdot \frac{10^{-pH} \cdot HCO_3}{K_a} \tag{3}$$

Fig. 1 depicts this aspect, where small excursions of pH (ΔpH = 0.03) result in disturbances of the CO_2 volume fraction in the off-gas.

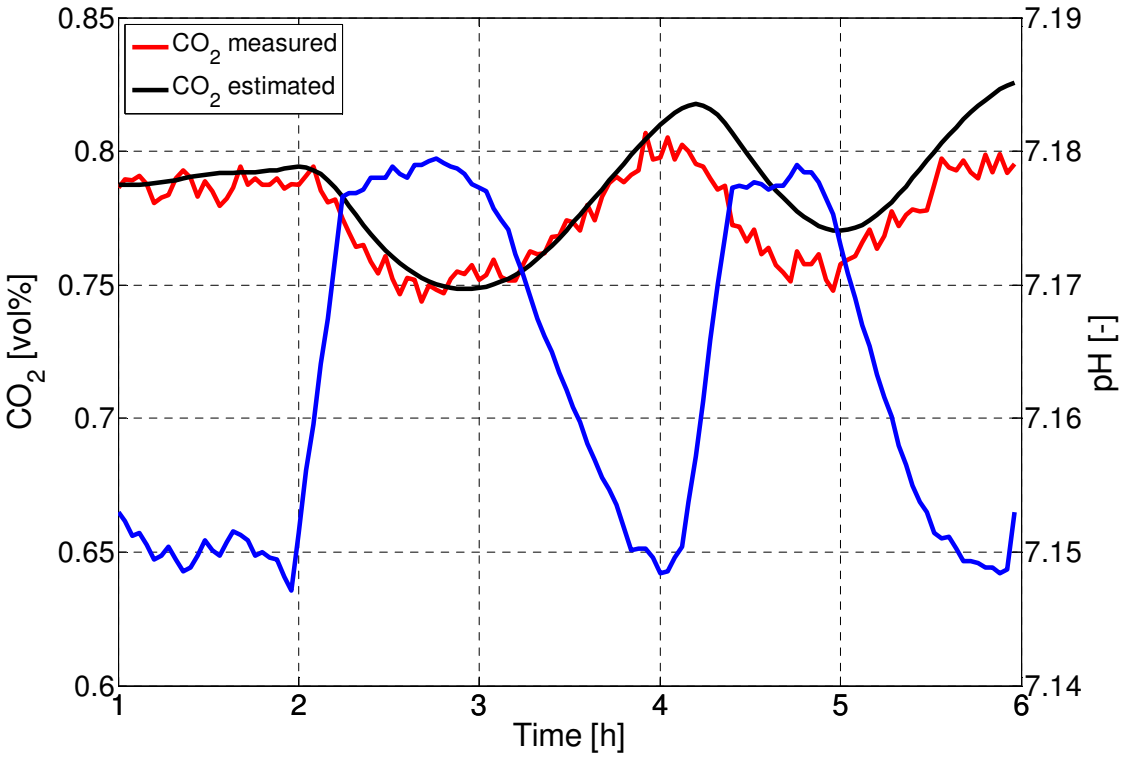

Figure 1. Measured (*red line*) and estimated (*black line*) off-gas CO_2 volume fractions on changes in pH (*blue line*). The sensitivity becomes obvious as the pH is raised only in 0.03 pH units from its setpoint of 7.15.

It is of importance to understand the mechanisms and effects of changes in pO_2 and pH to avoid misinterpretation of the calculated OUR and CPR trajectories. Hence, a multivariate evaluation of the influencing parameters has to be applied. Figure 2 shows the controlled pO_2 and pH profiles of a representative fed-batch CHO cultivation to depict the performance of their control in

gaining reliable off-gas and base consumption data for process monitoring purposes as performed in this work.

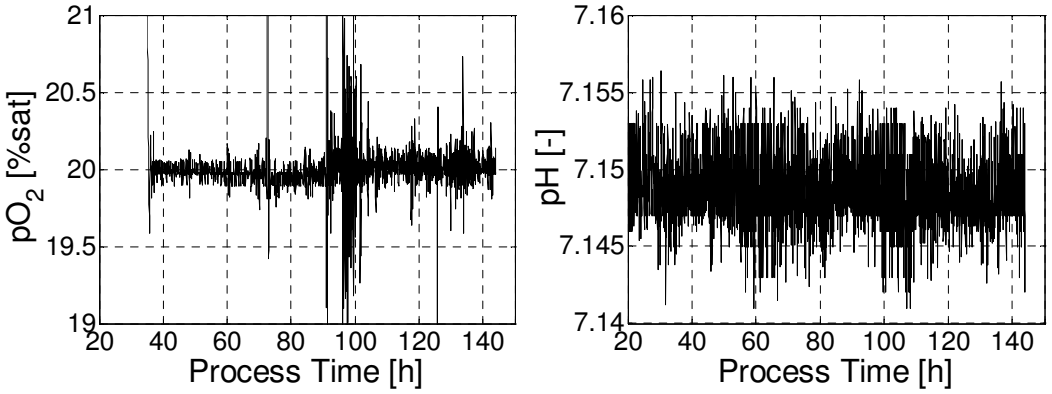

Figure 2. Controlled dissolved oxygen (*right*) and pH (*left*) profiles for a fed-batch CHO cultivation. Due to well tuned PID controller parameters, the noise of the signals around their setpoints is low for both parameters.

As reliable on-line data can now be obtained through OUR, CPR and consumed base with the respective inherent global information content, a multivariate approach using mathematical relationships to acquire process information, as recommended by the PAT framework, can be applied. For instance, this process information can be the cell density or the specific growth rate, two of the most important process attributes. The advantage that comes up is the possibility to estimate these quantities on-line, i.e. in real time, with multivariate inputs. Several multivariate techniques are available. These range from multiple linear or nonlinear regression techniques to hybrid models where a black-box approach, e.g. artificial neural networks (ANNs), and the system's mass balances are linked. Their application as on-line estimators necessitates the availability of historical data sets to prior train the estimator, but which is not problematic in industry as huge amounts of data have been produced.

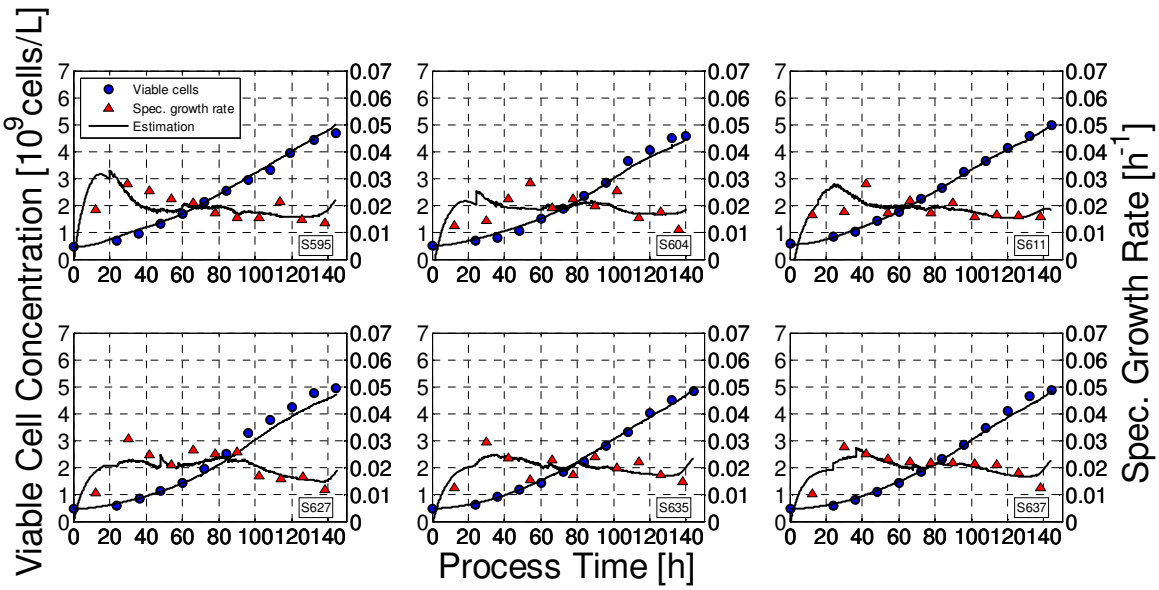

Figure 3. Estimation results (*lines*) of validation data sets using the hybrid model approach with total consumed O_2 (mmol), total produced CO_2 (mmol) and consumed base (g) as inputs. Outputs are the specific growth rate (*red triangles*) for the black-box part (ANN) and viable cell concentration (*blue circles*) for the mass balance part. The ANN consists of single hidden layer containing 4 nodes.

Figure 3 depicts the estimation results of validation data, meaning that the data are not used to train the ANN, using the hybrid model approach. This approach was already successfully applied in microbial cultures (Schubert et al. 1994) and now proves its applicability in mammalian cell cultures as well.

Recently, novel off-gas sensors have been commercialized with special emphasis on mammalian cell cultivations. The applicability of these sensors was investigated in parts of the experiments presented in this work. The measurement signals coming from these new devices were comparable to the already used mass spectrometer technique. These analyzers are easy to implement and robust in providing representative process data, thus, making them attractive to fit PAT analyzer requirements.

Advanced on-line monitoring or rather estimation of key process variables, which are generally assessed by periodic sampling followed by off-line analysis, can help to assure process performance and quality during the running process. It can support the operator to take decisions to keep the process within specifications with respect to the design space. But monitoring is just one tool to guarantee reproducible processes. Another part is the design of the process itself which should be robust and reproducible with regards to certain random or uncontrolled operational errors.

QUALITY BY DESIGN APPROACH

If an API is dedicated to become a commercial product, the manufacturer has to prove that the manufacturing process is reproducible and will consistently deliver the product quantity and quality. This is covered by several validation activities. The definition of the process design based on knowledge gained through development and scale-up activities, together with the capacity of a high batch-to-batch reproducibility (FDA 2011), is crucial to build quality into products by design.

The operational process conditions are typically fixed in process specific standard operational procedures (SOP) to guarantee a minimum of batch-to-batch reproducibility. As already discussed on microbial cultivations by Jenzsch et al. (2006a), applying the same SOP for batches/campaigns leads to a certain variation in the process outputs, especially in the biomass concentrations. For mammalian cell cultivations, it is exactly the same situation. The goal is to understand the sources of the observed process variability and to eliminate them by an appropriate process design.

Numerical simulation studies with mammalian cell process models facilitate the identification of sensitive variables. What becomes evident by setting up the model and by process understanding is that the cell concentration plays a central role for the performance and reproducibility of the process. In addition, this state variable is exposed to distortions since start of the cultivation. Firstly, the inoculum phase requires manual operations subjected to random deviations in the inoculated cell concentration X_0. Secondly, the current state of the cells (e.g. *in vitro* cell age, mid or late exponential growth phase) from the pre-culture is mostly not as reproducible as needed, thus leading to different starting conditions of the production bioreactor.

Common production processes are developed to yield high cell concentrations at time of harvest, mostly by fed-batch mode. This is obvious as the total amount of API is directly proportional to the amount of producing cells. Further, the total API amount is proportional to the cell specific product formation rate q_P as well, i.e. the performance of the living cell to produce the desired recombinant protein should be high. In such fed-batch processes, the substrates (mainly glutamine and glucose) are fed in excess to avoid substrate limitation. With respect to process robustness and reproducibility, such a feeding strategy results in unlimited growth, i.e. growth with maximum specific growth rate μ_{max}, which incorporates a higher sensitivity to different initial cell concentrations. From the simulated sensitivity analysis (Figure 4) it turns out that the specific growth rate μ has to be lower than μ_{max} to cope with initial cell concentration variations. The extent of the μ-decrease depends on the expected variation of the initial X_0. To maintain the smaller specific growth rate, an exponential feed rate, $F(t) = F_0 \cdot \exp(\mu_{set} \cdot t)$ with $\mu_{set} < \mu_{max}$ and $F_0 = f(X_0)$, of at least one main substrate has to be performed. Once μ_{set} and X_0 are chosen, the corresponding feed-profile is then fixed for the entire process time.

The mechanistic behind this strategy can be explained as follows: If the initial cell concentration is higher than expected, the substrate concentration is subsequently low leading to a reduced cell' specific growth rate. Thus, the cell concentration is tracked back to the desired value in a certain process time. If the initial cell concentration is lower than expected, the substrate concentration is high to allow the cells to increase their specific growth rate. Again, applying a specific growth rate lower than μ_{max}, it automatically compensates the deviation from the setpoint cell

concentration profile. Process robustness positively benefits from this simple approach. In contrast, when a variation in X_0 is expected and the feed profile is always calculated with $\mu_{set} = \mu_{max}$, cells cannot de- or increase their specific growth rate. As a consequence, different growth profiles will appear raising the standard deviation of the cell concentrations.

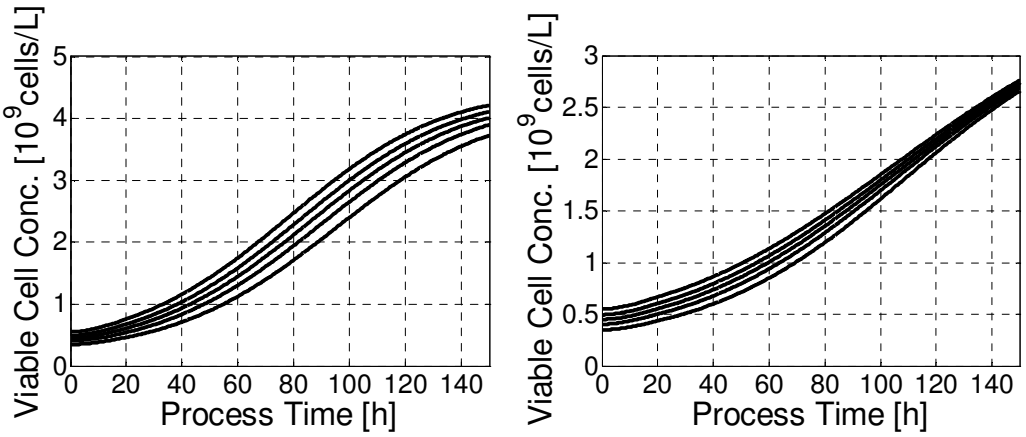

Figure 4. Different initial cell concentrations leading to variations in the entire cell concentration profile. *Left:* Fed-batch cultivation with exponential feeding for $\mu = \mu_{max}$. *Right:* Cultivation with a feeding strategy $\mu < \mu_{max}$ where glutamine was chosen as the limiting substrate.

This inherent control of variability by process design was already explained by Jenzsch et al. (2006) for *E.coli* fermentations but was not proven to be applicable for mammalian cell cultivations. As it is commonly stated that mammalian cells are more sensitive to changes in their environment (e.g. induction of apoptosis due to nutrient limitations) compared to microbial systems, the adoption of the strategy in applying specific growth rates lower than its maximum value is challenging. Results presented here utilizing CHO cells in glutamine limited fed-batches with $\mu_{set} < \mu_{max}$ indicate that is it indeed possible to reach a high batch-to-batch reproducibility without significant loss of viability (Aehle et al. 2011a).

With the simple control approach described before, process robustness can be significantly increased without any feedback from the process. This is generally referred to as open-loop control. From the FDA PAT initiative, process control strategies are intended to actively manipulate the state of a process to maintain the desired state. But active manipulation requires a certain feedback from the process. Taking the process characteristic into consideration, a decision about the variable that should be controlled has to be made. Different feedback control strategies for a mammalian cell culture fed-batch process are presented in this work.

SIMPLE CONTROL OF THE TOTAL CONSUMED OXYGEN

Closed-loop fully automated control strategies to reduce process variations using on-line signals are not yet established in industrial cell culture processes. Currently, a feedback is only obtained by daily sampling that provides information for the calculation of the corresponding substrate feed rate for a certain future time interval. But as the sampling rate is rather low (typically one or

two samples per day), only few data of the current state are available which hamper an adequate process control. For process safety reasons, nutrients are mostly fed in high amounts to avoid nutrient limitation. But non-limitation consequently leads to maximum possible specific growth rates and thus to an unfavourable batch-to-batch reproducibility.

A trend to control the cell concentration by using novel and marketed on-line sensors becomes an attractive option. But these sensors suffer from the fact that their signal output has to be directly correlated with the cell concentration, hence leading to high demands in their calibration. In addition, due to their installation in-situ they provide only local information and they are prone to be disturbed by the turbulent flow pattern, including bubbles or changing medium characteristics. Thus, global measurements representing the cultures' state, e.g. the cell concentration, have to be taken to implement an easy-to-use closed-loop control strategy for production bioreactors.

A very simple approach using a global measurement to keep the cell concentration along a pre-defined trajectory is the relation between cell's respiration activity and growth. The oxygen uptake rate is tightly related to the specific growth rate and cell concentration, $OUR = f(\mu,X)$. As a result, if the OUR of the validation run matches a predefined OUR setpoint profile, no differences between the corresponding cell concentration profiles are expected as well. One can now take the OUR(t)-profile, or even better, the total consumed oxygen profile of a golden batch or from an (simulated) optimization study as the controlled variable. To influence the process by the controller output, the feed rate of a limiting substrate serves as the control variable.

If the initial cell concentration is higher than its target, then cells consume more oxygen than expected. Hence, the controller reduces the feed rate leading to a lower substrate concentration in the bioreactor and cells reduce their specific growth rate. In contrast, if the initial cell concentration is lower than expected cells consume less oxygen forcing the controller to raise the feed rate and thus the substrate concentration in the bioreactor so that the cells can raise their specific growth rate accordingly.

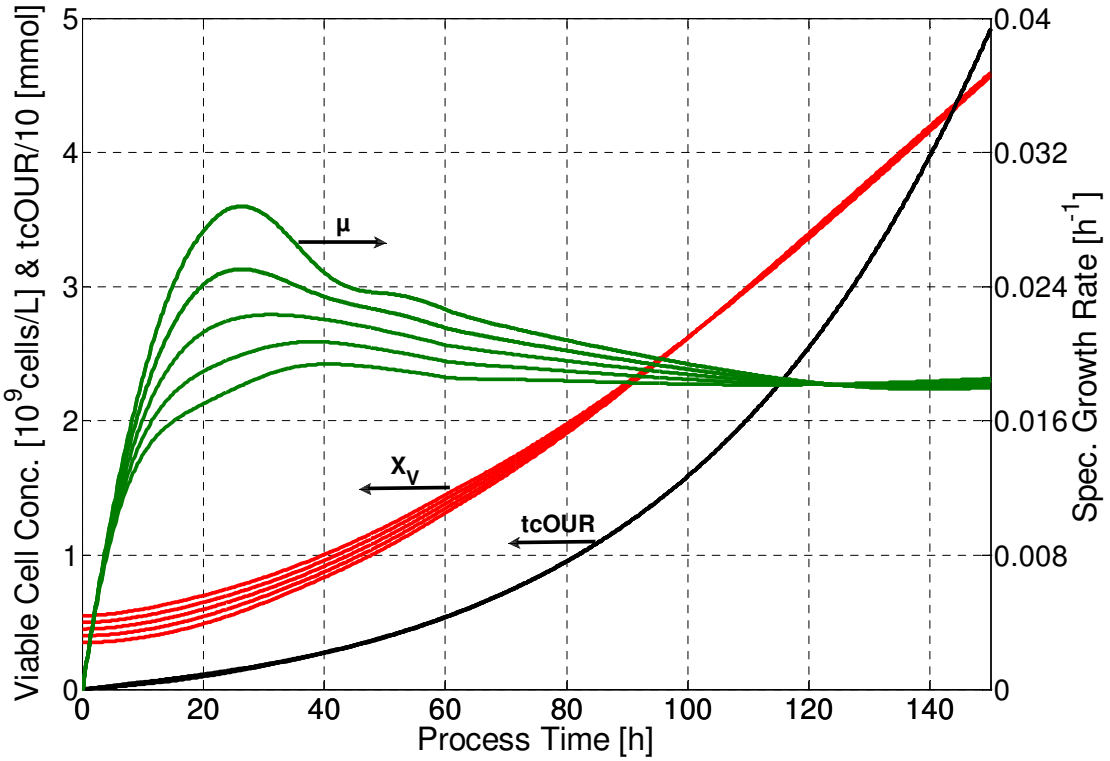

Figure 5. Closed-loop control simulation of fed-batch processes with a certain variation in the initial cell concentration. As the tcOUR is well controlled in each case the initial cell concentration variation is diminished by higher or lower specific growth rates at start and ending close to each other.

To allow robust process behaviour by using a closed-loop control strategy, the pre-defined biomass profile should correspond to a specific growth rate lower than μ_{max}. In addition, the controller should be designed in such a way that no sophisticated control parameter adjustments, according to the process state, have to be made as this would be more favourable in terms of controller qualification/validation in the production environment. This can be employed by a simple feedforward/feedback controller design as described in chapter 4.

MODEL SUPPORTED CONTROL OF THE TOTAL CONSUMED OXYGEN

Applying mechanistic process models for supervision and control purposes and their successful validation implies a high degree of process understanding. Knowledge gained from previous development or production scale data is compressed in mathematical equations connecting observations (inputs) and their potential influence on the entire process performance (outputs). A process model can estimate the process state and thus is able to predict the outcome, as would be intuitively done by an experienced human operator. Moreover, potential corrective actions can be evaluated and the optimal solution can be applied before the process runs into a critical path.

The driving force for model implementation is still the lack of robust and reliable on-line sensors for most of the common variables, e.g. the concentration of substrates, metabolites and most important of the target protein. Thus, their estimation could be done by mechanistic process models. Normally, the model is fitted to the data by model parameter identification after running the process. The more state variables to be estimated, the more the number of parameters contained in the model that have to be fitted and checked for their rational. Hence, the model quickly becomes quite complex, hence reducing the motivation to implement it in an industrial environment. On the other hand, the model structure and fitting algorithm can be simplified when an adaptive approach is used. In this case, the model is fitted to data while running process for defined time intervals, thereby raising the state representation accuracy and predictability.

For control purposes, an adaptive model predictive approach was used for a closed-loop control strategy in order to gain a high batch-to-batch reproducibility. The principle of this approach is described in Fig. 6. As for the previous control strategy, the total consumed oxygen and the glutamine feed rates serve as the controlled and control variables, respectively. One can now state that it would be possible to directly control the cell concentration as the model can estimate (within confidence intervals) this state variable. But it is evident that the sampling interval for the cell concentration is rather large, thus, the feedback from the process is not sufficient to guarantee a proper control. Therefore, control of the on-line determined total oxygen amount, as strictly related to the cell concentration, is advantageous since the measurement interval is very short. In addition, as the model is trained on historical data, faults or inconsistencies in the on- and off-line measurements for instance can be recognized.

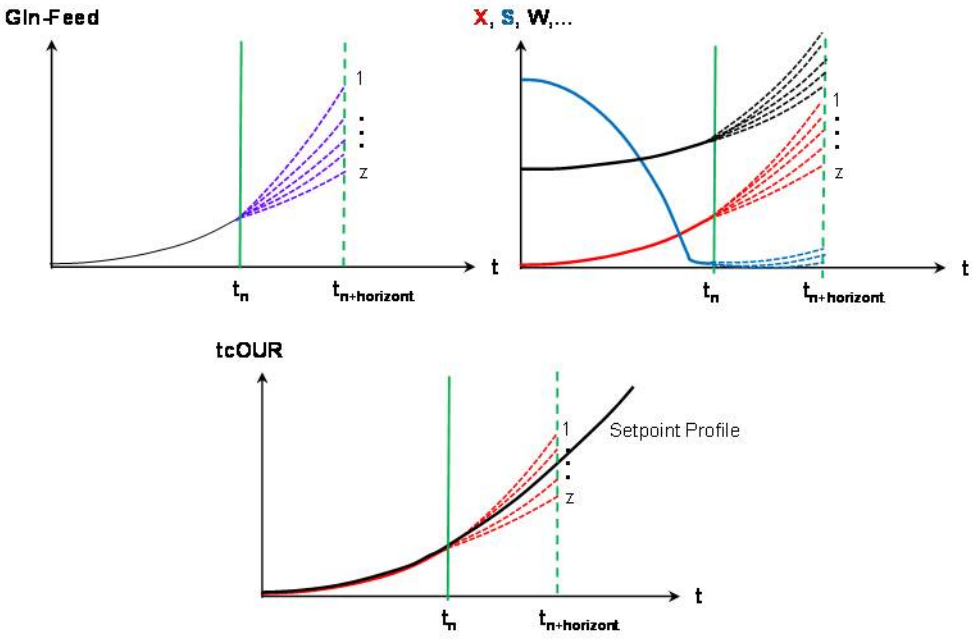

Figure 6. Schematic representation of the model predictive control strategy. The full model equations for 1…z different feed profiles of glutamine in the time interval $[t_n:\Delta t:t_{n+h}]$ are solved (*upper left*). Future state variable trajectories k(1...z) are predicted resulting from the different glutamine feed profiles (*upper right*). The k^{th} prediction

with the smallest RMSE between the predicted and setpoint tcOUR profile determines the new glutamine feed rate profile *(lower mid)*.

RECOMBINANT PROTEIN PRODUCTION

In the previous sections, much attention was given to the design and control of a robust mammalian cell culture process with emphasis on reproducible cell concentrations. Despite the fact that the cell concentration plays an essential role, the produced recombinant protein, or API, represents the economic benefit of each process. It is not only the quantity of the protein at the end but also its quality, measured by expression of the expected bioactivity. This is especially true for proteins produced by mammalian cells. The protein's quality is characterized by its correct post-translational modifications that primarily depend on the cultivation environment.

The mass of the product obtained at the end of the process is described by a time integral over three terms (Eq. 3).

$$m_P(t) = \int_0^t q_P \cdot X_V \cdot \frac{W}{\rho} \cdot dt \qquad (3)$$

From Eq. 3, maximum mass of the product can be obtained by increasing (i) the weight W or volume of the culture, (ii) the final viable cell concentration X_V, representative of cell longevity and (iii) the entire process time t. Another important parameter influencing the protein amount is the cell specific protein production rate q_P which should remain high during the entire process time, i.e. the stable transfection of a high productive clone is of tremendous importance. Besides this, it is important to note that q_P and X_V primarily depend on the specific growth rate μ which should therefore be controlled. But controlling the specific growth rate leads to inconsistencies in batch-to-batch reproducibility, as variations in the initial cell concentration lead to different biomass trajectories (Jenzsch et al. 2006b).

With the methods presented in this work, it is now possible to keep the biomass profile and subsequently the specific growth rate under control providing even the possibility to identify the relationship between q_P and μ resulting in additional process knowledge. In most cases of recombinant protein production, it is a compromise between maximum productivity and process robustness. A process design aiming at a high productivity by reaching high cell densities is hard to control and lacks robustness. Decreasing the specific growth rate to a too low value in order to gain robustness and reproducibility becomes inefficient as the cell concentration is consequently too low. Hence, an optimal μ respecting both economic aspects and a high batch-to-batch reproducibility has to be identified.

The influence of $q_P(\mu)$ on the protein quality has to be individually investigated for each host cell/ heterologous protein system. For the proof of concept, erythropoietin as a model protein and its sialylation distribution was used as the quality attribute in the experiments presented here.

The indirect control of the specific growth rate or the cell concentration by using the culture's total consumed oxygen as the controlled and easy to measure variable, results automatically in a high batch-to-batch reproducibility of both the recombinant protein concentration and quality. This is possible as these variables are tightly related to each other describing a deterministic system. In addition, it immediately has a positive impact on down-stream steps which can be adjusted more appropriately due to better up-stream predictability.

CONCLUSIONS

During the last 2 decades, much attention was put on the development of high cell and product titer cell culture processes. Intense improvements in expression systems and reactor operation modes have led to the current state-of-the-art industrial processes. In contrast, less effort has been put on the design of highly reproducible cultivation modes and their control. In the case of cell culture derived products, a reproducible process operation was shown to be of much higher value as the post-translational modifications were found to sensitively depend on details in the process operational procedure. Thus, monitoring and control activities have to be intensified as recommended by the FDA's PAT initiative, now adopted by other health authorities as well. In this regard, process understanding through mechanistic knowledge facilitates the design of robust operational procedures and consequently, the implementation of feedback control strategies.

The present work supports the FDA's initiative, with particular emphasis on cell culture processes. The following themes are discussed in the chapters herein: (i) methods to on-line estimate important process variables, namely the cell concentration and specific growth rate, (ii) strategies to significantly improve the batch-to-batch reproducibility of CHO-cell fed-batch cultures by open- and closed-loop control and (iii) the application of novel, low-cost sensors for off-gas analysis.

The basis for the listed points mentioned above is the establishment of efficient on-line process analyzers for highly informative quantities. These analyzers should be accurate, reliable, easy to implement (even in large scale bioreactor environment), do not possess a risk for contaminations and not adversely affect process performance or product quality. In this sense, off-gas analysis was successfully implemented and employed as shown by this work. It is important to mention that in order to use reliable off-gas signals with a high signal-to-noise ratio for monitoring and control purposes, the identification of critical cultivation parameters that mostly influence them is mandatory. Accordingly, pH and pO_2 signal quality is of crucial importance.

As the cell concentration and its evolution during the process plays an essential role, several multivariate on-line estimation techniques are shown to be very powerful tools. The choice of the techniques was made from an industrial perspective: The techniques are immediately applicable in production plants as the required measurements can be performed with well-established devices. These on-line soft sensors are important tools for fermentation operators as the process performance can be evaluated almost in real-time, as requested by PAT. From a comparison of the techniques, the best results could be obtained with hybrid models providing not only the cell concentration but also the specific growth rate estimation. A sufficient amount of data for a

reliable model parameter determination should be not an obstacle as industrial processes are running under the same operational procedures.

A very simple approach describing the design of a robust operational procedure is equally presented in this work. It includes the feasibility of decreasing the specific growth rate to a value below its maximum by limiting one of the main substrates using an appropriate feeding rate. As a consequence, this proposed fed-batch process design (open-loop design) enables to deviations from the nominal cell concentration profiles, hence leading to very reproducible trajectories and finally predictable states at harvest. In general, lowering the specific growth rate below its maximum is a prerequisite for all further control strategies presented in this thesis. Furthermore, it is shown that different values of the specific growth rate could be adjusted through different levels of substrate limitation without loss of cell viability, therefore giving the possibility to easily investigate the relationship between specific protein formation and cell growth rate. In this respect, it provides an efficient tool to identify the optimal strategy for mammalian cell culture processes.

Applying open-loop control suffers from the fact that no random distortions should occur during the process, which in practice is hard to avoid, especially with such long lasting processes. Thus, the goal is to establish feedback controllers that automatically eliminate deviations from a desired setpoint biomass or specific growth rate profile, quantities that can hardly or not measured on-line.

To follow that argumentation, a comprehensive experimental study was performed by setting up a closed-loop control strategy. For the controller design, the total cumulative OUR (tcOUR) was chosen to be the controlled variable and the glutamine feed rate as the control variable. The tcOUR can be easily determined with sufficient accuracy providing even global information. By controlling the tcOUR, the cell concentration and the specific growth rate can be thus indirectly kept on a desired track. This is possible through the deterministic nature of the cultivation system. In addition, sophisticated adaptation of the controller parameters, appearing necessary for such dynamic processes, is not needed when using the proposed controller design. Hence, the simple design of the controller and the well-established measurement techniques facilitate its practicability in the industrial GMP environment.

The idea of the tcOUR controller was further extended by incorporation of a mechanistic process model into the controller. The technique has the advantage compared to conventional feedback controllers that it takes changes in the process development into account that are to be expected on the base of à priori knowledge about the process behaviour. By using the model, different feeding profiles can be tested and their outcome on the state variables, particularly the OUR trajectory as the controlled variable, can be predicted. As a result, the optimal feeding profile for the future time interval can be automatically applied to obtain a high batch-to-batch reproducibility. On the other hand, as the model gives an estimation of the state variables, all on- and off-line measurements can be judged for their correct value and corresponding analytical faults can be recognized.

Finally, more simplified off-gas sensors already used for microbial systems were investigated for their applicability in mammalian cell cultivation systems. Due to the parallel installation of the well-established mass spectrometer and these sensors at the vent line of the bioreactor, a direct

on-line comparison of the derived OUR and CPR data was possible resulting in a very good conformity. In addition, the sensors proved to be devices fulfilling the requirements by PAT. Moreover, special know-how or trained personnel to operate these sensors are not necessary, further facilitating their utilization. From the experiences gained during these investigations, it is recommended to introduce the sensors for off-gas monitoring in development and industrial scale mammalian cell bioreactors.

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Chapter 2

Comparison of Viable Cell Concentration Estimation Methods for a Mammalian Cell Cultivation Process

ABSTRACT

Various mechanistic and black-box models were applied for on-line estimations of viable cell concentrations in fed-batch cultivation processes for CHO cells. Data from six fed-batch cultivation experiments were used to identify the underlying models and further six independent data sets were used to determine the performance of the estimators. The performances were quantified by means of the root mean square error (RMSE) between the estimates and the corresponding off-line measured validation data sets. It is shown that even simple techniques based on empirical and linear model approaches provide a fairly good on-line estimation performance. Best results with respect to the validation data sets were obtained with hybrid models, multivariate linear regression technique and support vector regression. Hybrid models provide additional important information about the specific cellular growth rates during the cultivation.

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Chapter 3

Increasing Batch-to-Batch Reproducibility of CHO Cultures by Robust Open-Loop Control

ABSTRACT

In order to guarantee the quality of recombinant therapeutic proteins produced in mammalian cell systems, the straightforward approach in industry is to run the processes as reproducible as possible. It is first shown that considerable distortions in the currently operated processes appear when the initial cell density deviates from its nominal value. Small deviations in the initial cell mass may lead to severe deviations from the desired biomass trajectory. Next, it is shown how to design a fed-batch production process in such a way that it is robust with respect to variations in the viable cell density. A simple open loop strategy is proposed for that purpose. Here we show for the first time at animal cell cultures (CHO cells) that by means of an appropriate glutamine feed rate profile F(t), which keeps the specific growth rate of the cells on a predefined value below its maximal value while maintaining the viabilities on a high level, the diverging viable cell count profiles change over into a robust converging set of profiles. The CHO cells used to validate the procedure could be focused to any specific growth rates below μ_{max}.

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Chapter 4

Simple and Efficient Control of CHO Cultures

ABSTRACT

Cell cultures must tightly be kept under control in order to guarantee a sufficiently small variability in the protein product quality. A simple and efficient technique for CHO-cell cultures is presented that allows keeping the viable cell count X_V and the specific growth rate μ of the cells on predefined trajectories. As X_V and μ cannot directly be measured on-line, they are controlled indirectly via the total mass of oxygen consumed. On-line values of the latter can precisely be estimated from off-gas analysis, i.e. from the O_2 volume ratio measured in the vent line and air flow rate measurements. In glutamine-limited fed-batch cultivations, the glutamine feed rate can be manipulated in such a way that the viable cell density and the specific growth rate are kept on predefined profiles for nearly the entire cultivation time. The viability of the cells is not affected by the closed loop control actions. The technique was validated with CHO-cells cultured in a 2.5 L fully instrumented stirred tank bioreactor. It is shown that the controller is able to run the process exactly on predefined tracks with a high batch-to-batch reproducibility. By means of six fed- batch cultivations of CHO cells it was shown that a remarkable reproducibility of viable cell concentration could be achieved throughout 140 h cultivation time.

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Chapter 5

Increasing Batch-to-Batch Reproducibility of CHO-Cell Cultures Using a Model Predictive Control Approach

ABSTRACT

By means of a model predictive control strategy it was possible to ensure a high batch-to-batch reproducibility in animal cell (CHO-cell) suspensions cultured for a recombinant therapeutic protein (EPO) production. The general control objective was derived by identifying an optimal specific growth rate taking productivity, protein quality and process controllability into account. This goal was approached indirectly by controlling the total oxygen mass consumed by the cells which is related to specific biomass growth rate and cell concentration profile by manipulating the glutamine feed rate. Process knowledge represented by a classical model was incorporated into the model predictive control algorithm. The controller was employed in several cultivation experiments. During the fermentations, the model parameters were adapted after each sampling event to cope with changes in the process' dynamics. The ability to predict the state variables, particularly for the oxygen consumption, led to only moderate changes in the desired optimal operational trajectories. Hence, nearly identical oxygen consumption profiles, cell and protein titers as well as sialylation patterns were obtained for all cultivation runs.

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Chapter 6

Simplified Off-Gas Analyses in Animal Cell Cultures for Process Monitoring and Control Purposes

ABSTRACT

Batch-to-batch reproducibility of animal cell cultures can significantly be enhanced using process control procedures. Most informative signals for advanced process control can be derived from the volume fractions of oxygen and carbon dioxide in the vent line of the reactors. Here we employed simple low-cost sensors, previously not considered for off-gas analysis at a laboratory-scale cell cultures, and compared them with a simultaneously used quadrupole mass spectrometer, i.e., the standard equipment. A decisive advantage is that the sensors did not need any calibration and are easy to use. We show that monitoring and advanced control of cell cultures can significantly be simplified using the devices tested here and that the same batch-to-batch reproducibility can be obtained with much less effort than before.

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Aehle M, Kuprijanov A, Schaepe S, Simutis R, Lübbert A (2011) Simplified off-gas analyses in animal cell cultures for process and control purposes. *Biotechnology Letters* 33(11): 2103-2110

Summary

The market for cell culture derived biopharmaceuticals is still growing as their demand will increase due to a broad range of new indications. In addition, intensive basic research by academic institutions and industrial companies has enabled remarkable developments towards new potential drugs. In contrast, the fermentation technology to produce the drug substances in a safe and efficient manner has been treated with less attention.

In addition, the quality of such processes plays a tremendous role with respect to the *in vivo* activity of the final drug. This is especially true for APIs expressed by mammalian cell systems as their correct post-translational modifications primarily depend on the cultivation conditions. In this sense, to obtain consistent quality throughout the entire production campaigns, the process design should guarantee a high batch-to-batch reproducibility.

To accommodate these requirements and to improve their processes, manufacturers are forced to invest into their process technology. Investments in monitoring technologies to analyze influencing variables on process critical parameters can improve the level of mechanistic process understanding. This is the prerequisite for the development of robust operational procedures, which will then lead to the implementation of feedback control to ensure predefined quality and reduce risk. Recommendations to manufacturers to adapt this argumentation have already been announced by the FDA's PAT initiative since 2004. The challenge is now to transfer the recommendations to sensitive cell culture processes with concrete methods applicable in industrial practice.

All methods and results presented in this work show that the batch-to-batch reproducibility of CHO cell cultivations expressing a recombinant therapeutic protein can significantly be improved. As the process variables are tightly related to each other, the control of one variable, here performed on the cells' oxygen consumption, automatically gives control over other variables like the viable cell density or target protein concentrations.

In a first approach, different software-based on-line estimation techniques for the most important variables, the viable cell density and specific growth rate, were compared with respect to their performances. Among the tested techniques, the hybrid model approach is shown to be the best. By using such soft-sensors that provide the data in almost real-time, executed changes to the process do not further rely on off-line measurements. Thus, critical process steps such as time of inoculation, start of feedings, initiation of pH/temperature shifts or the optimal harvest time, are under better control and consequently improve the reproducibility of the entire process.

In a next step, a critical source of process variability, the initial cell concentration, was identified and its sensitivity to the process explained. By using that knowledge, a robust operational procedure was developed so that deviations of the initial cell concentration to a nominal value were managed by the process itself. This was made possible as the specific growth rate could be lowered below its maximum value by an appropriate feeding strategy. Final cell concentrations of several fermentations could therefore be controlled to values close to each other and hence become more predictable. Furthermore, it is shown that different values of the specific growth

rate could be adjusted through different levels of substrate limitation without loss of cell viability. This provides the possibility to easily investigate the relationship between specific protein formation and cell growth rate. The proposed procedure, also referred to as open-loop control, provides a solution to the FDA's statement that quality should be built in or by design of the process.

The application of the open-loop strategy without the need of special measurement techniques proved to be a very powerful tool for mammalian cell culture processes. However, random or unpredictable distortions during such processes can occur and risk the release of the product. In addition, according to FDA's PAT initiative, process control strategies are intended to actively manipulate the state of a process to maintain a desired state.

For these reasons, several experimental studies based on developing different closed-loop control strategies were performed. The total consumed oxygen (tcOUR) by the culture was chosen to be the controlled variable, as this quantity is easy to determine on-line and provides global information. As a desired tcOUR profile corresponds to a certain cell density and specific growth rate profile, control of the tcOUR enables a high reproducibility in these process variables. In addition, expensive adaptation of controller parameters, appearing necessary for such dynamic processes, is not needed when using the proposed controller design. Hence, the simple design of the controller and the well-established measurement techniques facilitate its practicability in the industrial GMP environment.

The concept of the tcOUR controller was further extended by the incorporation of a mechanistic process model. The technique has the advantage, compared to conventional feedback controllers, that it takes changes in the process development into account that are to be expected on the base of à priori knowledge about the process behaviour. In other words, the process control, normally done by very skilled operators, can be taken over by this model supported controller.

Finally, off-gas sensors, already commercially available and used for microbial cultivations, were investigated for their applicability in mammalian cell cultivation systems. Due to the parallel installation of the well-established mass spectrometer and these sensors at the vent line of the bioreactor, a direct on-line comparison of the derived OUR and CPR data was possible resulting in a very good conformity. The determined OUR data by using these sensors were further used in another new control strategy. Based on the results of these investigations, it is recommended to introduce the sensors for determination and monitoring of the cell's respiratory activity from bench to large-scale mammalian cell bioreactors.

Zusammenfassung

Der Markt für Biopharmazeutika, hergestellt mittels Zellkulturprozessen, verzeichnet ein stetes Wachstum, da ihre Nachfrage aufgrund einer Vielzahl von neuen Indikationen zunehmen wird. Hinzu kommt, dass durch intensive Grundlagenforschung im akademischen und industriellen Umfeld bemerkenswerte Entwicklungen hinsichtlich neuer potentieller Medikamente ermöglicht werden. Im Gegensatz dazu wurde bis dato der Fermentationstechnologie, um diese Medikamente sicher und effizient herstellen zu können, weniger Aufmerksamkeit geschenkt.

Die Qualität solcher Prozesse spielt hinsichtlich der *in vivo* Aktivität des finalen Medikaments eine wesentliche Rolle, im Besonderen für APIs welche durch tierische Zellsysteme exprimiert werden, da ihre korrekten post-translationalen Modifikationen hauptsächlich von den Kultivierungsbedingungen abhängen. In diesem Sinne muss durch das Prozessdesign eine über die gesamte Produktionskampagne hohe batch-to-batch Reproduzierbarkeit gewährleistet werden, um eine gleichbleibende Qualität zu ermöglichen.

Um diesen Anforderungen und der Verbesserung ihrer Prozesse entgegenzukommen, sind die Hersteller gezwungen in ihre Prozesstechnologie zu investieren. Investitionen in Monitoring-Technologien, um Einflussvariablen auf prozesskritische Parameter analysieren zu können, wird der Grad an mechanistischem Prozessverständnis erhöht. Als Folge daraus ermöglichen diese die Entwicklung von robusten Prozessführungsstrategien sowie der Einsatz von Regelungen, um eine zuvor definierte Qualität unter reduzierten Risiken zu sichern. Vorschläge an die Hersteller, um diese Denkweise zu übernehmen, wurden durch die seit 2004 verkündete PAT Initiative der FDA von regulatorischer Seite bereits gemacht. Die Herausforderung besteht nun darin, diese Vorschläge auf die als sensitiv angesehenen Zellkulturprozesse mit konkreten, industrietauglichen Methoden umzusetzen.

Aus den entwickelten Methoden und Resultaten dieser Arbeit wird ersichtlich, dass die batch-to-batch Reproduzierbarkeit von CHO Zellkulturen, zur Herstellung eines rekombinanten therapeutischen Proteins, erheblich erhöht werden kann. Da die Prozessvariablen streng miteinander in Beziehung stehen, ermöglicht die Regelung einer Variablen, hier der Gesamtsauerstoffverbrauch der Zellen, automatisch die Kontrolle anderer Prozessvariablen wie die Lebendzell- oder Proteinkonzentration.

Zunächst wurden verschiedene Software basierte on-line Schätzmethoden für die wichtigsten Prozessvariablen, die Lebendzelldichte und spezifische Wachstumsrate, hinsichtlich ihrer Leistungsfähigkeit verglichen. Der Hybrid-Model Ansatz hat sich dabei als der beste Ansatz herausgestellt. Durch die Nutzung solcher Soft-Sensoren, welche Daten in nahezu Echtzeit liefern, ist man bei durchzuführenden Änderungen im Prozess nicht mehr auf off-line Messungen angewiesen. So sind kritische Prozessschritte, wie z.B. Inokulationszeitpunkt, Start der Zufütterung, Initiierung von pH- oder Temperaturprofilen sowie der optimale Erntezeitpunkt, deutlich besser unter Kontrolle und folglich wird die Reproduzierbarkeit des Gesamtprozesses erheblich verbessert.

Im nächsten Schritt wurde eine wesentliche Quelle für die Prozessvariabiliät, die Startkonzentration der Zellen, identifiziert und ihre Sensitivität auf den Prozess verdeutlicht. Mit Hilfe dieses Wissens wurde eine robuste Prozessführungsstrategie entwickelt, so dass Abweichungen der Inokulumzelldichte von einem vorgegebenen Sollwert durch den Prozess selbst ausgeglichen werden. Dies wurde möglich, in dem die spezifische Wachstumsrate unterhalb ihres maximalen Wertes durch eine entsprechende Zufütterungsstrategie eingestellt werden konnte. Das Zellwachstum von mehreren Fermentationen wurde damit so gesteuert, dass die Differenz der Endzellkonzentrationen sehr klein ist. Dies ermöglicht wiederum eine verbesserte Vorhersagbarkeit der zu erreichenden Zelldichten. Des Weiteren konnten verschiedene spezifische Wachstumsraten, ohne Verlust an Zellvitalität, durch unterschiedliche Limitierungsgrade des Substrates eingestellt werden. Damit werden Untersuchungen ermöglicht, welche die Beziehung zwischen spezifischer Produktbildungs- und spezifischer Wachstumsrate beschreiben können. Diese aufgezeigte Steuerungsstrategie bietet eine Lösung zu dem von der FDA gemachten Aussage, dass Qualität durch entsprechendes Prozessdesign erreicht werden soll.

Die Anwendung dieser Steuerungsstrategie hat den Nachteil, dass zufällige oder unvorhersehbare Störungen während der Prozesszeit nicht auftreten dürfen. Das ist bei tierischen Zellkulturen umso wichtiger, da die sich Prozesszeit über mehrere Tage erstrecken kann.

Aus diesem Grund wurden mehrere experimentelle Studien durchgeführt, in denen verschiedene Regelungskonzepte entwickelt worden. Der integrale Verbrauch an Sauerstoff (tcOUR), welcher on-line bestimmbar und eine globale Messgröße ist, diente dabei als Regelgröße. Da ein vorgegebener tcOUR Verlauf einem bestimmten Zelldichteverlauf bzw. einer spezifischen Wachstumsrate entspricht, konnte durch die hier aufgezeigte Regelungstrategie eine hohe Reproduzierbarkeit in diesen Prozessvariablen erreicht werden. Eine aufwendige Adaptierung der Reglerparameter, welche für diese dynamischen Prozesse notwendig erscheint, ist aufgrund des Reglerdesigns nicht erforderlich. Durch dieses einfache Reglerdesign unter Nutzung etablierter Messtechniken wird der praktische Einsatz in der industriellen GMP-Umgebung deutlich erleichtert.

Das Konzept des tcOUR-Reglers wurde durch die Einbindung eines klassischen Prozessmodels erweitert. Diese Variante hat gegenüber konventionellen Reglern den Vorteil, dass Änderungen im Prozessverlauf durch à-priori Wissen über das Prozessverhalten erwartet und damit berücksichtigt werden können. Die Prozesskontrolle, welche normalerweise durch ausgebildete und erfahrene Operatoren durchgeführt wird, kann somit durch die modelgestützte Regelung übernommen werden.

Zuletzt wurden Abluftsensoren, welche kommerziell erhältlich und schon bei mikrobiellen Systemen zur Anwendung kommen, auf ihre Tauglichkeit bei tierischen Zellkultivierungen hin untersucht. Da diese Sensoren zusammen mit dem bereits etablierten Massenspektrometer parallel in der Abluftstrecke installiert wurden, konnte somit ein direkter Vergleich on-line erfolgen. Die erhaltenen OUR und CPR Daten zeigten eine sehr gute Übereinstimmung, so dass auch im Weiteren die Sensoren für eine neue Regelungstrategie verwendet werden konnten. Durch diese Untersuchungen ist festzuhalten, dass ihr Einsatz in Zellkulturbioreaktoren unterschiedlichen Maßstabes zur Bestimmung, Überwachung und Regelung der respiratorischen Aktivität der Zellen zu empfehlen ist.

Publication List

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Oral Presentations & Posters

[10] Bioprocess Engineering Course, 2008, Supetar, Island Brač, Croatia

"On-line monitoring and state estimation in animal cell culture" (oral presentation & poster)

[11] BioProScale Symposium, 2009, Berlin, Germany

"Advanced process control strategies in animal cell culture" (poster)

[12] DECHEMA/GVC Vortrags- und Diskussionstagung „Bioprozessorientiertes Analgendesign", 2010, Nürnberg, Germany

"Highly reproducible processes via control of glutamine feeding in CHO-cultures" (poster)

[13] Gesamtarbeitsbesprechung Exzellenznetzwerk Biowissenschaften, 2010, Halle, Germany

"Evolutionary development of process control strategies in fermentation processes for recombinant therapeutic protein production" (oral presentation & poster)

[14] Bioprocess Engineering Course, 2010, Supetar, Island Brač, Croatia

"Robust recombinant therapeutic protein production in CHO cell cultivations" (oral presentation & poster)

[15] 8th European Symposium on Biochemical Engineering Science (ESBES), 2010, Bologna, Italy

"Is it possible to run animal cell cultures as reproducible as microbial cultures?" (oral presentation)

[16] 1st European Congress on Applied Biotechnology (ECAB), 2011, Berlin, Germany

"Running CHO-cell cultures on highest reproducibility levels as required by PAT" (poster)

Curriculum Vitae

General

Name:	Mathias Aehle
Date of birth:	December 30th, 1981
Place of birth:	Halle (Saale), Germany
Citizenship:	German

School Education

09/1988 – 08/1992	Polytechnische Oberschule, Grundschule, Halle (Saale)
09/1992 – 08/1994	Realschule, Halle (Saale)
09/1994 – 07/2000	Albert-Schweitzer Gymnasium, Halle (Saale)

Military Service

09/2000 – 07/2001	Basic military service, Hohenmölsen

University Education

10/2001 – 09/2006	Graduation in Bioengineering, Martin-Luther-University Halle-Wittenberg
09/2006	Degree “Diplom-Ingenieur”
since 10/2006	Ph.D. student at Centre of Bioprocess Engineering, Martin-Luther-University Halle-Wittenberg

Employment in Industry

since 11/2010	Novartis

Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Hiermit erkläre ich weiterhin, dass ich mich mit der vorliegenden Arbeit erstmals um die Erlangung des Doktorgrades bewerbe. Die Arbeit wurde noch keinem anderen Promotionsausschuss vorgelegt.

Weil am Rhein, den 08. Oktober 2011 Mathias Aehle