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Application Report

Report from

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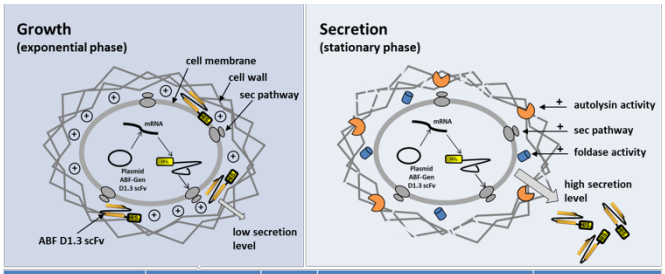
Soft-sensor based adapted feed control for rational secretion process optimization

1. Introduction

Antibodies and antibody fragments (ABF) are most important, highly demanded tools in diagnostic and therapeutic applications with predicted sales of 56 billion dollars by 2012 [1]. Smaller sized ABFs show certain pharmacokinetic advances like increased penetration into solid tumors, the possibility of local applications or rapid clearance from circulating blood serum [2]. Today for industrial antibody production mostly eukaryotic systems like CHO cells are used. These established systems produce especially whole size antibodies in high titers but exhibit long process times and high production cost. Therefore alternative bacterial systems with simple media compositions, short process times and high cell density processes are most desirable. Bacillus megaterium, the organism used in this project, is a promising alternative production system to Escherichia coli with it being an efficient and less cost intensive expression host with high secretion capacities [3]. Due to its lack of the outer membrane which is well known for Gram negative bacteria like E. coli, produced and functional secreted ABFs can directly be harvested from the culture supernatant. These secretion processes depend on a variety of parameters and are challenging in handling for an adequate process control. Recently it was shown that an increased ABF secretion in B. megaterium is coupled to cells being in the stationary phase under starving conditions [4]. Probable mechanisms associated to this stationary phase boosting protein secretion in general are illustrated in Figure 1. Under starvation the membrane potential (MP) is depolarized favoring an increased autolysin activity thereby loosening cell wall structures and facilitate the release of secreted proteins like ABFs into the supernatant [5, 6]. Despite these considerations some components of the secretion machinery (Sec-pathway) were reported to be temporarily controlled which resulted in a maximal level of expression in the onset of protein secretion in the early and ongoing stationary growth phase [7, 8]. Besides, genes coding for certain cell wall associated foldases with protein folding aiding functions like PrsA or HtrA were recently shown to be highly upregulated during starvation phases thus boosting the efficient

folding and release of secreted proteins like heterologous expressed ABFs.
The challenge in the following project was to implement this knowledge of a growth phase dependent secretion in an adequate rational process control to increase both biomass and the ABF titers. This was realized by a feeding strategy based on a control algorithm directly related to the exhaust gas analysis. This so-called soft-sensor was implemented as the basis for an accurate setting of the specific growth rate. By adjusting the specific growth rate the MP of cells influencing the particular secretion efficiency was controlled, thereby directly regulating and increasing the particular ABF secretion into the supernatant.



Phase	Growth rate μ	qCO_2	Membrane Potential (MP)	ABF Secretion
Growth (exp.)	high	high	high	low
Secretion (stat.)	low	low	low	high

Figure 1: Growth- and starvation phase related principles in *B. megaterium* cells having a direct effect on protein secretion. The table summarizes the particular effects on growth rate, qCO_2, membrane potential (MP) and antibody fragment (ABF) secretion, being the basis for an adapted process control strategy.

2. Aim of investigation

The essential idea behind the project is to optimize the production and secretion of the ABF D1.3 scFv anti-lysozyme in *B. megaterium* (YYBm1 (EJBmD1.3scFv) [9]) by means of optimizing the feeding strategy and the ratio of the growth time (t_{Growth}) to the secretion time (t_{Sec}) under stationary phase conditions. In previous experiments it was shown that a simple approach of an extended batch phase or exponential feeding at low growth rates only let to non-satisfying results of low product titers and reduced overall yields [4]. Therefore a repeating strategy with coupled growth and starvation/secretion phases with an optimized ratio (t_{Growth}/t_{Sec}) was implemented. Here growth phases accumulating precursors, increasing biomass and producing ABFs are followed by increased ABF secretion phases under stationary phase conditions. The technique of flow cytometry was used to investigate particular growth phase dependent effects on single cell level regarding the MP (polarization status) of cells as an additional process monitoring tool.

3. Experimental design

3.1 Oscillating fed-batch – concept

To implement a repeating, reproducible and automated growth and starvation/ABF-secretion phase coupled approach, a fed-batch strategy with a time-dependent μ-oscillating feed control was established. A brief overview of the control strategy is given in Figure 2. Its principle is founded on an exponential feed algorithm with a time dependent specific growth rate control which is based upon two cosine functions (equation 1, 2). This function was chosen to reproducibly automate the process and to avoid abrupt feed changes causing over-feeding or sudden increase in overflow metabolites like acetate. The current biomass concentration was estimated by CER and the particular time dependent specific growth rate μ. By this holistic approach the particular specific growth rate, thereby the polarization status of cells (MP) and secretion efficiency of ABFs is accordingly synchronized. Further detailed explanations concerning this control strategy are given in the following. The bioreactor control, defined medium composition, *B. megaterium* strain selection and flow cytometric assays were done accordingly David et al. 2011 [4].

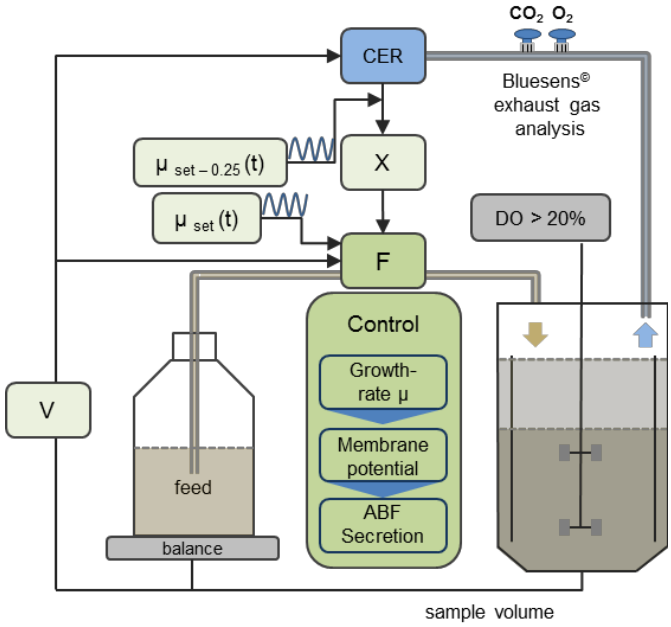


Figure 2: Overview of the control mechanism to create a μ-oscillating feeding (F) strategy adapted to the particular biomass concentration (X) present. The biomass is estimated via the exhaust gas analysis related to CO_2 evolution rate (CER) and to the current growth rate $\mu_{set-0.25}$. μ_{set} is time dependent and based on two cosine functions with a defined maximal growth rate μ_{max}. The underlying cultivation volume (V) in the bioreactor is corrected by sample and feed volume.

3.2 Oscillating fed-batch – mathematical background

The oscillating fed-batch strategy is based on the formula for exponential feeding strategy (1). The term $e^{\mu \times t}$ is assumed to be equal to 1 as the feed is steadily adapted making the time interval going to 0.

$$F = \left(\left(\frac{\mu_{set}}{Y_{x/s}}\right) + m_E\right) \times \frac{(X \times V)}{S_0} \times e^{\mu \times t} \quad \textbf{(1)}$$

X: biomass concentration (g/L) estimated by CER and $\mu_{set-0.25}$

m_E: maintenance coefficient; 0.0616 (g/g/h), calculated from chemostat cultivation

$Y_{x/s}$: biomass yield coefficient; 0.633 (g/g)

S_0: substrate concentration in the feed; 225 (g/L)

V: cultivation volume (L)

μ_{set}: growth rate (time dependent) (1/h)

The specific growth rate μ_{set} is time dependent based on two cosine functions leading to oscillating changes in growth and starving phases (Figure 4 (A)). To establish certain profiles the constant values of μ_{max} (amplitude) and k_{1-3} have to be chosen accordingly.

$$\mu_{set} = \mu_{max} \times k_1 \times \cos\left(cos(k_2 \times t)\right) + k_3 \quad \textbf{(2)}$$

t: time (h)

μ_{max}: maximal growth rate (process related); 0.3 (1/h)

k_{1-3}: constants

As the given specific growth rate continuously changes, a consistent adaptation of the current biomass concentration X and volume V is necessary. The biomass concentration is estimated via a soft-sensor derived from the CO_2 evolution rate (CER) and the particular growth rate, which is assumed to be 0.25 h time shifted before the growth rate μ_{set} taken for the feed calculation. This was done due to experiments showing a certain delay between the growth rate which is set by the feed and the actual growth rate present. The current CO_2 and O_2 exhaust gas values (BlueSens©) as direct in-process parameters are indispensable for a robust and reliable control. The linear correlation between CER/X and the corresponding growth rate μ was deduced from batch experiment data with a respiration quotient of 1.

$$X = \frac{CER}{(0.013 \times \mu_{set(t-0.25)} + 0.0024)} \quad \textbf{(3)}$$

X: biomass concentration, cell dry weight (CDW) (g/L)

CER: CO_2 evolution rate (mol/L/h)

The particular cultivation volume was determined by accounting for the fed volume and the sample volume taken.

$$V = \left(\frac{\text{feed}_{(\text{balance})}}{\rho_{\text{feed}}}\right) + V_{start} - \sum\left(V_{sample}\right) \quad \textbf{(4)}$$

V: volume (L)

feed (balance): weight of fed volume (g)

ρ_{feed}: feed density (g/L)
V_{start}: cultivation volume start (L)
V_{sample}: sample volume (L)

4. Results

4.1 Ratio screening: growth phase – starvation/secretion phase

The generation of an appropriate bioprocess control for reaching high cell densities with guaranteed high productivities of secreted ABFs was in the main focus of the underlying investigations. A fed-batch process with different durations of growth phases and starving phases was tested (Figure 3). An optimal ratio of growth (1.5 h) to starving phase (0.5 h) with a value of approximately 3 was determined and used for subsequent bioprocess control.

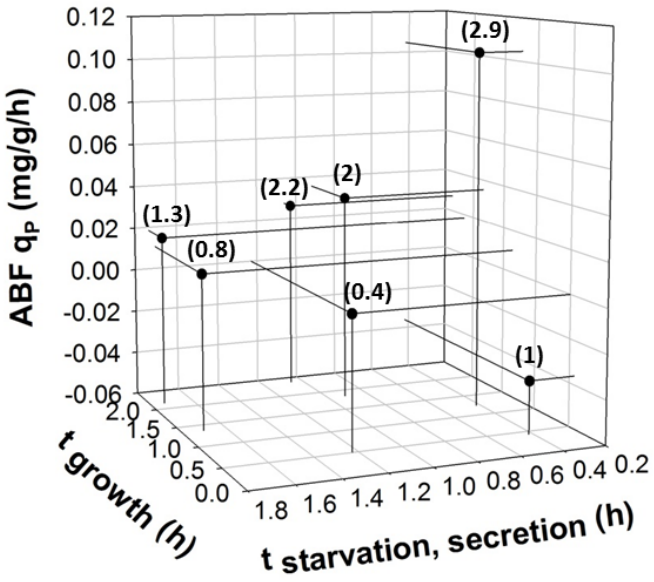


Figure 3: Screening of different time spends of growth phase and starving phase related to specific production rate of ABF D1.3 scFv (q_p) in *B. megaterium* YYBm1 (EJBmD1.3scFv). The number in brackets relates to the particular ratio ($t_{growth,(exp)}/t_{secretion,(stat)}$).

4.2 µ-oscillating fed-batch

For the secretion of ABF D1.3 scFv by *B. megaterium* an adapted feeding strategy based on a time-depending oscillating growth rate strategy was applied. As shown in Figure 4 (A), the particular cosine functions were modified to ensure a well-defined ratio of growth and starving/secretion phases, respectively. A growth phase of 1.5 h with a maximal growth rate of 0.3 (1/h) was followed by a starving phase of 0.5 h.

This strategy could successfully be implemented into the process represented by measured DO concentrations and exhaust gas analysis (O_2, CO_2; BlueSens©) based CER and OUR plots (Figure 4 (B)). As shown in Figure 4 (C) measured cell dry weight (CDW) values were directly comparable to the online CER-based estimated data. Based on this process information, a feed profile was successfully adapted (Figure 4 (D)) ensuring an automated robust control of comparable growth and starving/ABF-secretion phases. Measured fructose (main carbon source) and acetate (overflow metabolite) concentrations lay in a negligible range as the applied carbon limiting feeding strategy avoided any substrate over-feeding.

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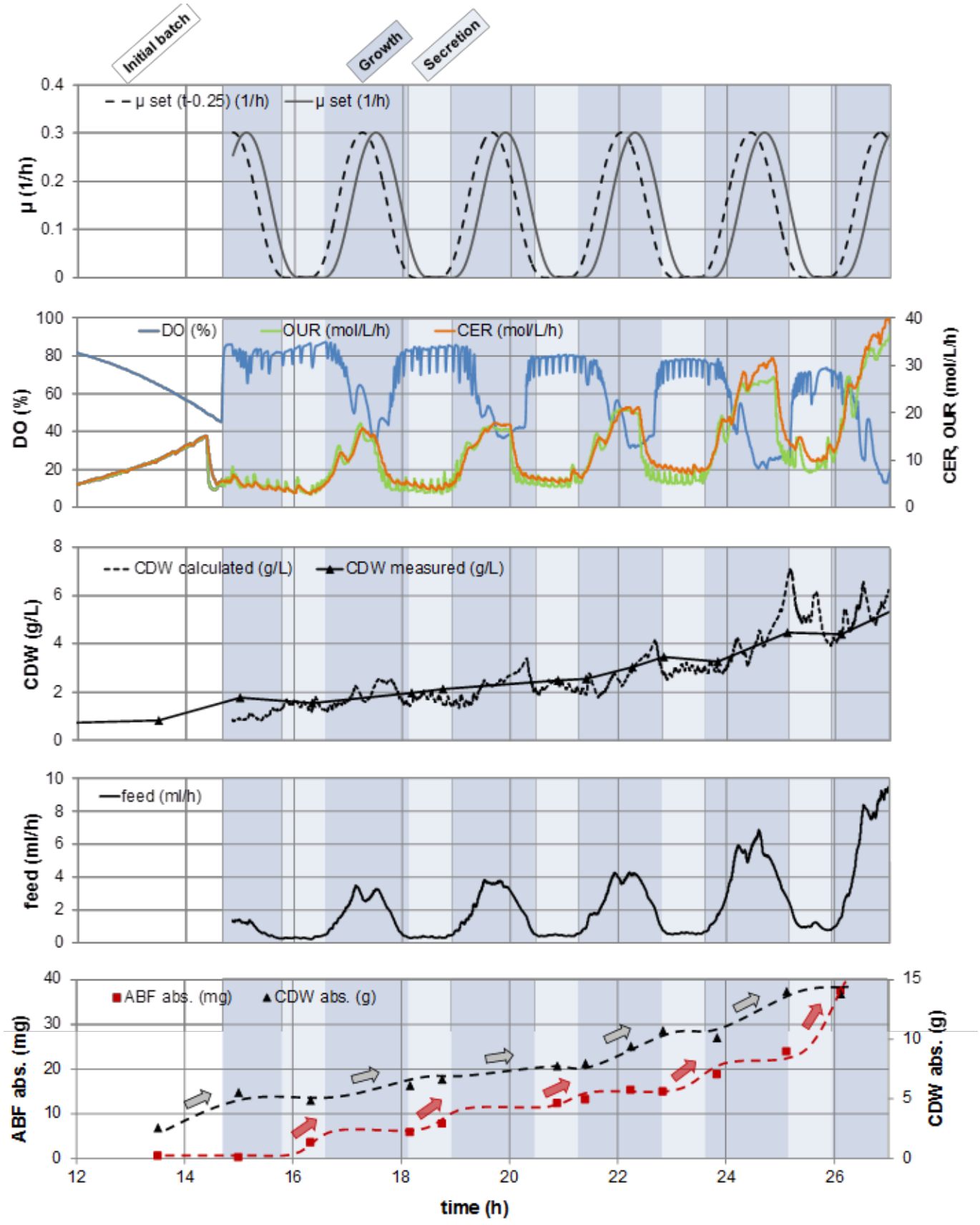

Figure 4: Process control of μ oscillating fed-batch strategy of a *B. megaterium* YYBm1 (EJBmD1.3scFv) cultivation; overview of process control variables during the oscillating fed-batch. **(A)** μ_{set} and $\mu_{set-0.25}$ were given by cosine functions with a defined μ_{max} value of 0.3 (1/h). **(B)** Dissolved oxygen (DO) concentration and calculated oxygen uptake rate (OUR) and carbon dioxide evolution rate (CER) values were measured online and show clear trends in growth and starvation phases. **(C)** Biomass was calculated based on μset-0.25 and CER and was related to the biomass concentration measured. **(D)** The feeding rate was successfully adapted and was the direct control parameter for particular phases of growth and starvation. **(E)** Absolute biomass and ABF amounts during the fed-batch process; arrows indicate the main periods of increased biomass formation (grey) and ABF secretion (red).

Having a closer look at the absolute biomass and ABF amounts during the oscillating fed-batch, certain dependencies become observable as shown in Figure 4 (E). While the biomass increases steadily in particular during the growth phases, the secretion of ABFs is mostly boosted during the controlled starving phases of the process (indicated by arrows, Figure 4 (E)). Comparing the overall yields to a similar batch process with same biomass concentrations 7-times higher ABF titers per g biomass were reached [4] (data not explicitly shown).

4.3 Single cell analysis

Another assessed parameter during the cultivation was the polarization status (MP) of cells using a previously established fluorescence labeling assay based on $DiOC_2$ staining [4]. Figure 5 gives a representative view on the repeating polarization and depolarization phases on single cell level in particular growth phases. The depolarization increased significantly during the starving phases and could be correlated to an improved secretion of ABFs.

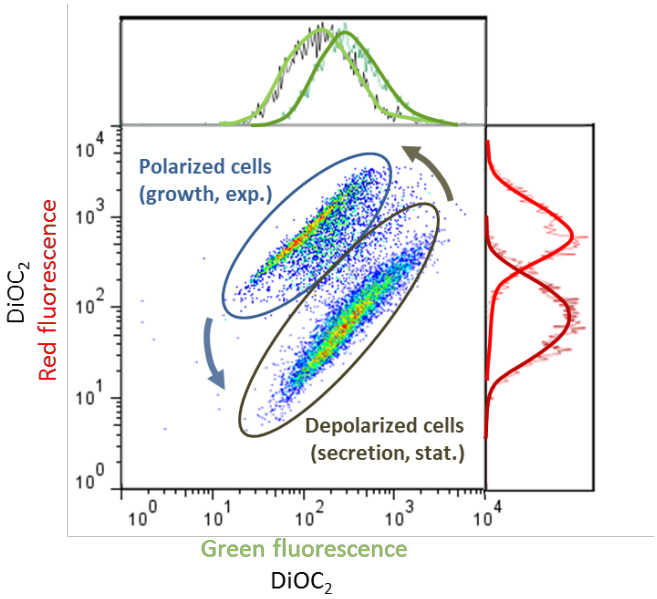

Figure 5: Membrane potential (MP) estimation at μ oscillating fed-batch strategy. Single cell analysis of the MP of *B. megaterium* cells (YYBm1 (EJBmD1.3scFv)) secreting ABF D1.3 scFv at different cultivation time points of exponential and stationary growth phase. Cells were stained with $DiOC_2$ and analyzed by flow cytometry in parallel to the cultivation. The dot plot presents red and green fluorescence values of single cells, where an increased red fluorescence indicates a higher polarization status. The arrows visualize the repeating shift from a polarized to a depolarized cell status.

5. Conclusions

The investigated bioprocess is both a production process and at the same time a secretion process of ABF D1.3 scFv. Secreted products have the big advantage of reducing the purification efforts as the product of interest is already functionally folded in the supernatant with less other contaminating proteins. This matter of fact should not be underestimated as downstream processing comprising up to 80 % of today's production costs [10]. Secretion processes in general are coupled to particular conditions and are affected by a variety of parameters like the current specific growth rate [11]. In this case an adequate process strategy based on the increased secretion of ABF D1.3 scFv coupled to the stationary/starvation phase was newly developed. By an adapted online

feeding profile controlled growth and starving/ABF-secretion phases were successfully established. The control algorithm used guaranteed comparable growth and starvation/secretion phases throughout the process with the feeding profile being founded on online estimated parameters of the particular CER based biomass concentration and the cultivation volume.

After establishing the oscillating fed-batch control at the lab scale, the strategy was successfully transferred to a pilot plant (data not shown). For scaling up the process to a 100 L bioreactor several parameters had to be taken into account in advance and thus were adapted to maximizing the number of growth/starvation cycles while simultaneously guaranteeing a sufficient change in CO_2 exhaust gas to ensure exact biomass estimation at the feed start. The used control parameters like CER for biomass estimation were not scale dependent and guaranteed a reliable process control. Equipped with exhaust gas analysis and an adequate pump system the method can be assigned to any scale and might also be interesting to accurately control other secretion processes. Besides the applied time dependent μ-oscillating cos-profile any other profile like linear changes in the particular growth rates, extended growth or starving phases with adapted μ_{max} values can be implemented into the process. Especially for quasi-stationary μ-dependent investigations in the field of systems biology the strategy of extended linear time dependent changes of the particular growth rate are most feasible for reliable process control.

Summarizing the approach it was possible to successfully implement a robust strategy for an optimized microbial ABF secretion process. Due to economic challenges of the public health care system, higher demands and the competitive global market it is most important to develop less cost intensive production platforms for ABF production like the presented *B. megaterium* as a production host. From the underlying approach it becomes clear that not only genetic strain engineering but also an adequate, rational bioprocess control and adapted process designs are necessary for further optimization. Thereby robust industrial scalable processes become implemented. Especially measurements of critical variables like the online-estimation of biomass concentration or investigations on single cell level lead to a better understanding and deeper insights into the particular bioprocesses as suggested by the Process Analytical Technology (PAT) initiative according to Food and Drug Administration (FDA) standards [12].

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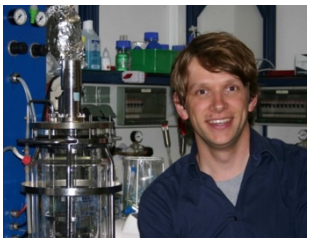

The group's main focus is on the holistic process optimization for the production of antibody fragments with *Bacillus megaterium* as production host. It is part of The Collaborative Research Center SFB 578 "Development of Biotechnological Processes by Integrating Genetic and Engineering Methods – From Gene to Product –". Here methods available both from the basic sciences and the fundamental engineering sciences are combined and integrated to obtain optimized bioprocesses for high value-added products. The Gram positive *B. megaterium* is used as host organisms for antibody fragment production being characterized by a high secretion capacity and plasmid stability. The holistic approach for bioprocess engineering ranges from cultivation media optimization and advanced process monitoring on single cell level to an integrated systems biological analysis incorporating various omics techniques. The aim is to uncover potential bottlenecks in secretion being the basis for a rational strain and process design making *B. megaterium* an industrial relevant production platform for various antibody fragments.