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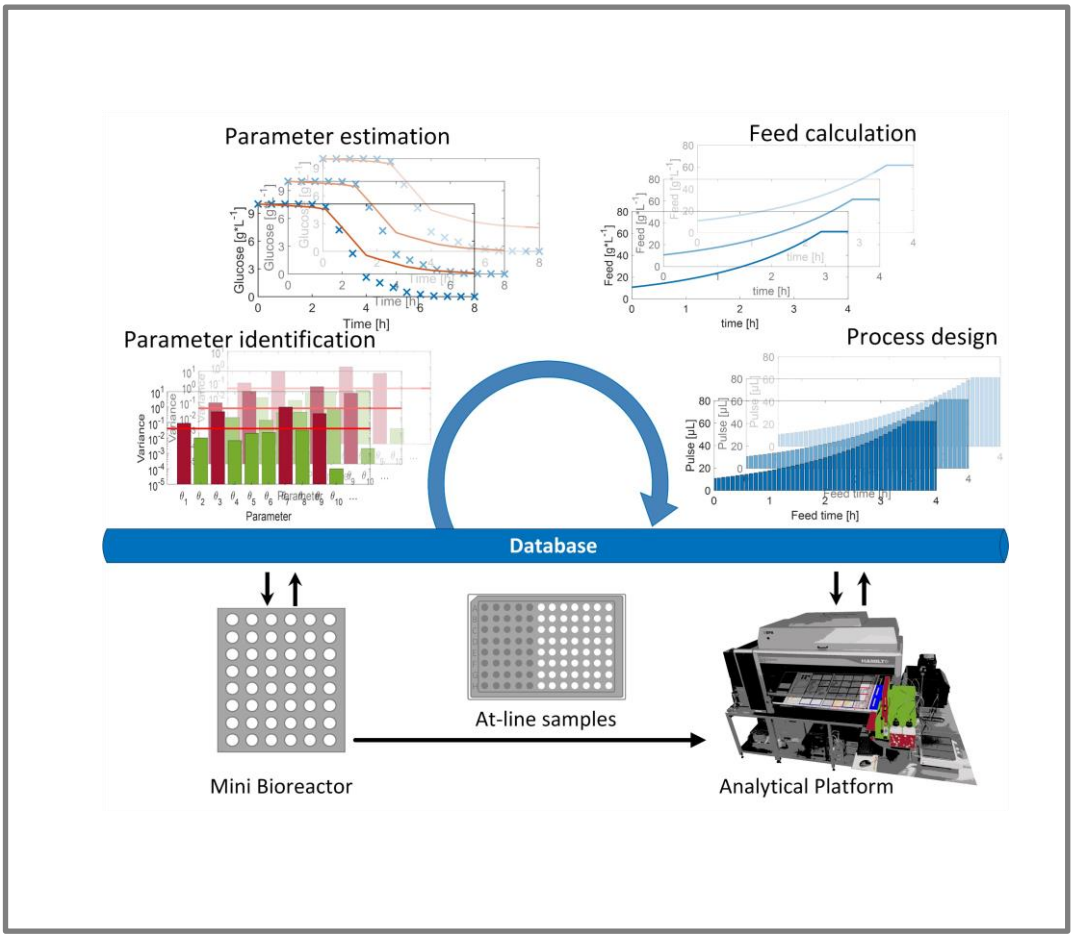

Accelerating biological screenings through the combination of high-throughput and modeling

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Accelerating biological screenings through the combination of high-throughput and modeling

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I Abstract

One of the greatest challenges facing our society today is the shift to a resource and environmentally friendly, green circular economy. Biotechnological products and processes play a central role in this transformation and acceleration and intensifying bioprocess development is an essential contribution to the economy of tomorrow. Automation, digitalization, and virtual prototypes are common tools of product development in many economic sectors. Their application in biotechnology, however, is hampered, mainly by the complexity of biotechnological processes and products. The majority of biotechnological cell and process models have to be adapted to the specific task at hand, which is time-consuming and data-intensive, and models are not universally applicable, as it is possible for example in mechanics.

This work encompasses the development of three major technologies of model-based operation of high throughput experiments to accelerate bioprocess development. First, operation of turbidostat cultivations in 96-well plates. This allows for: i) a better control of the pre-culture and a highly flexible inoculation strategy, ii) screening for fast growing mutants under controlled conditions, and iii) high throughput evolution experiments. Second, development of a model-based screening framework that enables an automated operation of multiple strains in parallel with significant different phenotypes. Third, tailoring of multivariate analysis tools to improve process monitoring during parallel fed-batch experiments and to automatically detect outliers and process failures.

The added value of the high-throughput turbidostat cultivations is demonstrated with a variety of *Escherichia coli* strains using a simple growth model in multi-well plates. Centralized data management, a minimal knowledge-based model and integration of advanced operation tools for automated task execution by Liquid Handling Stations (LHS) were the basis to produce multiple competent strains on a LHS and to transform them. For the model-based screening experiments a mechanistic model, consisting of five differential equations and 18 parameters, was used in a study with eight different *E. coli* strains in 24 parallel cultivations for process planning and on-line process adaptation. Through iterative online model fitting, it was possible to predict the optimal feed start with less than one minute difference. The model was suitable to predict process conditions to prevent overfeeding or starvation of cells and the experiment was sufficient to determine qualitative differences between the strains. The entire experiment was conducted in a fully automated bioprocess facility consisting of two LHS and one Mini-Bioreactor System (MBR). Finally, principal component analysis (PCA) was used to detect process and measurement errors and was able to identify the most important process failures. In this way, high quality of data can be guaranteed, which is imperative for successful model calibration.

II Zusammenfassung

Eine der größten Herausforderungen unsere Gesellschaft ist der Wandel hin zu einer ressourcen- und umweltschonenden Kreislaufwirtschaft. Biotechnologische Produkte und Prozesse spielen bei dieser Transformation eine zentrale Rolle und die Beschleunigung und Intensivierung der Bioprozessentwicklung ist ein wesentlicher Beitrag hierzu. Automatisierung, Digitalisierung und virtuelle Prototypen sind in vielen Wirtschaftsbereichen gängige Tools der Produktentwicklung, jedoch kaum in der Biotechnologie. Dies ist maßgeblich in der Komplexität biotechnologischer Prozesse und Produkte begründet. Eine Vielzahl biotechnologischer Zell- und Prozessmodelle haben gemeinsam, dass ihre Anpassung an die jeweilige Aufgabenstellung zeit- und datenintensiv ist und diese nicht universell einsetzbar sind, wie es beispielweise in der Mechanik möglich ist.

Diese Arbeit umfasst die Entwicklung von drei Haupttechnologien des modellbasierten Betriebs von Hochdurchsatz-Experimenten zur Beschleunigung der Bioprozessentwicklung. Erstens ermöglicht der Betrieb von Turbidostat-Kulturen in 96-Well-Platten: i) eine bessere Kontrolle der Vorkultur und eine hochflexible Inokulationsstrategie, ii) das Screening nach schnell wachsenden Mutanten unter kontrollierten Bedingungen und iii) ein Hochdurchsatz-Evolutionsexperiment. Zweitens wurde ein modellbasiertes Screening-Framework entwickelt, das eine automatisierte Untersuchung von mehreren Stämmen mit signifikant unterschiedlichen Phänotypen parallel ermöglicht. Drittens wurden multivariate Analysewerkzeuge angepasst, um die Prozessüberwachung von parallelen Fed-Batch-Experimente und die Erkennung von Ausreißern und Prozessfehlern zu verbessern.

Der Mehrwert des Turbidostat wird anhand der Transformation mehrerer *E. coli* -Stämme demonstriert. Mit einem einfachen Wachstumsmodell, einem zentralen Datenmanagement, und Automatisierung der nötigen Prozessschritte durch LHS wurden die Stämme parallel kompetent gemacht und transformieren. Für das modellbasierte Screening wurde ein mechanistisches Modell, das aus fünf Differentialgleichungen und 18 Parametern besteht, in der Case-Study mit acht verschiedenen *E. coli* Stämmen in 24 parallelen Kultivierungen zur Prozessplanung und Online-Prozessanpassung verwendet. Durch iterative online Modellanpassung war es möglich, den optimalen Feed-Start auf die Minute genau vorherzusagen. Das Modell war zudem geeignet, die Prozessbedingungen so einzustellen, dass Überfütterung oder Hunger der Zellen ausblieben. Das so designte Experiment war geeignet, um qualitative Unterschiede zwischen den Stämmen zu bestimmen und wurde in einer vollautomatischen Bioprozessanlage, bestehend aus zwei LHS und einem Mini-Bioreaktorsystem (MBR), durchgeführt. Schließlich wurde eine Hauptkomponentenanalyse (PCA) zur Erkennung von Prozess- und Messfehlern eingesetzt, um die wichtigsten Prozessfehler zu identifizieren. Auf diese Weise kann eine hohe Datenqualität gewährleistet werden, wie sie für eine erfolgreiche Modellkalibrierung notwendig ist.

III List of Contents

IV Acknowledgements

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V List of Abbreviations

μ	Growth rate
μ_{max}	Maximum Growth Rate
API	Application Programming Interface
DBMS	Data Base Management System
DO	Dissolved Oxygen
DoE	Design of Experiments
DOT	Dissolved Oxygen Tension
FBA	Flux Balance Analysis
GSM	Genome Scale Model
LHS	Liquid Handling Station
LTU	Linear Transfer Unit
MWP	Multi Well Plate
MVDA	Multivariate Data Analysis
NIR	Near Infrared
NN-ODE	Neural Network Ordinary Differential equation
OD_{600}	Optical Density at 600 nm
ODE	Ordinary Differential Equation
OPC	Open Platform Communication
PCA	Principal Component Analysis
RHS	Right-hand side
SiLA	Standard in Laboratory Automation
SQL	Structured Query Language
SSGSA	Steady-State Growth Space Analysis
STR	Stirred Tank Reactor
UV	Ultraviolet
VIS	Visual spectrum

VI List of Publications

First author publications:

i. **Hans S**, Gimpel M, Glauche F, Neubauer P, Cruz-Bournazou MN, Automated Cell Treatment for Competence and Transformation of *Escherichia coli* in a High-Throughput Quasi-Turbidostat Using Microtiter Plates. *Microorganisms*. 2018; 6(3). doi:10.3390/microorganisms6030060, accepted manuscript

ii. Haby B*, **Hans S***, Anane E, Sawatzki A, Krausch N, Neubauer P, Cruz Bournazou MN, Integrated Robotic Mini Bioreactor Platform for Automated, Parallel Microbial Cultivation With Online Data Handling and Process Control, *SLAS Technol. Transl. Life Sci. Innov*. 2019; 24(9). doi:10.1177/2472630319860775, accepted manuscript

iii. **Hans S**, Haby B, Krausch N, Barz T, Neubauer P, Cruz-Bournazou MN, 2020. Automated Conditional Screening of Multiple *Escherichia coli* Strains in Parallel Adaptive Fed-Batch Cultivations. *Bioengineering* 2020; 7(4) doi:10.3390/bioengineering7040145, accepted manuscript

iv. **Hans S***, Ulmer C*, Narayanan H, Brautaset T, Krausch N, Neubauer P, Schäffl I, Sokolov M, Cruz Bournazou M.N, Monitoring Parallel Robotic Cultivations with Online Multivariate Analysis, *Processes*. 2020; 8(5). doi:10.3390/pr8050582, accepted manuscript

*equal contribution

Further publications

v. Sawatzki A, **Hans S**, Narayanan H, Haby B, Krausch N, Sokolov M, et al. Accelerated Bioprocess Development of Endopolygalacturonase-Production with *Saccharomyces cerevisiae* Using Multivariate Prediction in a 48 Mini-Bioreactor Automated Platform. *Bioengineering*. 2018; 5(4). doi:10.3390/bioengineering5040101

vi. Gawin A, Peebo K, **Hans S**, Ertesvåg H, Irla M, Neubauer P, et al. Construction and characterization of broad-host-range reporter plasmid suitable for on-line analysis of bacterial host responses related to recombinant protein production. *Microb Cell Fact*. 2019; 18(1). doi:10.1186/s12934-019-1128-7

vii. E. Anane, Á.C. García, B. Haby, **S. Hans**, N. Krausch, M. Krewinkel, P. Hauptmann, P. Neubauer, M.N. Cruz Bournazou, A model-based framework for parallel scale-down fed-batch cultivations in mini-bioreactors for accelerated phenotyping, *Biotechnol. Bioeng*. 2019; 116(11). doi:10.1002/bit.27116.

viii. Krausch, N., **Hans, S.**, Fiedler, F., Lucia, S., Neubauer, P., Cruz Bournazou, M.N., 2020. From Screening to Production: a Holistic Approach of High-throughput Model-based Screening for Recombinant Protein Production, Computer Aided *Chemical Engineering*. 2020; 48. doi:10.1016/B978-0-12-823377-1.50288-3

1 Introduction

The development of biotechnological products is a compartmentalized and long-term process. Over the various stages of the product's lifecycle, such as discovery and development, bioprocess development, down streaming, and later production, - numerous professionals from bioinformaticians, molecular- and microbiologists, (bio-) process engineers to chemists and technicians are involved and the focus of development and optimization differs between the involved entities. While for a successful product development all stages of the product life cycle need to be efficient, bioprocess development plays an especially vital role. Here it is often decided whether a promising product is suitable for profitable production and marketing. Products with bad up-stream characteristics can often not be produced at all or only at very high prices. Through unaudited determination of crucial bioprocess parameters development becomes even more difficult. Bioprocess parameters like host and vector backbone are commonly already defined in the early product identification and development phase and partly not mandatory. Such decisions are often made based on expert knowledge (Neubauer et al., 2017) or lab internal blueprints.

Reasons for unaudited determination of process parameters can be personal experience or preferences as well as a lack of testing capacities and skills. The screening capabilities of many big players in biotechnology have advanced during the last decade through increasing automation, the spread of liquid handling stations and other laboratory robots, as well as the growing connectivity of screening and analytic devices. However, the expected advances in efficiency, robustness and speed during bioprocess development were not completely fulfilled. The laborious planning and evaluation of automated screening experiments could be one reason for this, as well as the requirement of preliminary information about the observation object (mostly the host), which is often not present. This leads to long idle times of expensive machines, experiments which are carried out with less informative value or screening campaigns which are considered as too costly. To overcome these high (entry) barriers, simple tools for planning experiments are needed and the dependency on preliminary information needs to be reduced as much as possible.

For the planning of an experiment a number of tools are available. Methods like Design of Experiments (DoE) are suitable to increase the information content of experimental data and to facilitate its evaluation. However, prior knowledge of the object of study is necessary to limit the design space. Knowledge based models can help to reduce the knowledge gap prior to the experiment. Unfortunately, such models are usually only useful if they have been previously calibrated to the specific system, a process that involves pre-testing and loss of time, even if the model calibration can be reduced to only one experiment with sufficient automation (Cruz Bournazou et al., 2017).

Therefore, the goal needs to be to design software that is able

i.) to plan experiments

ii.) to perform experiments autonomously

iii.) to monitor and, if necessary, adjust the experiments and

iv.) to evaluate the outcome.

The points i.)-iii.) are the core of this thesis using *Escherichia coli* and two selected phases of bioprocess development, namely the library screening and the conditional screening, as case study. The integration of expert knowledge into the executing facility in form of mathematical models was a decisive step for the success of this work.

2 Scientific Background

In the last decades, research has discovered an enormous amount of molecules, organisms and synthetic pathways and developed basic methods for their use. This leads to an almost infinite number of possible combinations on the road to new bioproducts. Even if the combinations are reduced to the supposedly reasonable ones, the number of necessary studies remaining cannot be mastered in traditional, manual, or partially automated manner. Reducing the parameters to be optimized to a manageable number leads to sub-optimal or unusable results. Automation can significantly expand the scope of testing. However, in bioprocess engineering, even with intensive automation not all desired combinations can be tested. High-Throughput Bioprocess Development (HTBD) addresses this issue by developing tools to intensify automated studies and intelligent study design selection.

This offers completely new sustainable solutions in the fields of pharma/medicine, chemistry, nutrition, environment, and energy. Among other things, biotechnological products can lead to the replacement of chemical syntheses of basic substances up to complex polymers (BMBF, 2010; EPSO, 2011; Zweck et al., 2015; Meyer, 2017).

2.1 Bioprocess Development

Product and process development in the field of biotechnology currently require an enormous expenditure of time, personnel and initial investment compared to other industries and are dominated by natural sciences at the beginning of the process, with an increasing engineering focus in the later phases of the product life-cycle. The missing interaction between the different fields, the changing focus and priority during development and varying applied methods are reasons for the missing efficiency and futile cycles (i.e., trial and error) in the development of biotechnological processes.

2.1.1 Cultivation methods

The kind of bioprocessing almost always has a major influence on product quantity and quality. Optimizing this is therefore a central element in any successful bioproduct development.

2.1.1.1 Batch cultivation

The cultivation of cells in a batch process is the simplest way to cultivate cells from picolitre (Agresti et al., 2010; Kaganovitch et al., 2018) to m^3 scale. All required cultivation components are filled into a container and incubated for a certain time. In biological batch cultivation gas and energy input is usually intended and desired. In general, a distinction needs to be made between batch cultivations in small scales (no process control, less observation) and in larger scales with common bioreactors (high process control, established online observation). Smaller cultivations are tempered and shaken from the outside. The shaking serves to set the

liquid in motion and increase the surface on which a gas exchange with the ambient air can take place. In larger scales, mechanical and thermal energy as well as gases are often supplied directly into the reactors.

Due to their simplicity, small-scale batch processes can be set up quickly, parallelized, automated, and scaled over a wide range. Therefore, they are very common in the early development and screening phase, with the focus lying on screening many variants. Batch processes are usually less productive compared to other cultivation methods due to limited reaction rate control (Enfors, 2019). Since all substrates are present in the medium at the beginning, it is not possible to add high amounts of substrates without inhibiting effects. In addition, the substrates are consumed by the cells at the maximum possible uptake rate and the cells enter the overflow metabolism. As a consequence, organic acids are produced, which inhibit growth and thus the biomass synthesis (Luli and Strohl, 1990; Nakano et al., 1997) and recombinant protein production (Bauer et al., 1990; Jensen and Carlsen, 1990; Shiloach et al., 1996; Ruano et al., 2016). By selecting the carbon source in the medium, the growth rate can be reduced, with positive effects on the maximum possible biomass concentration (Lee, 1996). Furthermore, sugar release systems can be incorporated into batch cultivation. By diffusion or enzymatic digestion glucose molecules are continuously released in the medium (Jeude et al., 2006; Panula-Perälä et al., 2008). This allows the cultivation of cells with a constant feed and leads to positive effects on biomass and recombinant protein production (Krause et al., 2010; Šiurkus et al., 2010; Toeroek et al., 2015) while keeping the simplicity of the batch process.

2.1.1.2 Fed-batch cultivation

A fed-batch cultivation differs from a batch cultivation by the continuous feeding of nutrients. This is accompanied by a significant increase in volume over the cultivation period. Significantly more equipment is required for a fed-batch cultivation, and the process cannot be reduced in scale and parallelized as easily as a batch process. However, the continuous feeding of a culture growing on limitation has enormous advantages for metabolic reaction control, biomass synthesis and the production of recombinant proteins. Due to the significantly better production properties, a majority of industrial bioprocesses are operated as fed-batch cultivation (Mears et al., 2017).

2.1.1.3 Continuous cultivation

In a continuous cultivation feed is added while culture liquid is removed simultaneously. As the culture medium is regularly diluted and replaced, the culture is kept in a constant state over a long period of time. A wide range of conditions can be kept stable. Depending on process control strategy, different types of continuous cultivation processes are distinguished.

The chemostat is the most used continuous cultivation method. The process control is based on the dilution rate and is adjusted to a desired growth rate (μ). Thereby, the composition

of the culture liquid (e.g., substrate, biomass concentration) is stable over a long period and can be used for intensive observation or sampling. Depending on which parameter the dilution rate is controlled by, the process is called a DO-stat (controlled by oxygen saturation), pH-auxostat (Martin and Hempfling, 1976), CO_2-auxostat (Watson, 1969), permittistat (electrical capacity of the culture) (Markx et al., 1991) or turbidostat (in the outlet) (Bryson and Szybalski, 1952). A special challenge in chemostat cultivation is the operation near the maximum growth rate (μ_{max}), with the risk that the dilution exceeds the possible maximum growth rate and the cells are washed out. The turbidostat takes exactly this problem into account and enables chemostat experiments close to or at μ_{max}.

However, after a change to a different growth rate the culture requires several generations to achieve extra- and intracellular stability. The screening of a broad steady-state growth space analysis (SSGSA) is therefore a very time-consuming process. Changestat cultivations keep the change in growth rate constant (ascending μ: accelrostat, A-stat; descending μ: decelerationstat, De-stat;) (Paalme et al., 1995). With Changestat cultivations, SSGSA can be performed in a reasonable time (Subramanian et al., 2017) and are a suitable tool for process optimization (Glauche et al., 2017). Further Changestat variants are the D-Stat (one cultivation parameter is changed, dilution rate remains constant) and the Auxoaccelerostat.

Apart from research for industrial applications of continuous cultivation, sustained operation with consistent product quality, reduced equipment size, high-volumetric productivity, streamlined process flow, low-process cycle times, and reduced capital and operating cost (Konstantinov and Cooney, 2015) can be facilitated with the chemostat. Furthermore, process dependent issues like product toxicity for the host, product inhibition or a rapid catabolism of the product (Villadsen et al., 2011) can be addressed and solved as well.

2.1.2 Liquid Handling Stations and data handling

Liquid Handling Stations (LHS) are complex facilities for transporting and manipulating liquids. Their development dates back to the 1990s (Freeman and Yeudall, 1986; Pfost et al., 1989). They have one or more pipetting units and are often also capable of moving labware such as Multi Well Plates (MWP). Some pipetting units can be installed stationary within the LHS (e.g.: CyBiWell Analytik Jana). In this case, the labware is transported to the pipetting unit. Alternatively, the pipetting units can be movable (e.g.: Beckman Coulter, Eppendorf, Hamilton, Tecan, m2pLabs, OpenTron). In these instruments, the ability to transport labware is no longer necessary, but often present. The pipetting units can comprise of 1 to 1536 channels. Generally, units with two to eight channels can process individual volumes in parallel, while pipetting units with more than eight channels can process only one volume at a time. Devices that can process 96 individual values are available but not widely used (e.g.: DynamicDevices). While interacting with liquids is the core functionality of LHS, further

functions can be implemented. These are mostly enabled by devices placed next to or directly in the LHS and can include almost the entire spectrum of laboratory devices.

LHS are very good at processing previously defined tasks. However, they reach their limits if details of the task are not fixed at the time of the process start (call of a method) or change during its run. There are several workarounds to solve the problem of missing input at the start of the method. Almost all LHS control software have the possibility to read from external files. A static reference to a file in a LHS method can be used as a pointer to the respective information. Regardless of the LHS software, the file can be constantly manipulated to provide missing or updated information. However, this requires a good management of the read and write rights and network access to the file system, in particular if the information is generated by applications that are not executed on the actual computer. In addition, traceability is more difficult because the old information is overwritten.

The storage of process control information and all kinds of measurements with a traceable history is mandatory for the evaluation of bioprocesses. In networked systems consisting of LHS, laboratory devices, microservices and software solutions (e.g.: Electronic Lab Journals (ELN), Manufacturing Execution Systems (MES) or Laboratory Information and Management System (LIMS)), centralized and uniform collection of all relevant data is a challenge. Even if a number of these entities run on the same system, a data and data exchange concept is necessary. Databases provide a good tool for this purpose. Depending on the task there are different types of databases available. The storage and retrieval of data is handled by an interface, the Data Base Management System (DBMS). The user or the application directs the queries to the DBMS. For relational databases the standardized Structured Query Language (SQL) is mostly used. In relational databases, complex data and relationships can be easily mapped using data models. For retrieving or writing data from a LHS method, separate interfaces (drivers) are necessary to exchange data with the DBMS. Rarely are database queries directly possible in the LHS software.

However, designing SQL queries for reading and storing data can be difficult and requires detailed knowledge of the underlying data model and still requires writing specific drivers for software and devices. Communication Standards like SiLA or OPC has already entered the laboratory environment and are design for network communication. Together with data standards such as AnIML or Allotrope, progress is being made towards easier networking of future laboratories.

2.1.3 High-throughput mL cultivation systems

Microbial cultures are carried out in all scales which are able to host a cell. The cultivation systems differ greatly in regard to process control, monitoring, technical equipment, and handling. Generally speaking, the degree of process control and monitoring increases with the size of the system and the possibilities of automatization and parallelisation decrease.

2.1.3.1 Multi Well Plate based bioreactors

Multi Well Plates (MWP) are widely used in the early screening phase (Junker and Wang, 2006; Funke et al., 2009*)* and have become standard practice in the industry (Duetz, 2007).As automated handling of MWP is the original purpose of LHS it is not surprising that LHS are also suitable for the cultivation of microbes in MWP (Cancer et al., 2004; Zimmermann and Rieth, 2006; Knepper et al., 2014; Glauche et al., 2015). Nevertheless, cultivations performed in this environment are hardly comparable to industrial processes, since for example active pH control, fed-batch or continuous process conditions are missing (Buchenauer et al., 2009; Scheidle et al., 2010). Due to the small volume of MWP, sampling is limited, too. Only optical measurements (e.g. optical density (OD) or fluorescence) can be determined non-invasively. With adapted MWP for non-invasive pH or DO measurement, these parameters can be determined without sampling, but simultaneously contact-free OD determination is lost (John et al., 2003; Arain et al., 2006).

Gas exchange in open and shaken systems like MWPs is limited (Duetz and Witholt, 2004). To avoid evaporation, the plates are sealed with foils and the oxygen transfer decreases further, which can present a problem for aerobic growth. However, there are notable differences in regard to gas permeability of different MWP foils and caps (Duetz et al., 2000; Zimmermann et al., 2003). In addition, alternative well geometries have been developed to increase the gas transfer rate (Funke et al., 2009).

A complete cultivation system based on MWP with integrated gas supply, temperature and control as well as integrated monitoring DO, pH and OD was developed as bioLector® (Kensy et al., 2009) and also integrated into LHS (Huber et al., 2009; 2011; Unthan et al., 2015; Blesken et al., 2016). By using MWP with microfluidic channels, continuous feed and pH control have been applied (Buchenauer et al., 2009; Funke et al., 2010) and this system is commercially available as the bioLector® Pro (Blesken et al., 2016).

The described systems have a similar functionality as benchtop stirred tank reactors, with a significantly higher throughput and the number of possible and performed experiments constantly increasing in the last decade. A disadvantage of the MWP based bioreactor is the comparatively small cultivation volume, which limits sampling. This could also be a reason why the expected acceleration in bioprocess development has not happened despite the development of complex MWP systems.

2.1.3.2 Milliliter stirred tank reactors

The Stirred Tank Reactor (STR) is the industrial standard in later bioprocess development stages. The bioprocess within a STR can be automated very well, which does only partly apply to the tasks outside of the bioprocess (e.g.: inoculation, sampling, sterilization). The process control and monitoring in STRs is high, as well as the effort required for parallelization and miniaturisation.

To increase the degree of automation outside the bioprocess, bioreactor integration into a LHS is beneficial. The HEL BioXplorer100 system is a minimization of an STR and has already been successfully integrated into LHS (HEL, 2019). With up to 150 mL working volume and 8 parallel reactors it is one of the larger systems that can be integrated into LHS. The bioREACTOR 48 from 2mag with its 48 parallel 10 mL reactors is also an attempt to mimic a benchtop STR that can be integrated into LHS (Puskeiler et al., 2005; Weuster-Botz, 2005; Knorr et al., 2007; Haby et al., 2019). However, its compact dimensions change the possibilities of process control and monitoring since the conventional actuators and sensors cannot be used. Although probes for online DO and pH are available, no other online measurements can be made, and the addition of feed or other supplements is only possible on a pulse basis. Linear feed approaches are very complex and have widely not been accepted (Gebhardt et al., 2011; Schmideder et al., 2015). The degrees of freedom in process control are further limited. Temperature, stirrer speed and gassing have to be the same for all included bioreactors. This disadvantage has been reduced with the bioREACTOR 8.

A compromise between the two systems mentioned above is the ambr15® system. Temperature, gas supply and stirrer speed are fixed for all 24 cultures, but pH control and feed can be added continuously to the vessels available at working volumes from 10 ml to 15 ml. Other miniaturized bioreactor systems that cannot be integrated into an LHS, whose operating effort is high and the volume is less comparable to benchtop STRs, have disappeared from the market. Examples are the DASbox System (Eppendorf) or the 24microreactor from PAL, although both were used in a wide range of bioprocess developments (Warr et al., 2013; Warr, 2014; Vital-Jacome et al., 2016; Koenig et al., 2018; Sousa et al., 2018; Kopp et al., 2019). The ambr250® system is comparable to the DASbox in terms of equipment and technology, but it is delivered directly with its own automation system and is very successful on the market for industrial bioprocess development (Ritz et al., 2017; Manahan et al., 2019).

2.2 Modelling in Bioprocess Development

From the philosophers of antiquity to the discovery of DNA by Watson and Crick to the most complex biochemical pathways, models have always been an important tool to make reality more tangible. Models never represent the whole reality, but represent it in a simplified way and are characterized by illustration, reduction and pragmatism (Stachowiak, 1973). Helpful models are characterized by a balanced relationship between simplification and complexity without ignoring important points of the respective problem. This explains why there is no single model for any organism, but rather a multitude of models that are meticulously tailored for different purposes.

Mathematical models are no exception in this context. They can help to represent knowledge and observations in equations. A distinction must be made between knowledge-based approaches, such as mechanistic or flux balance models, and data driven approaches, e.g. statistical regressions, principal component analysis or neural networks. The advantage of knowledge-based models is that they try to represent the existing state of knowledge mathematically. Pathways, mass balances, kinetic reaction rates or regulatory mechanisms can be mathematically formulated and calculated. Therefore knowledge-based models can be calibrated with relatively few data and a meaning can be assigned to the model parameters. This makes knowledge-based models very well suited to support screening processes and to facilitate the selection of clones.

Furthermore, depending on the application and the state of knowledge, different models can be created and compared. Model discrimination compares different models and evaluates them to a specific question. During model discrimination, different hypotheses on the same subject are investigated. The different outputs of the models are compared and, depending on how well the fit matches with the measured data, a hypothesis can be disproved or supported. Model discrimination can also be used to decide which models in a set to apply to a given dataset to avoid overfitting and ill conditions in the parameter set.

2.2.1 Mechanistic Models

Mechanistic models in chemistry and biochemistry consist of mass balance equations and specific (kinetic) rates to map metabolic interactions *(Almquist et al., 2014).* The concentration changes of the observed metabolites are usually formulated as ordinary differential equations (ODE) in the form of $\frac{dt}{dt} = r$, where r is the right-hand side (RHS) of the equation, representing the kinetic reaction rate. The knowledge-based approach is very well suited to predict the behavior of cells in the bioprocesses. A complete picture of the whole biochemical network is usually not necessary to obtain a good representation of the bioprocess. For this reason, reduced or less complex mechanistic models, which can be calibrated with a manageable amount of data and are computed quickly, are most often used in bioprocess development. As

early as 1973, Yoshida et al. published a first fed-batch model showing the growth of *Candia tropicalis* as a function of the feeding rate (Yoshida et al., 1973). Since then, many different bioprocess models have been published, which describe the cell behaviour with different focuses.

The organism this work focuses on is *Escherichia coli. E. coli* is probably the most utilized procaryotic organism in biotechnology and therefore well understood and examined. *E. coli* is used as a host for gene expression (Wang and Kushner, 1991; Hannig and Makrides, 1998; Rydenfelt et al., 2015), production of recombinant proteins (Hsu et al., 2014; McKinstry et al., 2014; Rosano and Ceccarelli, 2014) and other biochemical products like biofuels (Dugar and Stephanopoulos, 2011; Hollinshead et al., 2014). *E. coli* features many characteristics that make it especially applicable in biotechnology. It is one of the fastest growing bacteria with a generation time of 20 min (Sezonov et al., 2007) be cultivated in high cell density up to 190 $g * L^{-1}$ (Shiloach and Fass, 2005) and various tools for easy and fast transformation (Pope and Kent, 1996).

The overflow metabolism of *E. coli* is associated with inadequate bioprocesses and is undesirable in industrial applications (Axe and Bailey, 1995). Therefore, a trade-off between fast growth and high production while mantaining substrate limited conditions is required. Different model approaches focus on reflecting the production of acetate. In 1999, Xu el. al. published a model in which the production of acetate always starts if the glucose uptake rate exceeds a fixed assumed maximum respiratory capacity (Xu et al., 1999). The concept was further developed in 2001 by Lin et al (Lin et al., 2001b). In this model, the authors assume a dynamically changing capacity limit for glucose and oxygen uptake. By considering the metabolic changes of *E. coli* during fed-batch cultivation, the dynamics of acetate measurements were followed better. The concept of Basan et al. also describes the overflow metabolism of *E. coli* depending on the availability of a single carbon source (Basan et al., 2015). The acetate production is shown as a function of the cost for proteome formation and the energy requirement is calculated as a function of the growth rate. The shift of the overflow metabolism towards lower growth rates due to increasing metabolic burden by recombinant protein production can be well represented in this model. However, only batch data were used for the model development. A shift of the acetate production during a long cultivation period as assumed by Lin et al. 2001b was not shown.

All these models have in common that constraints were built in to simulate the different phenotypic effects. The models of Cruz et al. and Anane et al. substitute these discontinuities by a cyclic acetate production (Anane et al., 2017; Cruz Bournazou et al., 2017) adapted from the Xu and Lin models. This model is easier to apply in the planning of new bioprocesses, since unphysiological jumps in the simulation results are excluded. This model assumes that

acetate is constantly formed and absorbed in an intracellular cycle. Phenotypic acetate formation occurs only when acetate production exceeds the absorption capacity.

For industrial applications, models that incorporate protein production are of high interest (Lin et al., 2001a). Neubauer et. al published a model with recombinant protein production as early as 2003, and included the effects of plasmid free populations to the product formation rate (Neubauer et al., 2003). Whereas here the protein production rate is solved via a yield coefficient, Calleja et al. modelled the kinetic dependencies of recombinant protein production on the inducer IPTG (Calleja et al., 2016).

The models described so far consist of relatively few parameters and state variables and are less complex. For the simulation of bioprocesses, they represent the most important information and interrelationships are covered. The calibration can be carried out with moderate computation power and data. However, the macro-kinetic formulations are insufficient if we aim a system-wide approach, which includes metabolic engineering among others. A first approach in this direction was made by Chassagnole et al. already in 2002 by modelling the glycolysis and the pentose-phosphate pathway (Chassagnole et al., 2002). With the increasing knowledge about metabolic pathways, interrelationships and the emergence of the omics technologies, the size of the system wide modelling approaches continually increases (Costa et al., 2010; Tohsato et al., 2013; Khodayari et al., 2014). With 337 state variables, the model of Khodayari and Maranas is currently the most complex kinetic model for *E. coli* (Khodayari and Maranas, 2016).

2.2.2 Flux Balance Models

In the flux balance analysis (FBA), stoichiometric relationships are considered in the form $S * \vec{v} = 0$. The matrix S contains the stoichiometric relations and the vector $\vec{v}$ the conversion rates. Solving these systems for an unknown $\vec{v}$ usually represents the solution of the metabolic network in steady state conditions. Metabolic networks used for FBA usually are complex networks with more than one solution for $S * \vec{v} = 0$. Therefore, the vector $\vec{v}$ is mostly constrained and such models are called constraint based metabolic models. Depending on the research questions, genetic knockouts can be simulated, mass flows can be optimised or unphysiological solutions can be avoided by limiting the reaction rates of $\vec{v}$.

Partly, the metabolic network can be generated automatically based on the genome sequence data with sufficient accuracy (Henry et al., 2010). Therefore, established models can include much larger metabolic networks compared to mechanistic models. The largest metabolic networks can comprise over 1000 reactions and summarise the entire known knowledge of metabolic networks. The development of new models goes hand in hand with the creation of new knowledge. The development of the omics technologies in the last two decades has extremely accelerated this process. Between one of the first large *E. coli* genome scale models (GSM), the *i*JE660 GSM, and the current largest model, the *i*ML1515, an

extension or new GSM was published on average every three and a half years and the number of genes considered doubled in this time. (Edwards and Palsson, 2000; Reed et al., 2003; Feist and Palsson, 2008; Orth et al., 2011; Monk et al., 2017). The use of GSM is broad and includes applications in systems biology such as metabolic engineering, prediction of gene function, discovery of new microbial community-wide metabolic activities, but also the identification of drug targets in microbial pathogens (Kim et al., 2017).

Flux balance analyses and pure metabolic network models have some disadvantages compared to kinetic modelling. They represent the steady state and cannot calculate metabolite concentrations (Orth et al., 2010; Øyås and Stelling, 2018). This limits the applications of FBA for questions in bioprocess development, especially in conditional screening and for the use of model predictive control. Dynamic FBA tries to overcome the issue of pure steady state calculations (Mahadevan et al., 2002).

The fitting of kinetic models can be improved if FBA results are used as additional constraints or to estimate initial values (Feng et al., 2012; Khodayari et al., 2014). Furthermore, metabolic network models are used to train machine learning (ML) models. Here, GSM serves as an additional input (fluxomic) together with other omics data. The application of GSM in the field of ML has been summarised in detail in the past (Camacho et al., 2018; Zampieri et al., 2019).

2.2.3 Data driven modelling approaches

Data driven models do not require any previous knowledge of the system under investigation. However, previous knowledge is advantageous here as well. In supervised learning, the input data are evaluated, labelled or classified according to existing knowledge. The aim is to correlate input and output data as precisely as possible and to be able to draw conclusions about new, unknown data. If the expected results are not made available to the model in any way for training purposes, this is referred to as unsupervised learning (Tarca et al., 2007).

Principal components analysis (PCA) belongs to the unsupervised data-driven modelling methods and is a special form of factor analysis. The correlations of the variables of the input data set are minimised by multiple orthogonal transformations and represented with a few new factors, the principal components. It is therefore a suitable method for presenting and clustering complex multidimensional data, and to reduce dimension and complexity of datasets. PCA is used in almost all areas of biotechnology and bioprocess engineering: in the examination and evaluation of omics and multi-omics data (Argelaguet et al., 2018), as soft sensors for bioprocesses (Randek and Mandenius, 2018) as well as for bioprocess evaluation (Kroll et al., 2017; Sawatzki et al., 2018; Narayanan et al., 2020) and monitoring (Rajamanickam et al., 2017), for downstream optimisation (Edwards-Parton et al., 2008), and

for the evaluation of sensor data especially with spectra methods (Claßen et al., 2017; Doppler et al., 2020).

Supervised learning methods also have a wide range of applications in biotechnology and especially in bioprocess engineering. Support vector machines (SVMs) and artificial neuronal networks (ANN) are frequently used e.g. for the prediction of product formation (Cui et al., 2017), as soft sensors (Vaitkus et al., 2020; Zhu et al., 2020) and for the interpretation of complex spectral measurements like UV/VIS , RAMAN or NIR (Reddy and Bhargava, 2010; Takahashi et al., 2015; Zareef et al., 2020)

2.2.4 Hybrid models

Not all aspects of a metabolic network or bioprocess can always be represented with one single type of model. Knowledge-based models often reach the limits of complexity or knowledge. Data driven modelling approaches often lag in sufficient data for high quality models. The use of hybrid models enables the best possible model to be generated from different methods and available data (Feyo De Azevedo et al., 1997).

One example is the before mentioned use of GSM as fluxomic data in the establishment of multiomics models. Hybrid models were already used in the past millennium to monitor and optimise bioprocesses (Schubert et al., 1994; Feyo De Azevedo et al., 1997). Kinetic models combined with ANN have been used several times for the modelling of bioprocesses (Fellner et al., 2003; Zhang et al., 2019). Stosch et al. used a lean metabolic network, in which the material flows are determined via PCA, for bioprocess monitoring and optimisation (Stosch et al., 2012). Oscillations in yeast glycolysis was the object of study by Yazdani et al. Here, the ODE system was directly incorporated into the deep learning framework by including the ODE residues in the loss function (Yazdani et al., 2020). In all these cases, the fit or prediction of the model was significantly improved compared to the pure knowledge-based approaches.

However, in the previews mentions studies, the information of the knowledge-based models is used as inputs for the data driven approaches and the states are directly estimated. Neural Network ODE (NN-ODE) represent a different approach. The basic model structure is similar to mechanistic models, however, some (or all) reaction rates of the RHS are formulated as NN outputs instated of kinetic formulations (Lagaris et al., 1998). Even though NN-ODE have the power to improve lean mechanistic bioprocess models, they have never been applied in this field so far.

3 Research questions (RQ)

The overall aim of this thesis is to accelerate bioprocess development through combination of automation and modelling towards smart bioprocess facilities. The basic consideration is that only smart facilities can achieve the throughput required for today's bioprocess engineering in a reasonable amount of time. How to efficiently introduce well-adapted and tailored models into the different stages of bioprocess engineering is one of the driving motives of this work. For this purpose, the following subproblems are identified:

RQ 1: How can dynamic data input within a statically working LHS be achieved?

Even though the use of models is the path to intelligent process automation, hardware automation is a fundamental prerequisite. For isolated systems with static tasks, such as LHS, this is a solved problem. However, it becomes more complex when fundamental process dynamics have to be adapted during the process. The possibilities of LHS are manifold and can range from very simple to highly complex operations. However, the used control software of the LHS is not suitable for reacting to new inputs and adapting the running protocols. It is designed for tasks where all necessary steps and pipetting volumes are fixed at the start of the method.

RQ 2: How can multiple parallel bioprocess be monitored?

Bioprocess monitoring is a common application of mostly data-driven models. Based on historical data, models are trained and used for process prediction or observation. Errors in process control are thus quickly detected. For new strains, the necessary historical data are not available to create these models. However, especially in screening, process errors should be detected quickly to intervene in time and to avoid having to repeat the experiment.

RQ 3: Can online model adaptation and dynamic redesign be used for continuous growth alignment?

To accelerate bioprocess development the automation of library screening is an essential step. While automated transformation of *E. coli* is not a major challenge anymore, generating competent cells simultaneously for multiple strains is. Here, the growth of the different strains must be aligned to create a uniform harvest time.

RQ 4: How can high-quality parameter fitting with potentially inadequate data be ensured?

Effective parameter adaptation requires sufficient data, which is often not available in the early stages of of a bioprocess. However, many crucial predictions must be made as precisely as possible, especially at the beginning of the process. The model calibration must therefore produce a robust result even without sufficient data.

RQ 5: An autonomously working bioprocess development facility.

The design of bioprocesses is a complex task, dependent on the possibilities of process monitoring, information on the system used (host, vector, bioreactor, etc.) and experience of the operator. For fast and efficient screenings, robust bioprocesses should be scheduled and executed as automatized as possible without the need of historical data.

4 Results

In this work, the added value of combined automation and modelling is shown. A large number of parallel cultivations is managed without the need for preliminary experiments or knowledge about the used strains. This was made possible by the development of a system composed of two LHS and one MBR, which enables complex operations for bioprocess control.

Paper I (*Automated Cell Treatment for Competence and Transformation of Escherichia coli in a High-Throughput Quasi-Turbidostat Using Microtiter Plates*) shows the successful application of a growth model to keep the cells in the exponential growth phase. The model is used to predict the biomass concentration and the needed operations are directly executed by the LHS.

The used model is quite simple, the prediction horizon is short and biomass is taken into account. The entire experiment can be executed on a single LHS, which makes the requirements for data handling and automation manageable. In Paper II (*Integrated Robotic Mini Bioreactor Platform for Automated, Parallel Microbial Cultivation With Online Data Handling and Process Control*) a facility is described composed of two LHS, one MBR, full integrated data flow and process automation.

This facility is used for strain characterisation in paper III (*Automated conditional screening of Escherichia coli knockout mutants in parallel adaptive fed-batch cultivations*). Here, the complexity of the model used and the forecast period increases. The presented cycle of data collection, model adaptation and recalculation of the bioprocess design allowed a robust experiment for 8 different strains simultaneously without a manual review.

The adaption of mechanistic models to specific hosts is a computationally intensive and time-consuming process. This is fine for forecasting applications with a target duration of several hours, but not for process monitoring with the aim to detect accidents or operation failures. For such purposes models are needed that require minimal data and can be adapted rapidly. Such an application is descripted in paper IV (*Monitoring Parallel Robotic Cultivations with Online Multivariate Analysis*) with the use of a PCA model for process observation.

4.1 Automated Cell Treatment for Competence and Transformation of *Escherichia coli* in a High-Throughput Quasi-Turbidostat Using Microtiter Plates

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Abstract: Metabolic engineering and genome editing strategies often lead to large strain libraries of a bacterial host. Nevertheless, the generation of competent cells is the basis for transformation and subsequent screening of these strains. While preparation of competent cells is a standard procedure in flask cultivations, parallelization becomes a challenging task when working with larger libraries and liquid handling stations as transformation efficiency depends on a distinct physiological state of the cells. We present a robust method for the preparation of competent cells and their transformation. The strength of the method is that all cells on the plate can be maintained at a high growth rate until all cultures have reached a defined cell density regardless of growth rate and lag phase variabilities. This allows sufficient transformation in automated high throughput facilities and solves important scheduling issues in wet-lab library screenings. We address the problem of different growth rates, lag phases, and initial cell densities inspired by the characteristics of continuous cultures. The method functions on a fully automated liquid handling platform including all steps from the inoculation of the liquid cultures to plating and incubation on agar plates. The key advantage of the developed method is that it enables cell harvest in 96 well plates at a predefined time by keeping fast growing cells in the exponential phase as in turbidostat cultivations. This is done by a periodic monitoring of cell growth and a controlled dilution specific for each well. With the described methodology, we were able to transform different strains in parallel. The transformants produced can be picked and used in further automated screening experiments. This method offers the possibility to transform any combination of strain- and plasmid library in an automated high-throughput system, overcoming an important bottleneck in the high-throughput screening and the overall chain of bioprocess development.

Own Contributions: Conceptualization, Methodology, Formal Analysis, Investigation, Resources, Data Curation, Writing-Original Draft, Visualization

Author Contributions: Conceptualization, MNC-B, SH and MG; Methodology, SH; Validation, MG, FG and MNC-B; Formal Analysis, SH; Investigation, SH, MG and MNC-B; Resources, SH; Data Curation, SH; Writing-Original Draft Preparation, SH; Writing-Review & Editing, MG, FG, MNC-B and PN; Visualization, SH; Supervision, MG, FG, MNC-B and PN; Project Administration, MNC-B and PN

4.1.1 Introduction

The vast number of factors that influence the expression of recombinant protein production in bioprocesses makes screening a challenging task in bioprocess development [1]. The choice of the strain is typically made at early stages in product development and is therefore excluded in the following steps [2,3].

With the increasing number of tools to manipulate DNA, new options are available in the field of metabolic engineering, and genome editing [4,5]. On that node, the expression host gets more in focus of the optimization processes [6]. Metabolic engineering to increase the production of small molecules is a common task for various hosts [7,8,9,10]. The availability of a variety of expression plasmids with low (e.g., pSC101 [11]), medium (pBR322 [12]) or high (e.g., pUC18/19 [13]) copy numbers as well as different constitutive and inducible promoter systems (e.g., PT7 [14], Plac, [15], PBAD [16], Pm/Xyls System [17]), controlling target gene expression, enlarge the search region for the optimal bioprocess even further.

Beyond the field of bioprocess development, studying knockout mutants helps to get a deeper understanding of gene functions and regulatory processes. With the use of fluorescent reporter systems, genetic networks can be studied. The largest available set of *Escherichia coli* (*E. coli*) strains with unknown behavior is the Keio Collection [18,19]. The Keio Collection is a library of 3864 *E. coli* K-12 single knockout strains. Similar collections are also described for Bacillus subtilis [20], Pseudomonas aeruginosa [21], Acinetobacter baylyi [22] and Saccharomyces cerevisiae [23]. With the use of fluorescent reporter systems, genetic networks can be studied in these strain libraries in vitro online and without extensive analytics [24]. Nevertheless, a systematic study of these collections with a reporter system is very difficult without automated treatment. Hence, there is a need for automated and high throughput treatment for cell competence and transformation.

The easy handling and the well-established molecular and microbiological methods made *E. coli* into one of the most commonly used organisms for heterologous protein production. Until the end of 2011, over 200 biopharmaceuticals have gained regulatory approval, nearly one third of them are produced in *E. coli* [2], demonstrating its importance for biotechnology. The first step in the process of manipulating cells is their treatment for competence. In *E. coli* there are mainly two different methods for competent cell preparation available: chemical treatment with CaCl2 [25,26,27,28] or the use of electricity [29,30]. As the competence depends on the physiological state of the cell, for both methods, the cultures must be harvested at a certain turbidity (optical density, OD) during the exponential growth phase. What is a basic task in laboratory with a well-known strain, could be a challenging task in a high throughput screening with a vast number of strains of unknown growth behavior. Whereas the problem of automated competence treatment has been solved when using a single strain [31,32],

completely different problems arise when using entire strain libraries. Normally, a batch culture is chosen to start the treatment of cells for competence. The cells are more or less monitored until a certain OD is reached. Even though automated frameworks exist to harvest the culture at a desired biomass concentration [33], different growth rates, starting ODs and lag-phases make it difficult to reach the same OD at the same time when transforming different clones (Figure 1a). From the perspective of bioprocess engineering, the method of choice to maintain the cells at a given condition would be a continuous cultivation [34]. The most used system (due to its simplicity and robustness) is the chemostat, where the growth rate is determined by the dilution rate. An extension of the chemostat is the turbidostat method. Here, the OD is continuously monitored and the dilution is controlled by the OD signal. Such a system enables cultivation close to the maximum growth rate at a specified OD (Figure 1b). However, the experimental setup for such a system is complex, consumes relatively high amounts of media, is prompt to faults in pumps or sensors, and its miniaturization and parallelization is challenging [35]. Even though miniaturized turbidostats have been realized [36,37] the experimental setup is still laborious and the parallelization does not reach the throughput of a 96-microwell plate.

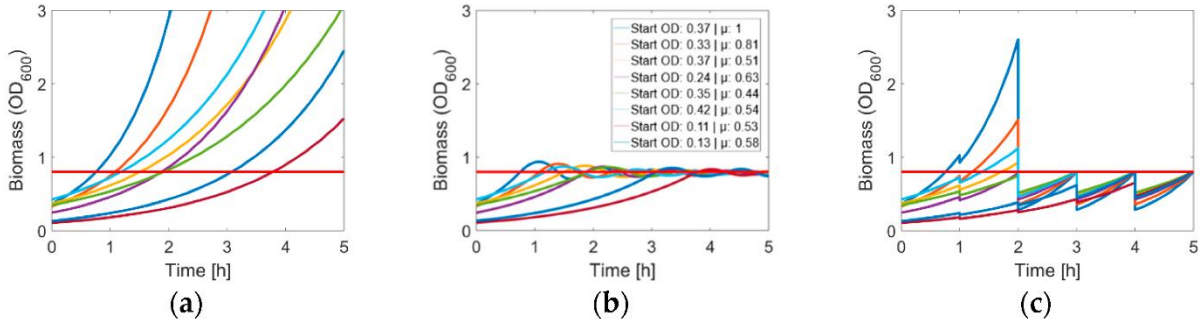

Figure 1. Illustrated overview of possible cultivation modes for the preparation of competent cells. Values for initial biomass and μ were chosen randomly; red line: threshold for harvesting (Optical Density (OD) = 0.8). The used models could be seen in the appendix. (a) Batch cultivation; (b) chemostat cultivation; (c) quasi-turbidostat cultivation.

To ensure a constant quality of DNA transformation, we developed a new strategy for optimal preparation of competent *E. coli* cells based on a CaCl2 treatment. Here, optimal means that all cells are in the exponential growth phase, the OD is equal, and the desired conditions are maintained up to the selected harvesting time. This new method is an automated, high throughput quasi-turbidostat, developed for 96 well plates (Figure 1c). Furthermore, as proof of concept, we compare the results obtained from manual and automated transformation of different *E. coli* strains.

4.1.2 Materials and Methods

4.1.2.1 Experimental Platform

As experimental platform a Hamilton Mircolab Star (Hamilton Bonaduz AG, Bonaduz, Switzerland) is used as described in [38]. Figure 2 gives an overview of the deck layout; the method is archived in the Supplementary Materials (Source Code S1/ Source Code S2). A freedom EVO 200 liquid handling platform from Tecan (Tecan, Männedorf, Switzerland, see Figure S3) is placed back-to-back with the Hamilton platform. Both liquid handlers are connected by a linear transfer unit, controlled by the Hamilton Venus ONE software.

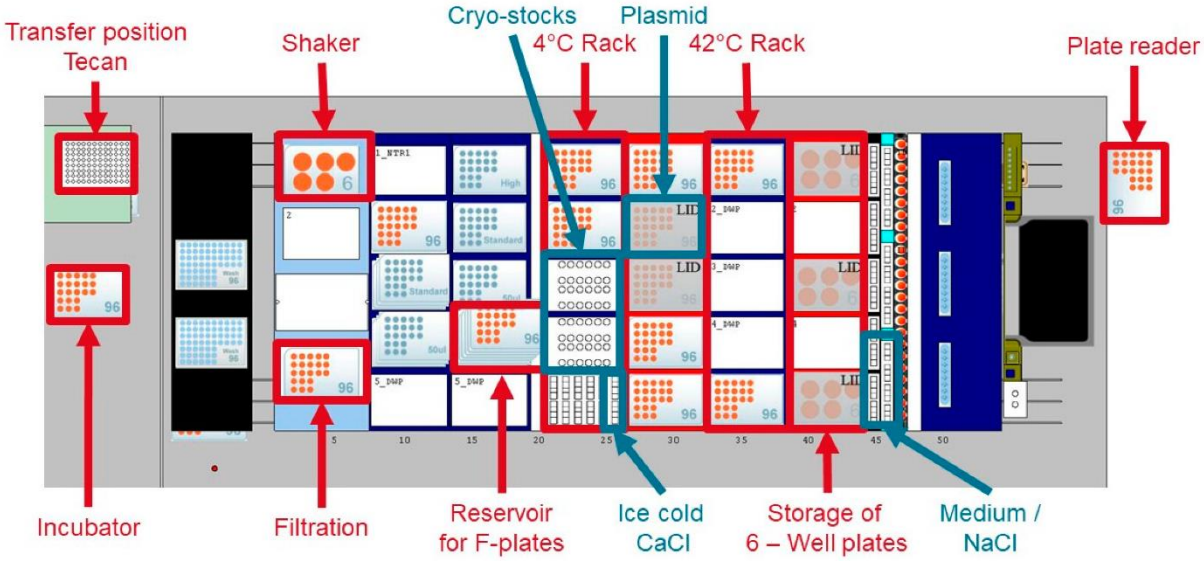

Figure 2. Deck layout of the used Hamilton Microlab Star liquid handling station. In this platform a FAME incubator (Hamilton), a Shaker (inheco Industrial Heating and Cooling GmbH, Planegg, Germany), a vacuum station, two terminable racks (each for five SBS labware) and a Synergy MX II plate reader (BioTek, Bad Friedrichshall, Germany) are mounted. Red: used Labware/Hardware; Blue: provided liquid solutions.

All cultivations are carried out in U-shaped microtiter plates (Greiner Bio-One, Frickenhausen, Germany), incubated at 37 °C and aerated by shaking at 1000 rpm at an amplitude of 2 mm in a FAME incubator (Hamilton). TY medium (16 g/L tryptone; 10 g/L yeast extract; 5 g/L NaCl) is used for all cultivations. The cellular growth is monitored by measuring the OD at 600 nm, in 96 well plates as described earlier [38].

The platform is connected to the iLab-Bio database (infoteam Software AG, Bubenreuth, Germany). All generated data and needed set points are stored in and read from this database [39].

4.1.2.2 Strains, Cell Competence and Transformation

For the manual preparation of competent cells, *E. coli* TG1 (see Table 1 for all strains) was cultivated in 10 ml of TY medium at 37 °C until an OD_{600} of approximately 0.8. Cells were harvested from 200 µL culture by centrifugation, resuspended in 200 µL ice-cold CaCl2

solution (100 mM CaCl2, 50 mM Tris-HCl pH 7.5), and incubated on ice for 30 min. Finally, the cell pellet was obtained by centrifugation and resuspended in 100 µL ice-cold CaCl2 solution and left on ice for at least 2 h.

Table 1. List of used strains.

Strain	Genotype	Source
E. coli TG 1	*K-12 supE hsd Δ5thi Δ[lac-proAB] F' [traD36 proAB lacIq lacZ ΔM15]*	[40]
E. coli TG 90	*K-12 supE hsd Δ5thi Δ[lac-proAB] F' [traD36 proAB lacIq lacZ ΔM15] pcnB80 zad::TnlO*	[41]
E. coli BW25113	*K-12 lacI^{+} rrnB$_{T14}$ ΔlacZ$_{WJ16}$ hsdR514 ΔaraBAD$_{AH33}$ ΔrhaBAD$_{LD78}$ rph-1 Δ(araB–D)567 Δ(rhaD–B)568 ΔlacZ4787(::rrnB-3) hsdR514 rph-1*	[42]

As no ice is available at the liquid handling platform, the competence protocol was adjusted as follows: 4 °C cold solutions were used and all incubation steps were carried out at 4 °C. Automated cell treatment is described in the results section (Section 3.2, Section 3.3 and Section 3.4).

Manual transformation was carried out as follows: 20 µL competent cells were incubated with 1 ng pUC19 [13] for 30 min, on ice or at 4 °C, respectively. Afterwards, cells were heat shocked for 2 min at 42 °C, 180 µL TY added, shaken at 37 °C for one hour, spread on an agar plate with 125 µg mL−1 ampicillin and incubated at 37 °C overnight. For the automated transformation protocol see the results in Section 3.5.

For the determination of the optimal harvesting point for the preparation of competent cells, the *E. coli* strain BW25113 was cultivated in 10 mL TY medium at 37 °C. After 0.5; 1; 1.5; 2; 2.5; 3; 4; 5; 6; and 16 h OD600 was measured and equal numbers of cells were harvested (200 µL × 0.8 OD600). Competent cells were prepared as above. 50 µL of competent cells were used for transformation with 1 ng pUC19 as described above.

4.1.2.3 Computational Methods

All computation steps were performed in MATLAB 2016a (Natick, MA, USA). Based on the equation given by Enfors and Häggström 2010 [43] the growth rate is calculated by Equation (1)

$$\mu = \frac{ln\left(\frac{X_k}{X_{k-1}}\right)}{t_k - t_{k-1}} \quad (1)$$

where µ is representing the specific growth rate [h^{-1}], X the biomass as OD600 and t the time [h]. Based on Equation (1), the biomass for the next hour is estimated with

$$X_{k+1} = X_k \, e^{\mu(t_{k+1} - t_k)} \quad (2)$$

The biomass (X_k) at t_k to reach the desired biomass at t_{k+1} ($X_{Threshold}$) is calculated with Equation (3).

$$X_k = \frac{X_{threshold}}{e^{\mu(t_{k+1}-t_k)}} \quad (3)$$

The dilution factor is calculated with X_k from Equation (3) divided by the actual measured biomass.

4.1.3 Results

The aim of this study is the development of a protocol on the liquid handling platform, to generate competent cells, and transform them directly with one or more plasmids. One of the main problems when transforming different strains with unknown growth characteristics in parallel is the correct harvesting point for all clones. Additionally, this point must be reached at the same time by all cultures to allow running subsequent procedures in parallel.

We compare the quasi-turbidostat method against the traditional batch cultivation to reach the optimal harvesting point. Due to the complexity and fault promptness of integrated high throughput robots, the focus is set on robustness (maximizing the production of competent cells for sufficient transformation). For the development of our method, we used *E. coli* as a case study.

4.1.3.1 Determination of the Optimal Harvest Conditions

The use of highly competent cells facilitates transformation. Due to their high transformation efficiency, low plasmid DNA concentrations or ligation mixtures result in a high number of CFUs. The optimal harvest point for preparation of competent *E. coli* cells is assumed to be from the early to mid-exponential growth phase [44]; probably the best competence is obtained with cells growing at their maximum specific growth rate. This can also be seen by the number of colonies obtained after transformation of *E. coli* BW25113 harvested at different growth phases in a typical batch shake flask experiment (Figure 3a). As expected, mid log phase cultures with an OD600 between 0.7 and 1.4 proved to be optimal. In contrast, cells from the early or late exponential phase (OD600 0.3 and 2.7) resulted in slightly less colonies and cells from either the lag phase or the stationary phase resulted in significantly less colonies. Interestingly, at the same time the number of satellite colonies resulting from not transformed cells growing in the vicinity of real transformants increased over the growth curve (Figure 3b).

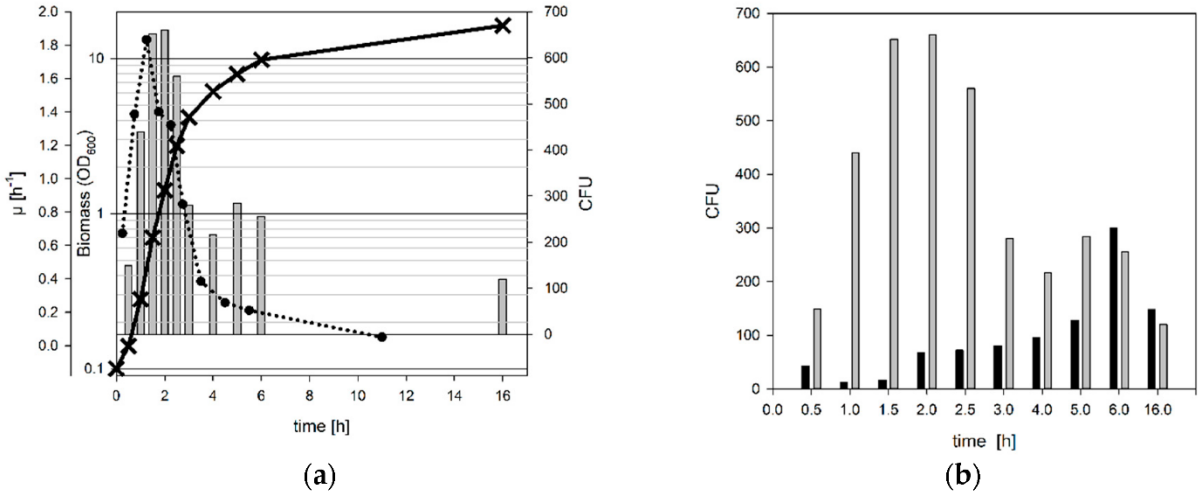

Figure 3. *E. coli* BW25113 was grown in TY medium, equal numbers of cells were harvested at different time points, competent cells were prepared and transformed with 1 ng pUC19 as described in materials and methods. (a) Black line: growth curve; dotted line: growth rate [h^{-1}]; grey bars: number of transformants. Resulting colonies were counted after overnight incubation at 37 °C. A correlation between transformation efficiency and growth rate can be detected (b) grey bars: number of transformants; black bars: number of satellite colonies. Comparison of transformants and satellite colonies obtained after transformation of competent cells as in (a). A lower number of satellite colonies indicates higher quality of the competent cells.

In other words, by creating a system where cells are maintained at maximum growth rate until the harvesting point has been reached, we obtain a highly effective transformation system that is also flexible to cope with the needs of other units, personnel, or sudden faults in the system. As shown in materials and methods, the setpoint for biomass concentration was set to 0.8 to assure no oxygen limitation minimized the cultivation time but also to assure that the cell density was high enough to compensate for low transformation efficiencies. Nevertheless, high transformation efficiencies are not necessary for most applications as for all further steps a few positive transformants are sufficient.

4.1.3.2 Competent Cells with Batch Cultivation

First, batch cultivations with an adapted sampling were performed. To solve the problem of different lag-phases and stating ODs, precultures were performed for 8 h. Afterwards, the OD600 was measured and a new cultivation with fresh medium was prepared. From the precultures, 10 µL were taken by the robot as inoculum for the new main cultivation plate. In this second plate OD600 was monitored every hour. If the mean of all cultivations reaches a threshold of 0.4, the monitoring interval was shortened to 30 min. After the mean of all performed cultivations reached a threshold of 0.7, the cells were harvested.

Examples for measured OD600 values are shown in Figure 4. The OD600 after the precultures were 2.50, 2.48, and 3.36 for the *E. coli* strains TG1, TG90, and BW25113, respectively. For the main cultivation, 10 µL of each culture were taken. This corresponds to a

1:20 dilution. After the second measurement, during the main cultivation, the mean OD of all cultures was 0.37 and therefore below the threshold for adapting the sampling mode. Hence, the next sample was taken one hour later. However, the *E. coli* TG1 and TG 90 were already over the threshold of 0.4. For these two strains the sampling point one hour later was already suboptimal. At the third measurement, the *E. coli* strains TG 1 and TG 90 were—with 1.11 and 1.02—out of the optimal range for harvesting, only the *E. coli* BW25113 strain was at the intended point with a mean of 0.82. Since only one of the strains reached an optimal threshold of 0.8 with the batch cultivation method, the adaptation of the experiment to the strains' growth conditions must have been insufficient. Obviously, the chosen thresholds were not optimal, leading to a suboptimal harvest time. Additionally, this method requires continuous re-tuning, making it time intensive and not proper for a high-throughput system.

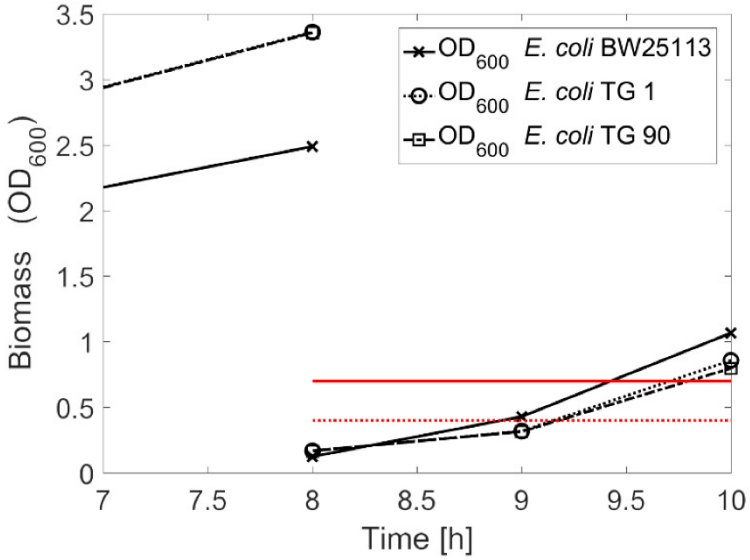

Figure 4. Batch cultivation approach for harvesting of competent cells with Preculture (0–8 h) and a main cultivation (8–10 h). Lower dotted red line: threshold of sampling interval adaptation; upper red line threshold for harvesting. The square of *E. coli* TG 90 is mostly hidden under the circle of *E. coli* TG 1.

4.1.3.3 Competent Cells in the Quasi-Turbidostat

As mentioned before, the ideal solution to this issue would be a parallel turbidostat system. Therefore, we developed a quasi-turbidostat on a 96-well plate using the liquid handling platform. For the quasi-turbidostat, no preculture is needed. The cultivation is operated in a loop with one-hour cycles containing the following steps (I) sampling, (II) OD600 measurement, and (III) dilution (Figure 5).

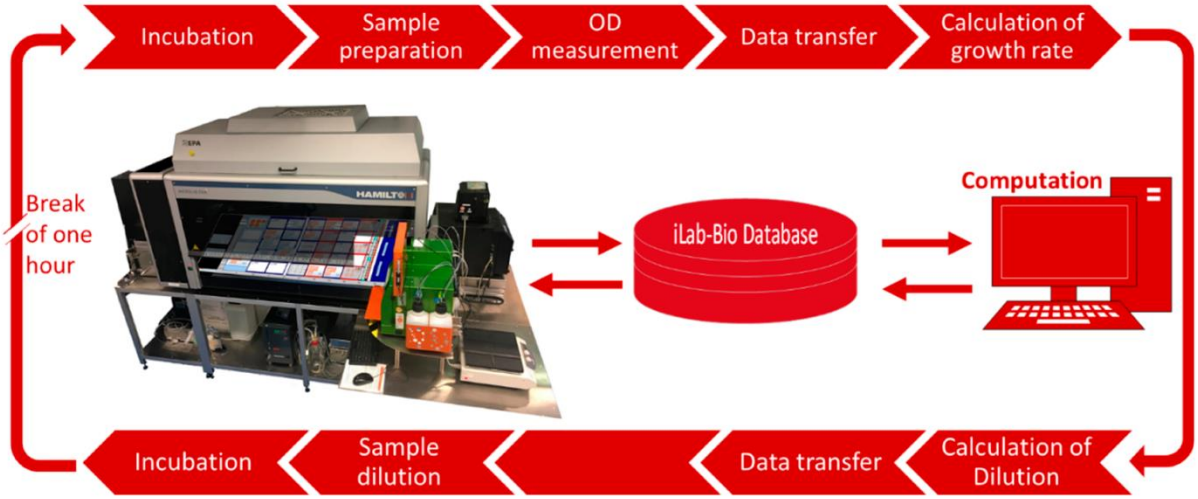

Figure 5. Robotic platform and workflow used for the quasi-turbidostat cultivation for the preparation of competent cells. The incubator is mounted on the left site of the liquid handling station, the plate reader is mounted on the right site. During the quasi-turbidostat cycle, the cells are transferred from the incubator to the liquid handler, a sample is taken, diluted, and measured in the plate reader. Afterwards, the OD_{600} values are transferred into the database and the execution of the script is triggered. The script calculates the current dilution of the quasi-turbidostat cultivations and sends the set points back to the database. Subsequently, the liquid handler reads the set points for the database and executes the dilution step. The cells are incubated for one hour until the next cycle is started.

The first 20 µL sample is taken directly after inoculation, 1:5 diluted, OD600 is measured, and the measured values are transferred into the database. Afterwards, the execution of a MATLAB (Mathworks) script is triggered (Source Code S4). During the execution of the script, the program reads the OD600 values from the database and the required biomass to reach the targeted OD600 in one hour is calculated based on Equation (3). Depending on the calculated OD600 values (X0), volumes for removing cell suspension and adding fresh medium are sent as setpoints to the database. These setpoints are read out and used by the pipetting robot to perform the dilution step. If no dilution is necessary, only the sampling volume is added to assure a constant cultivation volume of 170 µL over the whole cultivation.

In Figure 6, exemplary the OD600 measurements of *E. coli* TG1, TG 90, and BW25113 cultures are shown. After the second measurement, the first dilution was calculated. The growth rates at this time were 1.37, 1.28, and 1.32 h−1 with OD600 values of 0.30, 0.28 and 0.44, respectively. Assuming a constant growth rate, one hour later the OD600 was estimated to be 1.17, 0.99, and 1.65 h−1, respectively, by applying Equation (2). Therefore, from the beginning, a dilution for all strains was needed. At the third measurement, the OD600 of *E. coli* BW15113 was 0.76 and thus very close to the threshold. On the contrary, the growth rates of *E. coli* TG 1 and TG 90 were lower than assumed, as the OD600 values of these strains were only 0.68 and 0.56, respectively, and thus clearly below the targeted OD600 value. The same behavior is observed at the fourth sampling point after three hours. However, the distances to

the target value were lower. The observed OD600 values were 0.74 and 0.73 for E. coli TG 1 and TG 90, respectively. The *E. coli* BW25513 strain was, with an OD600 of 0.82, again the best matching strain. During samplings four and five (hours three to four) all growth rates stayed constant compared to the former interval. The OD values at the last sampling point were 0.80, 0.79, and 0.78 for *E. coli* TG 1, *E. coli* TG 90, and *E. coli* BW25113, respectively. This was a very low deviation from the threshold for all strains and the signal to continue with the cell treatment was given.

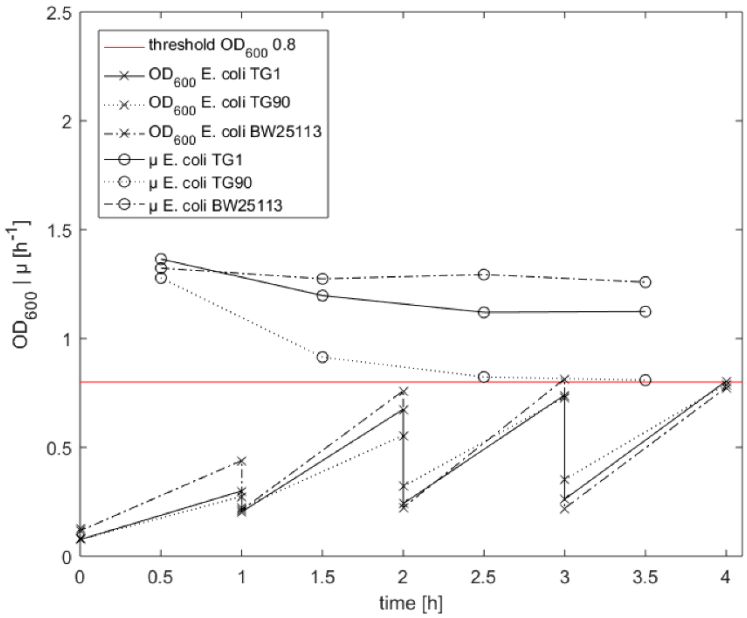

Figure 6. Quasi-turbidostat approach for harvesting of competent cells in a defined physiological state. Biomass is measured every hour. Depending on μ and biomass a dilution of the culture is performed.

4.1.3.4 Cell Treatment for Competence

For automated cell harvest the cultivation plate was transferred to a position of the 4 °C rack on the liquid handler (Figure 2) to cool down the cells. The whole available culture volume was taken and transferred to a 0.2 µm 96 well filter plate. This filter plate was placed on a vacuum station, integrated on the liquid handler (Figure 2). Over a time of 60 sec, a vacuum of 300 mbar was created to remove the medium from the cells. Afterwards, the cells were resuspended with cold CaCl2. This step was repeated three times before transferring the cells into a 96 well PCR plate to enhance the temperature transfer.

3.5. Transformation

After the incubation time of two hours at 4 °C, plasmid DNA was dispensed into the incubated cell suspension. The cells were further incubated for 30 min at 4 °C, and subsequently a heat shock at 42 °C for 2 min was performed by moving the PCR plate from the 4 °C rack to the 42 °C rack. And afterwards back to the position on the 4 °C rack. While keeping the cells cooled, a new U-shaped plate with fresh medium was prepared and the whole

batches from the PCR plate were transferred into this medium. The U-shaped plate was then cultivated for one hour. Finally, 200 µL of each culture were dispensed on TY agar, which was prepared in 6 well plates. These agar plates were shaken to spread the liquids even over the whole area of the wells. Afterwards, the agar plates were transferred into an incubator and cultivated for at least 12 h at 37 °C.

On all plated wells, the number of observed colonies was high enough for colony picking. In Figure 7, the wells of (a) a manual transformation on ice and (b) with 4° C treatment, (c) the automated treatment with the batch cultivation, and (d) the treatment with the quasi-turbidostat method is shown to be exemplary for the *E. coli* BW25113 strain.

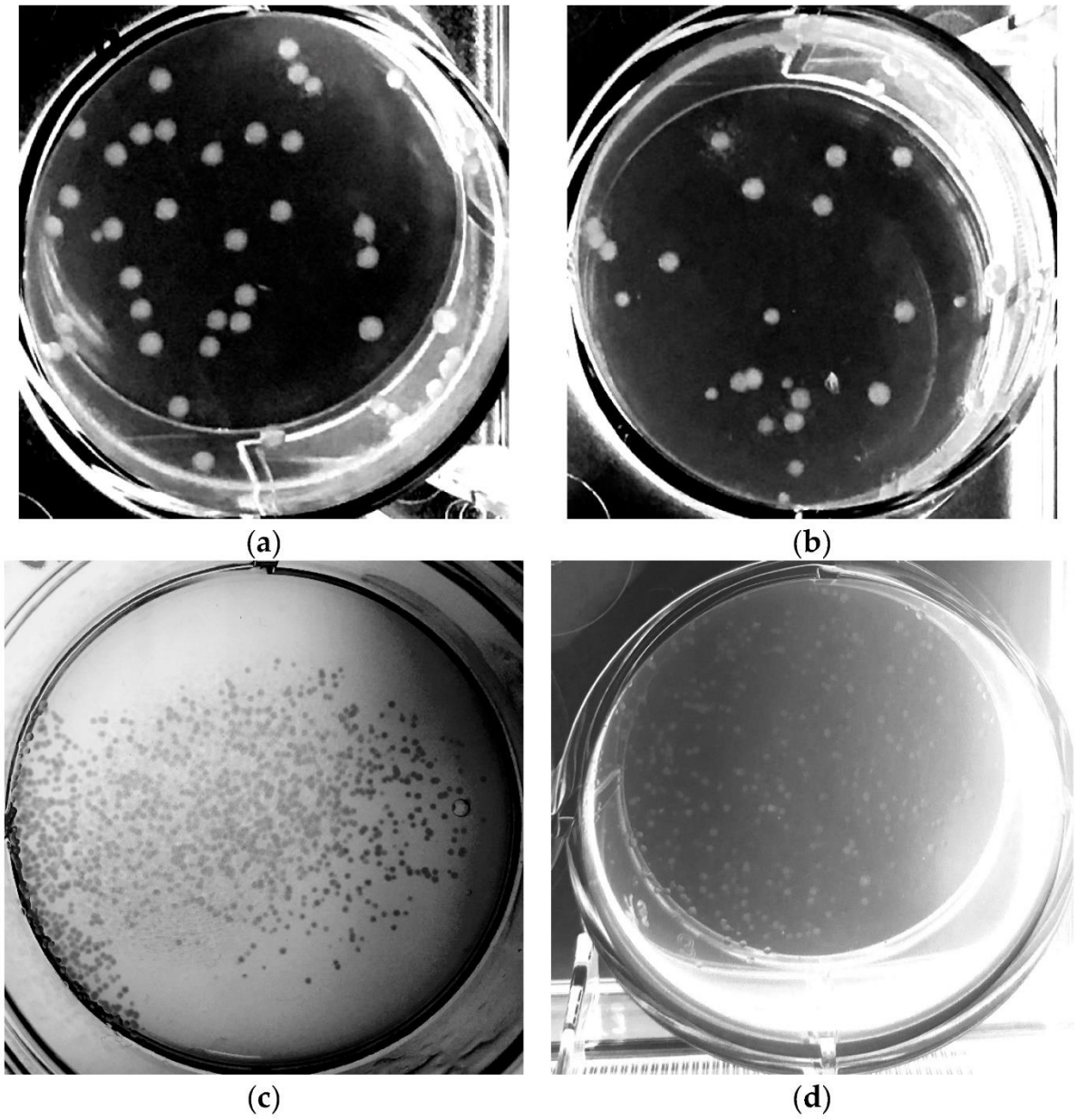

Figure 7. Spread cells after treatment for competence and transformation in 6-well plates (**a**) manually on ice; (**b**) manually at 4 °C instead keeping cell on ice; (**c**) automated treatment with a batch for cell harvesting; (**d**) automated treatment with quasi-turbidostat for cell harvesting.

No significant differences were observed by comparing the manual cell treatment on ice with the storage at 4 °C. Both methods lead to 30–50 transformed cells per well and thus we conclude that the use of 4 °C instead of keeping the cells on ice has no negative influence on the transformation efficiency. Moreover, the results show that with the applied shaking protocol the cells are equally spread over the area of the well without the use of a spatula, indicating that shaking the plates only in a one-dimensional movement is sufficient for spreading.

Differences between the automated and manual treatments (comparison of Figure 7b with Figure 7c or Figure 7d) are mainly caused by the different volumes used for plating. Both methods on the liquid handling platform are suitable for the treatment of competent cells and the transformation. Accordingly, as the results of both automated approaches show, there are enough colonies for colony picking. Apart from this, there were no significant differences observed for the *E. coli* BW15113 between the batch treatment and the quasi-turbidostat method. However, the quasi-turbidostat provides a way to guarantee that clones with different specific growth rates are harvested in the growing state at similar cell densities.

4.1.4 Discussion

The development of an automated method to obtain competent cells when whole strain libraries need to be transformed is a decisive step towards fully automated screening processes. This closes the gap between existing strain and vector libraries and high-throughput screening processes. To our knowledge, this is the first description of a turbidostat implementation in a 96 well plate and therefore, also an important step for the development of advanced screenings and phenotyping applications. The system was tested in an automated robotic facility, so it can be directly included in a broader process development framework reaching up to scale-down experiments at mL scale [45,46]. In order to increase a liquid handling facility towards an automated bioprocess development platform by [47,48], this method can be further connected to automated image analysis, colony counting, and clone picking.

For the development of this method we compared two different strategies for cell harvesting, a classical batch approach and a novel quasi-turbidostat approach. With the latter one we were able to harvest strains with different growth characteristics at the best harvesting point (i.e., cell density and growth stage), independent from the source of the strains; e.g., an agar plate, cryo-stock or another liquid culture.

The batch approach is simple to implement, but due to the different growth characteristics of the hosts, changing harvesting points, sensitivity to faults in the system as well as initial concentrations, and low time flexibility it is not suitable for use in a high throughput.

The use of the quasi-turbidostat provides some important advantages compared to the batch method. The sampling point for all strains was reached perfectly after four hours. The only adjustable parameter is the cycle time, which can automatically be adjusted to each well for a wide coverage of fast and slow growing cells. In the case study, the cycle time was one hour, therefore a minimal growth rate of 0.1 h−1 is needed, but an automated adjustment of the cycle time, to cover also slower and faster growing cells is straight forward.

However, the samples had to be diluted already after the first analytical cycle. Therefore, we expected to reach the threshold for all strains directly after the first cycle. As shown above (Figure 2b), this was not the case. Our method requires a constant growth rate to get optimal results. During the first two cycles, until hour three, we saw a decrease in the growth rate of the *E. coli* strains TG1 and TG90 mainly caused by inaccuracies of the OD measurement at low values. Our method calculated the dilutions with an unprecise μ and therefore reached suboptimal results. Whether the decrease in the growth rate is caused by measurement errors or by a physiological background could be in the focus of further investigations. It is known that acetic acid has a negative influence on the glucose uptake rate at high levels and therefore is known to reduce the maximum specific growth rate [45,49]. Hence, the acetic acid concentration is also diluted in every cycle and should not reach a critical concentration. It has been reported that the maximum glucose uptake rate decreases significantly over the time in glucose limited fed-batch cultivation with a constant feed profile: i.e., at lower specific growth rates [50,51].

To ensure a sufficient transformation efficiency especially when using low plasmid concentrations or ligation mixtures, it is important to harvest all cells during the optimal growth phase. The benefit of the quasi-turbidostat compared to the batch method is that the former ensures this because it keeps strains with different growth rates in the logarithmic growth phase until all cells reach the optimal harvesting point for the preparation of competent cells and their subsequent transformation.

To maximize our throughput and keeping the system as simple as possible, we used 96 well plates for cultivation and cell treatment. The use of enhanced high throughput cultivation systems like minibioreactor systems (MBRs) [52] could be alternatively considered if higher titers are required, and applications based on the online biomass signal have already been reported [33]. Nevertheless, this would increase the complexity of the system. Apart from that, our developed quasi-turbidostat method could be adapted to MBR with online biomass monitoring. Other systems that are very well suited for the quasi-turbidostat method are: BioLector [53], m2p, HEL minireactors, etc.

In addition, the development of a high-throughput method protocol for competent cell treatment and transformation, also describes a method that does neither need ice nor centrifugation. In contrast to the manual preparation of competent cells where centrifugation is

used for harvesting and washing steps, no centrifuge is needed for the automated protocol. Also, filtration has several advantages compared to centrifugation; (1) washing steps are more accurate as the media can be removed thoroughly without touching the cell pellet; (2) a vacuum station can be easier integrated in a liquid handling station as it needs less space and no hardware modifications and thus (3) investment costs are significantly lower.

The protocol is also very useful for laboratories with limited equipment or for a parallel treatment of cells manually.

4.1.5 Conclusions

The developed method is able to treat *E. coli* cells for competence and transform them with a certain vector and could be adapted to other strains with a similar protocol.

As show in Figure 4, the results are good enough to transfer the cells for further colony picking. Our automated method ended with the incubation of the spread agar plates. The created strain library is the basis of further automated screening and strain engineering methods [46,54]. Furthermore, the method could be adapted to other organisms.

Supplementary Materials

The following are available online at https://www.mdpi.com/2076-2607/6/3/60/s1, Source Code S1: Hamilton Script Overview, Source Code S2: Hamilton Script detail, Figure S3: Tecan LHS, Source Code S4: MATLAB Source Code.

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Conflicts of Interest

The authors declare no conflict of interest.

4.1.6 References

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

2. Berlec, A.; Štrukelj, B. Current state and recent advances in biopharmaceutical production in *Escherichia coli*, yeasts and mammalian cells. J. Ind. Microbiol. Biotechnol. 2013, 40, 257–274.

3. Schmidt, F.R. Optimization and scale up of industrial fermentation processes. Appl. Microbiol. Biotechnol. 2005, 68, 425–435.

4. Sun, J.; Wang, Q.; Jiang, Y.; Wen, Z.; Yang, L.; Wu, J.; Yang, S. Genome editing and transcriptional repression in Pseudomonas putida KT2440 via the type II CRISPR system. Microb. Cell Fact. 2018, 17, 41.

5. Maeda, T.; Sanchez-Torres, V.; Wood, T.K. Metabolic engineering to enhance bacterial hydrogen production. Microb. Biotechnol. 2008, 1, 30–39.

6. Sagt, C.M.J. Systems metabolic engineering in an industrial setting. Appl. Microbiol. Biotechnol. 2013, 97, 2319–2326.

7. Kim, S.-J.; Kim, J.-W.; Lee, Y.-G.; Park, Y.-C.; Seo, J.-H. Metabolic engineering of Saccharomyces cerevisiae for 2,3-butanediol production. Appl. Microbiol. Biotechnol. 2017, 101, 2241–2250.

8. Erb, T.J.; Jones, P.R.; Bar-Even, A. Synthetic metabolism: Metabolic engineering meets enzyme design. Curr. Opin. Chem. Biol. 2017, 37, 56–62.

9. Stefanovic, E.; Fitzgerald, G.; McAuliffe, O. Advances in the genomics and metabolomics of dairy lactobacilli: A review. Food Microbiol. 2017, 61, 33–49.

10. Kruyer, N.S.; Peralta-Yahya, P. Metabolic engineering strategies to bio-adipic acid production. Curr. Opin. Biotechnol. 2017, 45, 136–143.

11. Wang, R.F.; Kushner, S.R. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene 1991, 100, 195–199.

12. Bolivar, F.; Rodriguez, R.L.; Greene, P.J.; Betlach, M.C.; Heyneker, H.L.; Boyer, H.W.; Crosa, J.H.; Falkow, S. Construction and characterization of new cloning vehicle. II. A multipurpose cloning system. Gene 1977, 2, 95–113.

13. Yanisch-Perron, C.; Vieira, J.; Messing, J. Improved Ml3 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 1985, 33, 103–119.

14. Studier, F.W.; Moffatt, B.A. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J. Mol. Biol. 1986, 189, 113–130.

15. Gronenborn, B. Overproduction of phage lambda repressor under control of the lac promotor of *Escherichia coli*. Mol. Gen. Genet. 1976, 148, 243–250.

16. Guzman, L.-M.; Belin, D.; Carson, M.J.; Beckwith, J. Tight Regulation, Modulation, and High-Level Expression by Vectors Containing the Arabinose P BAD Promoter. J. Bacteriol. 1995, 177, 4121–4130.

17. Gawin, A.; Valla, S.; Brautaset, T. The XylS/Pm regulator/promoter system and its use in fundamental studies of bacterial gene expression, recombinant protein production and metabolic engineering. Microb. Biotechnol. 2017, 10, 702–718.

18. Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, Y.; Baba, M.; Datsenko, K.A.; Tomita, M.; Wanner, B.L.; Mori, H. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. Mol. Syst. Biol. 2006, 2, 2006.0008.

19. Yamamoto, N.; Nakahigashi, K.; Nakamichi, T.; Yoshino, M.; Takai, Y.; Touda, Y.; Furubayashi, A.; Kinjyo, S.; Dose, H.; Hasegawa, M.; et al. Update on the Keio collection of *Escherichia coli* single-gene deletion mutants. Mol. Syst. Biol. 2009, 5, 335.

20. Kobayashi, K.; Ehrlich, S.D.; Albertini, A.; Amati, G.; Andersen, K.K.; Arnaud, M.; Asai, K.; Ashikaga, S.; Aymerich, S.; Bessieres, P.; et al. Essential Bacillus subtilis genes. Proc. Natl. Acad. Sci. USA 2003, 100, 4678–4683.

21. Jacobs, M.A.; Alwood, A.; Thaipisuttikul, I.; Spencer, D.; Haugen, E.; Ernst, S.; Will, O.; Kaul, R.; Raymond, C.; Levy, R.; et al. Comprehensive transposon mutant library of Pseudomonas aeruginosa. Proc. Natl. Acad. Sci. USA 2003, 100, 14339–14344.

22. De Berardinis, V.; Vallenet, D.; Castelli, V.; Besnard, M.; Pinet, A.; Cruaud, C.; Samair, S.; Lechaplais, C.; Gyapay, G.; Richez, C.; et al. A complete collection of single-gene deletion mutants of Acinetobacter baylyi ADP1. Mol. Syst. Biol. 2008, 4, 174.

23. Giaever, G.; Nislow, C. The yeast deletion collection: A decade of functional genomics. Genetics 2014, 197, 451–465.

24. Cox, R.S.; Dunlop, M.J.; Elowitz, M.B. A synthetic three-color scaffold for monitoring genetic regulation and noise. J. Biol. Eng. 2010, 4, 10.

25. Singh, M.; Yadav, A.; Ma, X.; Amoah, E. Plasmid DNA Transformation in *Escherichia Coli*: Effect of Heat Shock Temperature, Duration, and Cold Incubation of CaCl 2 Treated Cells. Shock 2010, 6, 561–568.

26. Mandel, M.; Higa, A. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. 1970, 53, 159–162.

27. Hanahan, D.; Jessee, J.; Bloom, F.R. bacteria. In Bacterial Genetic Systems; Methods in Enzymology; Academic Press: Cambridge, MA, USA, 1991; Volume 204, pp. 63–113.

28. Cohen, S.N.; Chang, A.C.Y.; Hsu, L. Nonchromosomal Antibiotic Resistance in Bacteria: Genetic Transformation of *Escherichia coli* by R-Factor DNA. Proc. Natl. Acad. Sci. USA 1972, 69, 2110–2114.

29. Forster, R.L.S.; Bevan, M.W.; Harbison, S.; Gardnerl, R.C. High efficiency transformation of *E.coli* by high voltage electroporation. Nucleic Acids Res. 1988, 16, 291–303.

30. Taketo, A. DNA transfection of *Escherichia coil* by electroporation. Biochim. Biophys. Acta (BBA)-Gene Struct. Expr. 1988, 949, 318–324.

31. Hughes, S.R.; Dowd, P.F.; Hector, R.E.; Riedmuller, S.B.; Bartolett, S.; Mertens, J.A.; Qureshi, N.; Liu, S.; Bischoff, K.M.; Li, X.-L.; et al. Cost-Effective High-Throughput Fully Automated Construction of a Multiplex Library of Mutagenized Open Reading Frames for an Insecticidal Peptide Using a Plasmid-Based Functional Proteomic Robotic Workcell with Improved Vacuum System. JALA J. Assoc. Lab. Autom. 2007, 12, 202–212.

32. Finley, J.B.; Qiu, S.H.; Luan, C.H.; Luo, M. Structural genomics for Caenorhabditis elegans: High throughput protein expression analysis. Protein Expr. Purif. 2004, 34, 49–55.

33. Rohe, P.; Venkanna, D.; Kleine, B.; Freudl, R.; Oldiges, M. An automated workflow for enhancing microbial bioprocess optimization on a novel microbioreactor platform. Microb. Cell Fact. 2012, 11, 144. [Green Version]

34. Neubauer, P.; Cruz-Bournazou, M.N. Continuous Bioprocess Development: Methods for Control and Characterization of the Biological System. In Continuous Biomanufacturing: Innovative Technologies and Methods; John Wiley & Sons: Hoboken, NJ, USA, 2017.

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

36. Hoffmann, S.A.; Wohltat, C.; Mu, K.M.; Arndt, K.M. A user-friendly, low-cost turbidostat with versatile growth rate estimation based on an extended Kalman filter. PLoS ONE 2017, 12, e0181923.

37. Takahashi, C.N.; Miller, A.W.; Ekness, F.; Dunham, M.J.; Klavins, E. A low cost, customizable turbidostat for use in synthetic circuit characterization. ACS Synth. Biol. 2015, 4, 32–38.

38. Knepper, A.; Heiser, M.; Glauche, F.; Neubauer, P. Robotic Platform for Parallelized Cultivation and Monitoring of Microbial Growth Parameters in Microwell Plates. J. Lab. Autom. 2014, 19, 593–601. [Green Version]

39. Schmid, I.; Aschoff, J. A scalable software framework for data integration in bioprocess development. Eng. Life Sci. 2017, 17, 1159–1165.

40. Baer, R.; Bankier, A.T.; Biggin, M.D.; Deininger, P.L.; Farrell, P.J.; Gibson, T.J.; Hatfull, G.; Hudson, G.S.; Satchwell, S.C.; Seguin, C.; et al. DNA sequence and expression of the B98-8 Epstein-Barr virus genome. Nature 1984, 310, 207–211.

41. Gonzy-Tréboul, G.; Karmazyn-Campelli, C.; Stragier, P. Developmental regulation of transcription of the Bacillus subtilis ftsAZ operon. J. Mol. Biol. 1992, 224, 967–979.

42. Datsenko, K.A.; Wanner, B.L. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA 2000, 97, 6640–6645.

43. Enfors, S.-O.; Häggström, L. Bioprocess Technology: Fundamentals and Applications; Royal Institute of Technology: Stockholm, Sweden, 2000; ISBN 91-7170-511-2.

44. Szostková, M.; Horáková, D.; Němec, M. The influence of the growth phase of enteric bacteria on electrotransformation with plasmid DNA. Folia Microbiol. 1999, 44, 177–180.

45. Anane, E.; Lopez Cárdenas, D.C.; Neubauer, P.; Cruz Bournazou, M.N. Modelling overflow metabolism in *Escherichia coli* by acetate cycling. Biochem. Eng. J. 2017, 125, 23–30.

46. Cruz Bournazou, M.N.; Barz, T.; Nickel, D.B.; Lopez Cárdenas, D.C.; Glauche, F.; Knepper, A.; Neubauer, P. Online optimal experimental re-design in robotic parallel fed-batch cultivation facilities. Biotechnol. Bioeng. 2017, 114, 610–619.

47. Neubauer, P. Editorial: Towards faster bioprocess development. Biotechnol. J. 2011, 6, 902–903

48. Neubauer, P.; Glauche, F.; Cruz-Bournazou, M.N. Editorial: Bioprocess Development in the era of digitalization. Eng. Life Sci. 2017, 17, 1140–1141.

49. Kleman, G.L.; Strohl, W.R. Acetate metabolism by *Escherichia coli* in high-cell-density fermentation. Appl. Environ. Microbiol. 1994, 60, 3952–3958.

50. Lin, H.Y.; Mathiszik, B.; Xu, B.; Enfors, S.O.; Neubauer, P. Determination of the maximum specific uptake capacities for glucose and oxygen in glucose-limited fed-batch cultivations of *Escherichia coli*. Biotechnol. Bioeng. 2001, 73, 347–357.

51. Brand, E.; Junne, S.; Anane, E.; Nicolas, M.; Bournazou, C.; Neubauer, P. Importance of the cultivation history for the response of *Escherichia coli* to oscillations in scale-down experiments. Bioprocess Biosyst. Eng. 2018, 1–9.

52. Hemmerich, J.; Noack, S.; Wiechert, W.; Oldiges, M. Microbioreactor Systems for Accelerated Bioprocess Development. Biotechnol. J. 2018, 13, 1–25.

53. Kensy, F.; Zang, E.; Faulhammer, C.; Tan, R.; Büchs, J. Validation of a high-throughput fermentation system based on online monitoring of biomass and fluorescence in continuously shaken microtiter plates. Microb. Cell Fact. 2009, 8, 31.

54. Glauche, F.; Pilarek, M.; Bournazou, M.N.C.; Grunzel, P.; Neubauer, P. Design of experiments-based high-throughput strategy for development and optimization of efficient cell disruption protocols. Eng. Life Sci. 2017, 17, 1166–1172.

4.2 Integrated Robotic Mini Bioreactor Platform for Automated, Parallel Microbial Cultivation With Online Data Handling and Process Control

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Abstract During process development, the experimental search space is defined by the number of experiments that can be performed in specific time frames but also by its sophistication (e.g., inputs, sensors, sampling frequency, analytics). High-throughput liquid-handling stations can perform a large number of automated experiments in parallel. Nevertheless, the experimental data sets that are obtained are not always relevant for development of industrial bioprocesses, leading to a high rate of failure during scale-up. We present an automated mini bioreactor platform that enables parallel cultivations in the milliliter scale with online monitoring and control, well-controlled conditions, and advanced feeding strategies similar to industrial processes. The combination of two liquid handlers allows both automated mini bioreactor operation and at-line analysis in parallel. A central database enables end-to-end data exchange and fully integrated device and process control. A model-based operation algorithm allows for the accurate performance of complex cultivations for scale-down studies and strain characterization via optimal experimental redesign, significantly increasing the reliability and transferability of data throughout process development. The platform meets the tradeoff between experimental throughput and process control and monitoring comparable to laboratory-scale bioreactors.

Own Contributions Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing-Original Draft and Visualization

Author Contributions: Conceptualization, SH, BH and MNC-B; Methodology, SH and BH; Validation, SH and BH; Formal Analysis, SH; Investigation, SH, BH and AS; Resources, SH, B-H AS and NK; Data Curation, SH and BH; Writing-Original Draft Preparation, BH and SH; Writing-Review & Editing, NK, AS, PN, and MNC-B; Visualization, SH and BH; Supervision, MNC-B and PN; Project Administration, MNC-B and PN Funding: PN

Abbreviations

DOT, Dissolved Oxygen Tension
HT, High-Throughput
LHS, Liquid Handling Station
MBR, Mini-BioReactor
MWP, Micro Well Plate
OD_{600}, Optical Density;
SiLA, Standardization in Lab Automation

4.2.1 Introduction

High Throughput Screening (HTS) facilities have evolved to perform thousands of experiments in parallel generating experimental data at incredible speed. These Liquid Handling Stations (LHS) have accelerated drug discovery, sequencing, protein design, and analytics among others. Unfortunately, typical screening procedures do not provide sufficient information to assess cell performance at industrial process conditions [1]. The big difference in environments during cultivation in Micro Well Plates (MWPs) and biomanufacturing in large fed-batch reactors is an important factor for failure in bioprocess development [2]. It is essential that strain and process engineering are done considering industrial conditions, since cultivation regimes have a significant effect on the metabolism of the producer strain [3]. Hence keeping conditions comparable to the production scale while going through the development stages (from µL to L systems) decreases the risk of failure during scale-up [4].

In contrast, early process development is currently mainly focused on miniaturization and throughput maximization sacrificing monitoring and control of the cultivations [5]. By this, development often follows a trial and error strategy based on batch experiments with end-point evaluations [6,7]. Today MWPs are the preferred vessels for small scale microbial cultivation [8] and its application for screening has become standard practice in industry [9]. Using MWPs as cultivation systems has several drawbacks, as are unknown conditions inside the wells (by the lack of sensors) and a limited sampling capacity, due to the small volumes (mostly one sample). Such experiments are hardly comparable to industrial processes, since monitoring and active controls (e.g. pH) [10] as well as typical perturbations at industrial scale (e.g. reactor heterogeneities) [11] are missing. Furthermore, cultivations in batch are known to lead to different outcomes in the screening phase compared to typical fed-batch in biomanufacturing.

There are different automated approaches, which are tackling HT screenings in MWPs for early process development. Some examples are: the automated enzyme characterization of mutant libraries, with cell growth and protein production in MWPs [12]; the integration of the BioLector® (m2p-labs, Baesweilter, Germany), for up to 48 parallel cultivations in a modified MWP with feeding, online monitoring, event triggers [13], and analysis after cultivation [14]; the BioLector® Pro with feeding and pH control via microfluidic addition of fluids for each cultivation

well in up to 32 parallel cultivations [15]; and parallel laboratory evolution in MWPs at different stress conditions [16].

Still, the experimental conditions in MWPs are far from emulating substrate, pH or temperature heterogeneities present in large bioreactors. The response of the cells to these environmental stresses has been shown to have a significant impact on the substrate uptake and the intracellular fluxes [17,18]. Moreover cell-to-cell heterogeneity is increased having also a severe impact on productivity [19]. For a robust scale-up the physiological conditions inside the cell and the space-time dynamics of the environmental parameters have to be investigated [20]. Therefore, it is necessary to have dynamic experiments and high frequency data to understand the behavior of a biological process [21].

Fed-batch processes require monitoring and control, which are difficult to implement as ultra HT, as performed in molecular target screenings [22] or droplet single-cell [23-25], On the other hand, liter-scale bioreactors consume high material volumes and require manual intervention hampering parallelization in large numbers. Miniaturized stirred-tank bioreactors offer a good tradeoff between the experimental throughput and quality of the generated process data. Examples are the ambr® 15 system (Sartorius AG, Göttingen, Germany), the bioREACTOR 48 or 8 systems and the explore HEL system [26,27]. Via the combination with automated liquid-handling systems, the throughput and parallelization of microbial cultivations in small - scale stirred tank systems is increased [28].

There is also work on automation of experimental facilities towards “smart” platforms. One way of achieving this is the sequential design method, by which experimental data is processed by algorithms to design the following experimental round [29,30]. Unfortunately, these technologies are still focused on static experiments with mainly endpoint measurements and no investigation of bioprocess dynamics. Fed-batch and scale-down experiments require some understanding of the dynamics of the cultivation and resulting cell responses [31]. To these end feedback operation methods have been developed [32] with online Optimal Experimental re-Design strategies to fit macro-kinetic differential equation model of *Escherichia coli* (*E. coli*) [33,34]. Nevertheless, these complex approaches require a fully automated and efficient workflow of different laboratory devices, data exchange and management, which is has not been achieve previously. In the previous the solutions were tailored to a specific process, less flexible, relied on proprietary data formats, one-way data transfer and included manual steps. In this article, we present a fully automated and highly modular facility based on a novel concept for the interaction of i.) laboratory equipment, ii.) data, iii.) smart services and vi.) the operator. The robotic facility presented here enables us for the first time to perform parallel small-scale experiments with fully automated process handling, at-line sample analysis and closed-loop process control. Additionally, optimal experimental designs, model-based screenings and scale-down experiments are possible [35,36]. This was achieved by the

physical and virtual interaction of two LHSs and a MBR system. A central database is used for the integration of all units into a continuous workflow, which allows for end-to-end automation [37] and operation of the parallel experiments. This automated “smart” experimental facility is a step towards advanced bioprocess development phasing towards the digitalization of biomanufacturing and Industry 4.0 [38].

4.2.2 Material and methods

4.2.2.1 Concept and structure of the platform

To ensure robustness, flexibility and dynamic of the MBR platform we used a central database as data master. All measurements, sampling ids, setpoints, process parameters or any other data generated or necessary during the cultivation are stored in a structured manner, independent of the source or scope. Data transfer lines were avoided as much as possible, all the data were transferred to the database on a direct way and can be read out from every entity involved in the framework. Direct communication between the MBR platform devices is needed for the execution of triggering events for certain tasks.

During the experiment all of the generated data sets from each MBR cultivation are saved in the database, such as the liquid-handling steps and the online and at-line values. On the other hand, set points for the culture handling regarding feeding, pH, stirring speed and aeration are written to the database and read by the corresponding devices. The database backbone was designed by infoteam (Software AG, Bubenreuth, Germany) during the BMBF AutoBio project [37].

The setup of the robotic MBR platform centers around the integration of the bioREACTOR 48 mini-bioreactor system (2mag AG, Munich, Germany), which enables 48 cultivations in parallel, with a Tecan Freedom EVO 200 LHS (Tecan Group Ltd, Männedorf, Switzerland). The Tecan robot system performs the cultivation handling, process control and sampling in an automated and parallel manner. Via the use of an electric linear transfer unit, the combination with a Microlab Star LHS (Hamilton Company, Bonaduz, Switzerland) is enabled (Figure 1). Samples of the parallel cultivations are taken into MWPs on the Tecan deck and carried via the transfer unit to the Hamilton deck, where the at-line analytics are performed. By the use of an integrated SynergyTM MX microwell plate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany) photometric measurements of the recently taken samples are performed. The Optical Density (OD600) of the parallel cultivations, enzymatic based tests, as well as fluorescence signals are measured.

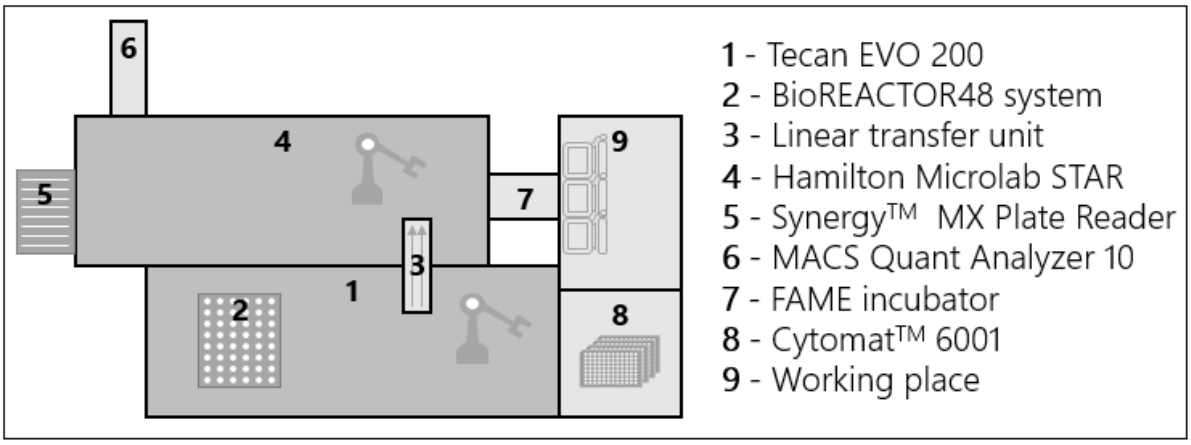

Figure 1 Setup of the robotic platform with the combination of two liquid handlers via a linear transfer unit. Further integrations of labware devices such as a plate reader for photometric analysis, a cytomat as plate storage, an incubator for plate cultivations and a flow cytometer for single cell analysis are shown. The working place contains three computers, two for the control of the LHSs and one for the monitoring of the parallel cultivations.

4.2.2.2 Devices used in the robotic platform

4.2.2.2.1 Parallel Mini-BioReactor System

Microbial cultivations are performed in the bioREACTOR 48 system, which enables HT parallel cultivation in up to 48 baffled single-use bioreactors, each with a working volume of 8-14 mL. The system can easily be integrated into liquid handlers and offers a good gas transfer, which is critical for microbial cultivations. Via the embedding of fluorometric sensor spots (PreSens Precision Sensing GmbH, Regensburg, Germany) in each MBR, the pH and Dissolved Oxygen Tension (DOT) is measured in real-time with an interval as low as 10 sec. This allows for a close culture monitoring and results in large data sets. During a cultivation with all MBRs 34 560 data points for every hour of the experiment are created, alone already for pH and DOT. The stirrer speed, aeration and temperature are controlled for the whole system uniformly. The temperature in the reactor block is measured via a Pt 100 sensor and controlled through the interplay of a water bath and a cooler. The mixing of the liquid in the MBRs is provided by hollow shafts with magnetically driven stirrers. This causes an induced draft mechanism, by which the air is sucked from the headspace into the liquid phase. The system can reach a volumetric mass transfer coefficient (kLa) of up to 0.4 s^{-1} [39]. The power input and local energy dissipation is comparable to standard stirred-tank bioreactors and CFD simulations showed similar hydrodynamics to bench-top stirred bioreactors on a liter scale [40]. In the lid of the system the supplied air is distributed to the headspace of each single bioreactor. The surplus of air leaves through ports in the lid above each bioreactor assuring sterility by a continuous outflow of sterile air. The lid of the bioreactor system is cooled to 4 °C in order to minimize evaporation during cultivations. In preliminary tests the maximal evaporation rate was

determined with 50 µL per hour (data not shown), which is in good agreement with literature [41]. The ports in the lid enable access to each MBR with the steel needles of the Tecan liquid handler, allowing automated culture handling and control as well as automated sampling.

4.2.2.2.2 Gas mixing station

A Red-y gas mixing station (Vögtlin Instruments GmbH, Aesch, Switzerland) was installed upstream of the bioREACTOR 48 system for flexible and precise control of the aeration supply. The exact inflow can be controlled as well as the composition of the aeration through the addition of pure nitrogen or pure oxygen to the pressurized air. This can be controlled via a Graphical User Interface (GUI) developed in LabView (National Instruments, Austin, USA), which enables manual control or automatic control through the database. Thus, an aeration profile can be automatically applied to the cultivations. The sterility of the system is guaranteed via a Midisart 2000 air filter (Sartorius AG, Göttingen, Germany), which is installed between the gas mixing station and the MBR system.

4.2.2.2.3 Liquid Handling Stations

On the deck of the Tecan LHS the bioREACTOR 48 system, trays for the liquids needed for sterilization of the needles, feeding, pH control, a cooled carrier for the sample plates, an Eppendorf tube carrier for offline samples and a washing station for the steel needles are placed (Figure 2A). Moreover, an adaptive carrier of the Cedex Bio HT analyzer can be placed for direct sampling into the corresponding tubes. The system is equipped with a Robotic Manipulator Arm (RoMa) for plate transport and a Liquid Handling arm (LiHa) with eight fixed steel needles. The Tecan liquid handler is used for performing all necessary steps for the parallel cultivation handling in the MBRs including feed addition, pH control, sampling and volume balance. Moreover, a cytomatTM 6001 (Thermo Scientific, Berlin, Germany) is connected to the robot, which functions as a plate storage for the sample plates. Via the Tecan Freedom Evoware, the cytomat can be controlled and a new plate is transferred to the deck when needed. The test for sterility of the housing of the Tecan liquid handler with a sterile hood and the test for sterility in place of the steel needles, to assure that cross contamination is avoided, were performed according to the method of Rohe et al. [13].

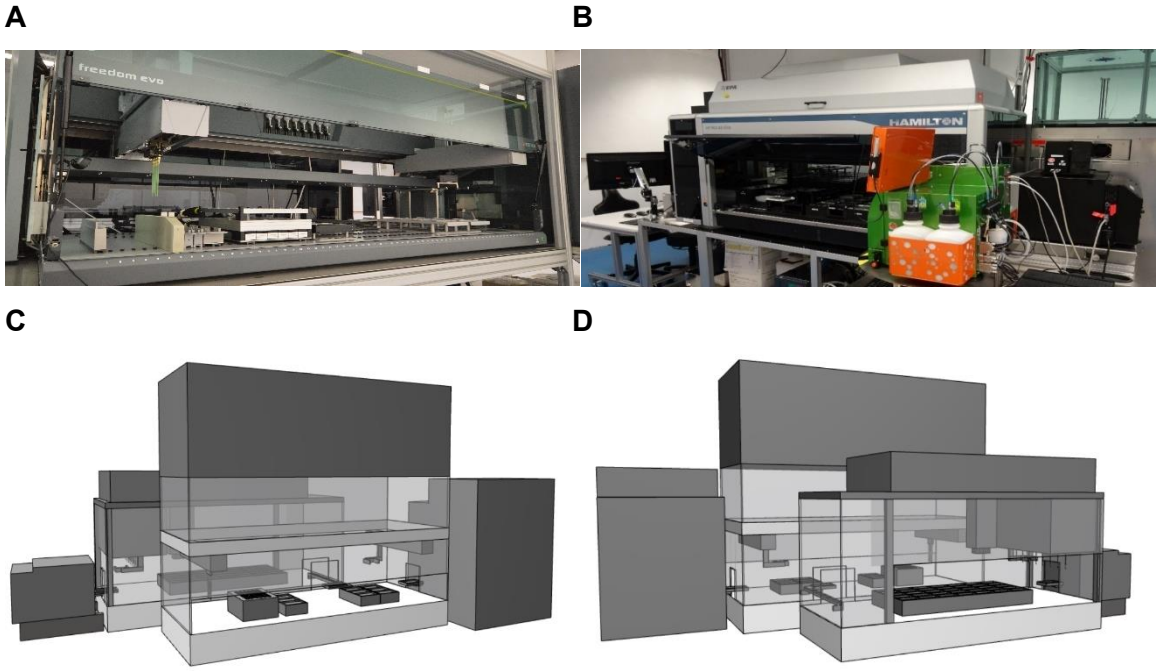

Figure 2 Presentation of the integrated robotic facility: The Tecan LHS with the MBR cultivation platform (A) is connected to the Hamilton LHS (B) on the other side of the platform with the incubator, the MACS Quant flow cytometer and the plate reader (spectrophotometer). Figure C and D show a schematic 3D representation of the platform from both sides.

The Hamilton is used for the at-line processing and analysis of the samples taken during the course of an experiment. The Hamilton system is suitable for sample analysis, since it can process a high number of samples in parallel through the 96 Multi Probe head with single-use tips, the integration of a vacuum station for filtration and the integration of a plate reader (Figure 2B). After a sample plate reaches the Hamilton deck via the linear transfer unit, the first operation is the measurement of the OD_{600}. For further analysis the rest of the sample is filtrated in order to separate the cells from the supernatant. Scale-down of enzymatic tests for glucose and acetic acid concentration in 96-well plates allows for fast HT analysis of samples in parallel to the cultivations [42]. Further downstream processing applications can be performed on the Hamilton LHS if needed, e.g. chemical cell lysis [43] or protein affinity purification in prepacked 96-well plates. Moreover, other enzymatic assays, which are evaluated photometrically, can be scaled-down and performed on the Hamilton liquid handler. Single cell at-line-analytics are possible with the MACS Quant® Analyzer.

4.2.2.3 Standard cultivation workflow

Prior to the inoculation of the MBRs a two-staged batch-phase is performed. The first batch-phase is executed in Ultra Yield™ Flasks (Thomson Instrument Company, Oceanside, CA, USA) directly after inoculation from cryo stocks. After adaptation to the medium for four hours, the cells are transferred to PreSens shake flasks with online monitoring of pH and DOT. The cells are collected during the exponential growth phase, which can be evaluated by the DOT

profile. The active cells are diluted with fresh medium to a low OD_{600} around 0.1-0.5 in one batch and subsequently each MBR is inoculated with 10 mL of that batch. This enables similar conditions between biological replicates to enable good batch-to-batch reproducibility. The number of biological replicates depends on the number of strains used. Until now, a maximum of eight strains was handled in parallel.

In the beginning of an automated experiment, a batch phase is performed to reach comparable ODs_{600} within the different. After a steep increase in the DOT, which signals the exhaustion of the substrate and end of batch phase, the fed-batch phase is started. Each MBR can be fed individually based on the OD_{600} in the bioreactor and the set growth rate (μ_{set}). With the use of computational modeling industrial feeding strategies can be performed: a batch-phase at the start of the experiment, followed by an exponential fed-batch phase and a constant feeding after induction. It is possible to use different types of substrates or substrate concentrations, therefore multiple cultivation conditions in one run can be executed. Feeding can be realized in two ways, either via a small bolus glucose addition every few minutes, or continuously via the use of a substrate release system, e.g. by the EnBase® technology (Enpresso GmbH, Berlin, Germany) [44]. This enables a slow glucose release over time based on enzymatic degradation of a polymer and is similar to a fed-batch with a constant glucose feed rate [45]. Also, exponential fed-batches can be performed with this system through a semi-continuous additions of enzyme.

The pH control is based on a PI controller programmed in LabView, which calculates the addition of base or acid based on the parameters entered in the database for each bioreactor individually. The control is performed for each bioreactor accordingly and the parameters for control can be changed over the course of a cultivation, such as the maximum amount of acid or base added in one control step. The pH control can be executed as a two-sided or a one-sided control, during the latter only base is added to the cultivations. This is especially useful during handling of microorganisms with cycling acid metabolism such as *E.coli* [46].

Sampling is performed in an alternating manner with culture handling to ensure a steady process control. In between two sampling steps substrate addition and pH control are executed. After sampling of five columns the plate is transferred to the Hamilton LHS for at-line analysis and a new plate is provided by the cytomat. It is possible to have different sampling procedures included in the script, whether offline samples are needed aside from the at-line samples. In order to keep the volume constant during a cultivation a subroutine for volume balance is executed. This LabView application considers all volume changes, namely i.) sampling, ii.) feeding, iii.) pH controlling additions and iv.) evaporation and calculates the volume deficiency which is corrected with fresh culture medium. The calculated volume

deficiency is saved in the database, read by a LabView application and performed by the Tecan LHS.

To control the DOT during the cultivations, two stirrer control modes are possible. Either a cascade control is applied, where the stirrer speed increases when the DOT in one of the MBRs reaches a critical lower threshold, or set points are given, which can be read directly from the database. In this way, pre-programmed agitation profiles can be accurately executed over the whole cultivation. The scripts in the Tecan Freedom EvoWare are written in a modular way, where each action of the LHS is saved in subroutines, so that scripts for each phase of the cultivation are easily modifiable. This modular setup of the robotic platform allows for fast execution of new experimental runs and adaptation of the developed scripts to new experimental requirements.

4.2.2.4 At-line analytics

For the at-line analytics 200-300 µL samples are taken out of each MBR into a sample plate. In advance, 15 µL 2 M NaOH is added to each well of the sample plate and dried. The addition of the sample results in the dissociation of the dried NaOH and an increase of the pH (pH approx. 10), which in combination with cooling of the MWP carrier to 4°C stops the metabolism of the cells. It could be shown that over the course of three hours there was no metabolic activity with this inactivation method 32. For the OD600 measurement a maximum of 20 µL of the sample are taken, which leaves at least 180 µL for filtration. Afterwards 2 x 20 µl are used for the enzymatic test in duplicate measurements, and the remaining 140 µL can be used for further offline analyses. The script for at-line analysis of the samples takes about 45 min, which includes the incubation times of the assays, the measurement of OD600, fluorescence signal, glucose and acetic acid concentration in up to 48 samples in parallel.

4.2.2.4.1 Optical density and fluorescence measurement

OD_{600} is the first at-line measurement performed on the Hamilton liquid handler. Before the measurement the samples are automatically diluted with 0.9 % sodium chloride in 96-well flat-bottom MWPs. Different dilution factors are applied, in order to stay within the linear range of the measurement over the course of the cultivation. The needed amounts of liquid to be pipetted are calculated by the Hamilton software with an upper limit of 10 µL per sample. Before the spectrophotometric measurement, the plate is shaken for 10 s in the plate reader to guarantee a good suspension of the cells. The samples are prepared in duplicates, measured at 600 nm, automatically corrected against the blank and multiplied by the correction factor. The correction factor for the path length, which is used to convert the OD_{600} measured in 96-well plates to a measurement in a cuvette, was calculated in advance [32]. If fluorescent proteins are used as reporters, the fluorescence is measured directly after the OD_{600}

measurement in the plate reader. For different fluorescence measurements, the scripts in the microwell plate reader software are adjusted to the correct excitation and emission wavelengths. The corresponding script is then executed by the Hamilton software.

4.2.2.4.2 Glucose and acetate measurements

After the OD_{600} and fluorescence measurements, the samples are further processed automatically on the Hamilton platform for the analysis of the metabolites and medium components. Glucose concentration is measured with the enzymatic Glucose Hexokinase FS kit (DiaSys Diognostics Systems GmbH, Holzheim, Germany), acetic acid concentration is measured with the Acetic Acid Assay kit (Megazymes, Bray, Ireland). Both measurements function via the enzymatic conversion of the analyte, by which NADH is oxidized 47 or NADPH is produced [48], both can be measured photometrically at 340 nm. For each measurement 10 µL of sample are needed and both are measured in duplicates. Two 96-well MWPs are prepared with pre-added master mixes of the test kids, the samples and standards are added and subsequently the plate is measured after the stated incubation times. The standards used for the determination of the analyte concentration were put in random order to avoid systematic errors and were analyzed for each sample plate repeatedly. The analysis is executed fully automatically and after each run the single-use plastic tips of the robot are replaced. The data is automatically processed and the measured absorption is subtracted by the blank and converted to the analyte concentration by regression of the standard samples.

4.2.2.4.3 Offline analysis

Direct samples from the MBRs as well as the surplus of the filtrated sample volume can be used for offline analyses, e.g. for metabolite and protein analyses. Over the course of a cultivation a lot of samples are generated. If all MBRs are used, nearly 100 samples are generated after two sampling rounds. Therefore, the offline analysis also needs to be performed in HT manner. The cultivation samples are automatically pipetted into tubes by the Tecan LHS and the filtrated samples are collected in 96-well plates, both are manually removed and stored in the fridge or directly frozen for subsequent analyses.

4.2.2.4.4 High-Throughput Metabolite Quantification

For the analysis of the produced metabolites the Cedex Bio HT analyzer (Roche Diagnostics International Ltd, Rotkreuz, Switzerland) is used. Tests are based on photometric and potentiometric measurements in combination with enzymatic tests for offline analysis of samples in HT manner. For our purposes glucose, acetate, formate, ethanol, lactate, ammonia, phosphate and magnesium were analyzed. For each measurement 2-20 µL are needed based on the test used, but the minimal liquid volume required for sample handling by the Cedex

pipettes is around 100 µL. The filtrated supernatant samples were diluted if needed and processed according to the user manual.

4.2.2.4.5 High-Throughput Protein Analysis

For the analysis of produced recombinant proteins during the cultivation, the LabChip GXII Touch HT (PerkinElmer Inc., Baesweiler, Germany) is used. The measurement is based on the microfluidic and electrophoretic separation in combination with detection via laser-based fluorescence of proteins in the offline cultivation samples. The analysis of one sample takes around 42 sec and per measurement 2-4 µL are required. The device is applied for protein quantification in the culture supernatant or after cell lysis. The Protein Pico Assay Reagent Kit was used, which enables detection of protein concentrations as low as 10 pg/µL. The filtrated supernatant samples were used and the preparation was performed according to the user manual.

4.2.3 Results

4.2.3.1 Data handling and communication

To ensure modularity the central database as data master for all tasks is used. Thereby all devices and programs are able to perform the tasks independently, in parallel and a modular fashion via the direct communication with the database. One tool to achieve this, is a .NET library that is used as a communication interface to the database and can be applied for nearly all Windows-based applications such as VenusFour (Hamilton), Python (Python Software Foundation) or LabView. applications. A mirror image version of this library is implemented in MATLAB to ensure functionality on Linux based systems. Complex feeding profiles based on process simulation and optimal experimental designs can be entered into the database for each individual bioreactor.

For manual operation during the experiments, the database can be accessed via a developed software. It can be used to write set points and parameters manually to the database. Moreover, the software offers a flexible real-time visualization of the generated data for process control. The offline data is entered via a MATLAB script that properly handles significant amount of data generated by the LabChip and Cedex Bio HT analyzer. By this all the data gained in one experimental run are saved together in a centralized manner, which is easily accessible by both, the model-based operation algorithms and the operators.

Through the database different software tools and laboratory devices can communicate and interact enabling an end-to-end data and device integration. Online data such as temperature, stirring speed, pH and DOT values are stored in the database using a LabView application, which also enables manual control or direct reading of process parameters, such as stirring speed or aeration rate. Set points for culture handling, such as feeding and pH control are

saved as set points in the database and read by different LabView applications. These applications are converted to executable files (exe), which can be run from the Tecan Freedom EVO software directly (Figure 3). Each set point in the database is connected to a timestamp and the most recent ones are written into General Writing Language (gwl) lists.

The server-client based communication between the two liquid handlers is assured by a Standardization in Lab Automation (SiLA 2) driver. This further developed tool serves as a general and modular interface for the communication between the two LHS. The Tecan robot sends a SiLA Client command via the network to the SiLA Server on the Hamilton site, which triggers the start of the Hamilton script for at-line analysis. The script on the Hamilton robot is started directly after samples are taken, so that the time frame between sampling and analysis is as short as possible. The at-line data are extracted from the Gen 5 BioTek software of the plate reader, read by the Hamilton software and saved in the database.

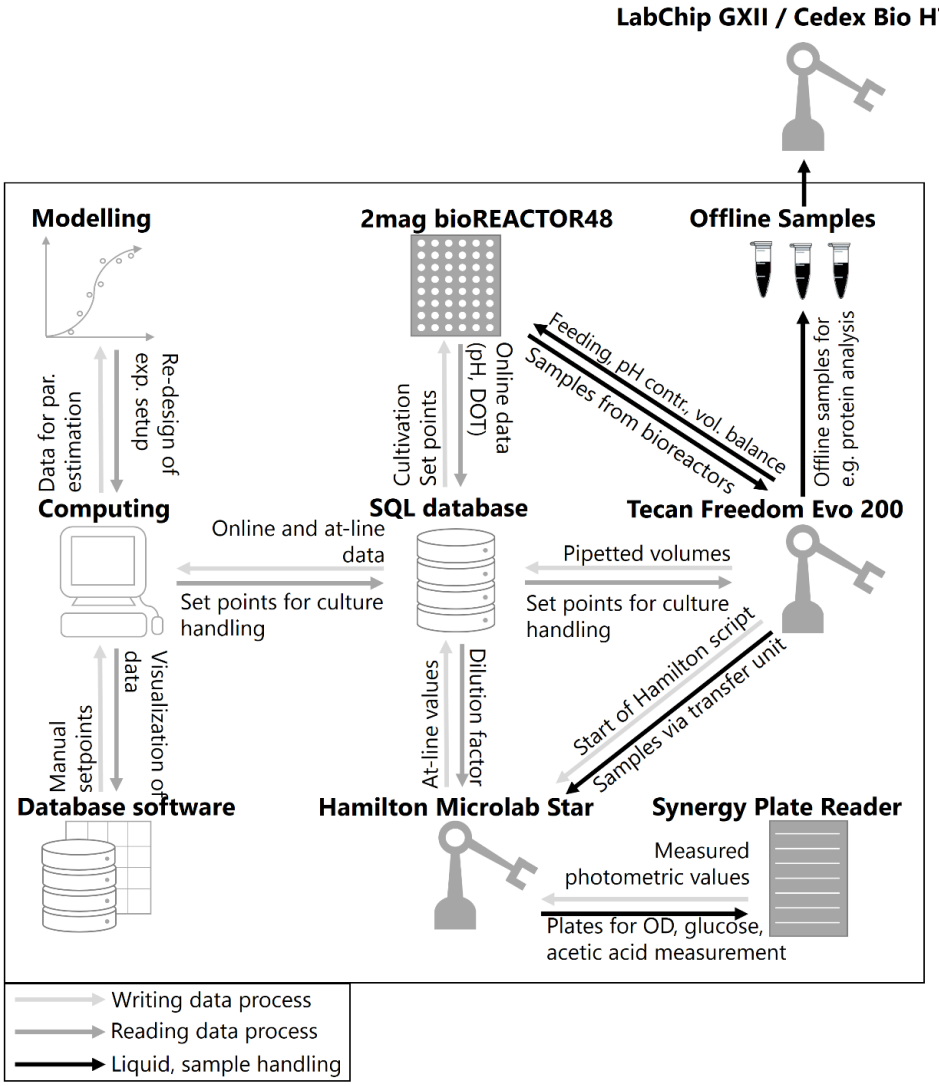

Figure 3 Representation of the schematic data exchange via the centralized database with end-to-end data communication over the entire robotic platform. On the one hand, the database is used for saving the data generated during the experiment as pH, dissolved oxygen tension and stirring speed. On the other hand it is used as an interface between the manual or computational control of the liquid handlers and the 2mag bioREACTOR 48 system. For the data transfer different software parts need to interact as the database software, LabView applications, Python scripts, model-based Matlab scripts and the corresponding liquid handler software.

4.2.3.2 Scheduling of automated experimental procedure

Scheduling of the automated liquid handling steps is a crucial part during the development of such a platform, since a lot of actions need to be performed within a limited amount of time. It needs to be evaluated which steps in which intervals should be performed for a steady culture handling. A script was developed, which meets the requirements for overall culture handling without longer breaks for sampling or at-line analysis (Figure 4A). For the addition of substrate an interval of 5 min was chosen, this ensures a semi-continuous feeding over the cultivation. After the start of the script in the Tecan Freedom Evo software, prior to the handling of the

sterile liquids, the fixed steel needles are sterilized. For sterilization the steel needles are treated twice with 70% ethanol during the process ethanol is aspirated and the needles placed inside the ethanol trays. Through this, the needles are sterilized from the inside as well as from the outside for 20 sec. After each ethanol handling step the residual liquid is blown out by an air dispense step. To avoid any carry-over of the ethanol to the cultures the needles are washed with sterile deionized water subsequently.

In the Block 0, all the necessary steps for culture handling are integrated as feeding, pH control and volume balance. Block 0 is designed so that the execution takes 5 minutes, it can be repeated any number of times before block 1, which contains the sampling, is triggered (Figure 4A). The duration of the specific liquid handling steps can differ depending on the number of bioreactors, which are controlled. Thus, each subroutine is ended by a timer to ensure that the timeframe is kept constant regardless of the experimental handling. Each time a Block is started and before every sampling the needles are sterilized, after the handling of each liquid, as base or glucose, the needles are washed with sterile deionized water. In this way, any carry over between the liquids is avoided. Sampling could be repeated in a grid of 5 minutes. Here it could be chosen independently between at-line (Block 1a) and offline sampling (Block1b). One limitation here is the analysis protocol on the second LHS, which takes 45 minutes for OD, fluorescence, glucose and acetate. As an example workflow Block 0 and Block 1 can be repeated regularly in a 10 min interval and 5 columns are collected before the sample plate is transferred for analysis (Figure 4B). In each sampling procedure one column (i.e. 8 reactors) of the MBR system is sampled. With respect to the analysis time of the at-line measurements this cycle is performed five times (Block 0 & 1 = 50 min). The next sampling cycle starts with the remaining column of the MBR system and proceeds again from the first column. Due to this modular interplay of subroutines both in process control (Block 0 & 1) and at the at-line analysis, the experiments can be quickly and easily adapted to new requirements. The separation of process control and at-line analysis clears a lot of robotic capacity on the Tecan liquid handler a steady culture handling is ensured, while at the same time maintaining the possibility of reducing, expanding or convert the at-line analyses.

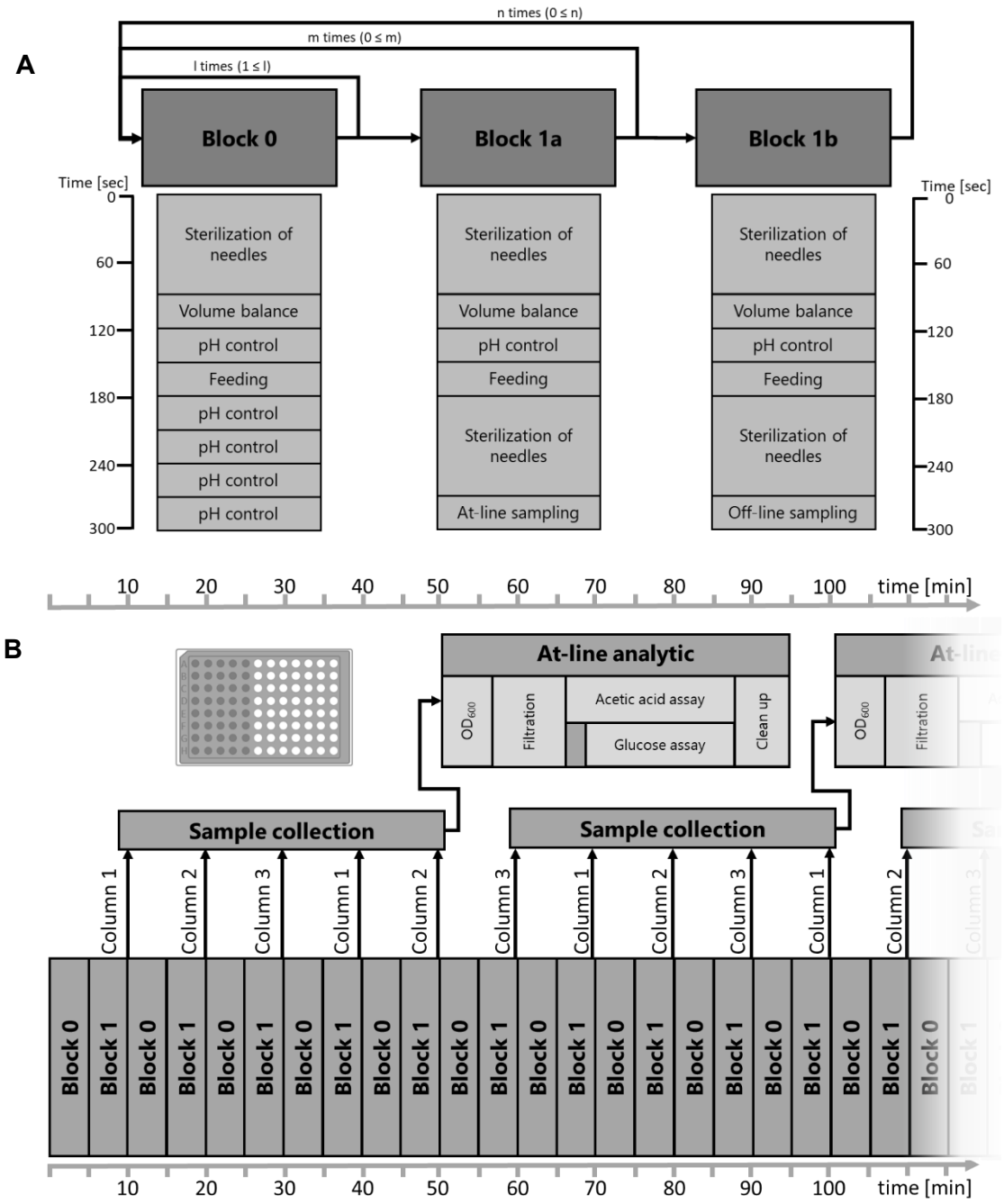

Figure 4 Schematic procedure of the culture handling in combination with sample handling. In figure 4A Combination of the liquid handling steps into blocks. In Figure 4B an exemplary experimental process is descripted. The script starts with the Block 0, which contains sterilization of the needles and all steps necessary for culture handling. After n cycles of Block 0 the Block 1 is entered, which combines culture handling with sampling procedure. Each time the Block 1 is executed one column of the MBR system is sampled, after five runs the sampling plate is forwarded to the second LHS for at-line analysis. Afterwards a new sampling plate is provided by the robot and the scheduled procedure is repeated. Thereby, the repletion of Block 0 could be one or higher and defines the sampling frequency(n=1; sampling every 10 min; n=2 sampling every 15 min and so on). The next sampling cycle starts with the remaining column in the MBR and proceeds again with the first column of the sampling plate. In figure 4B the time intervals, each Block and the individual subroutines take to be executed, are depicted.

4.2.3.3 Experimental results

As a proof of concept, a HT fed-batch cultivation of *E. coli* BW25113 expressing recombinant mini-proinsulin under the Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducible tac-promoter, is shown. The cultivation was performed with 24 MBRs and pulse-based feeding with different intervals, as a scale-down approach. The data of only six of the MBRs are depicted, to maintain a good visualization of the data (Figure 5A).

The batch phase was inoculated to an OD_{600} of 0.1 with an initial glucose concentration of five g L^{-1} in mineral salt media, prepared as previously shown 18. The peaks in the DOT profiles during the batch phase were caused by rises in the stirring speed, until 2400 rpm, in response to low DOT concentrations. The pH was controlled at a set point of 7 with a tolerance of 0.1, so that as soon as the measured pH dropped below 6.9 for one of the bioreactors, the pH controller automatically interacted. Over the course of the cultivation the pH dropped for some cultivations below 6.9, since the pH controller could not compensate for the produced acetate. Therefore, the amount of base per addition was increased from 10 µL to 50 µL at 12 h.

The batch-phase lasted around five hours and an OD_{600} of 6.74±0.5 was reached for the six shown bioreactors. This shows a good batch-to batch reproducibility for the parallel cultivations. After the exhaustion of the substrate, which results in a steep DOT increase, the pH increased gradually to 7.2 with a steady decline in DOT. This indicates the consumption of the acetate, which was produced as a result of substrate overflow in the batch-phase. At 6 h and 13 min the pulse-based exponential fed-batch phase was started, where a glucose pulse was added by the Tecan LHS every 5 min for MBR 1-3 and every 10 min for MBR 4-6. The size of the glucose pulses was derived from a time-based integration of a continuous exponential feed that was calculated based on the physiological state of the culture at the end of the batch phase (OD600 and residual glucose). The glucose pulses caused a cycling of the DOT and pH, due to the changes in the metabolism of the cells in response to the availability or limitation of glucose. The drop in the DOT increased with the amount of substrate added per addition and the growing biomass, therefore the stirrer speed was increased to 2600 rpm at 8 h and 18 min to avoid anoxic conditions. During the exponential growth phase a µ of 0.925±0.05 (Bioreactor 1-3) and 0.75±0.1 (Bioreactor 4-6) was reached. Despite the different pulse intervals the cumulative amount of substrate added to the bioreactors was the same over the course of the cultivation. After 9 h and 13 min the feed was switched to constant feed by maintaining the last value of the exponential phase for every subsequent substrate addition.

Moreover, the cells were induced with an IPTG concentration of 0.5 mM (Bioreactor 4-6) and 1 mM (Bioreactor 1-3), which is indicated by the vertical, black dotted line (Figure 5A and 5B). The glucose and acetate measurements were performed after the cultivation with the

Cedex Bio HT analyzer, since no readjustment of the experiment was performed. The cultivations with

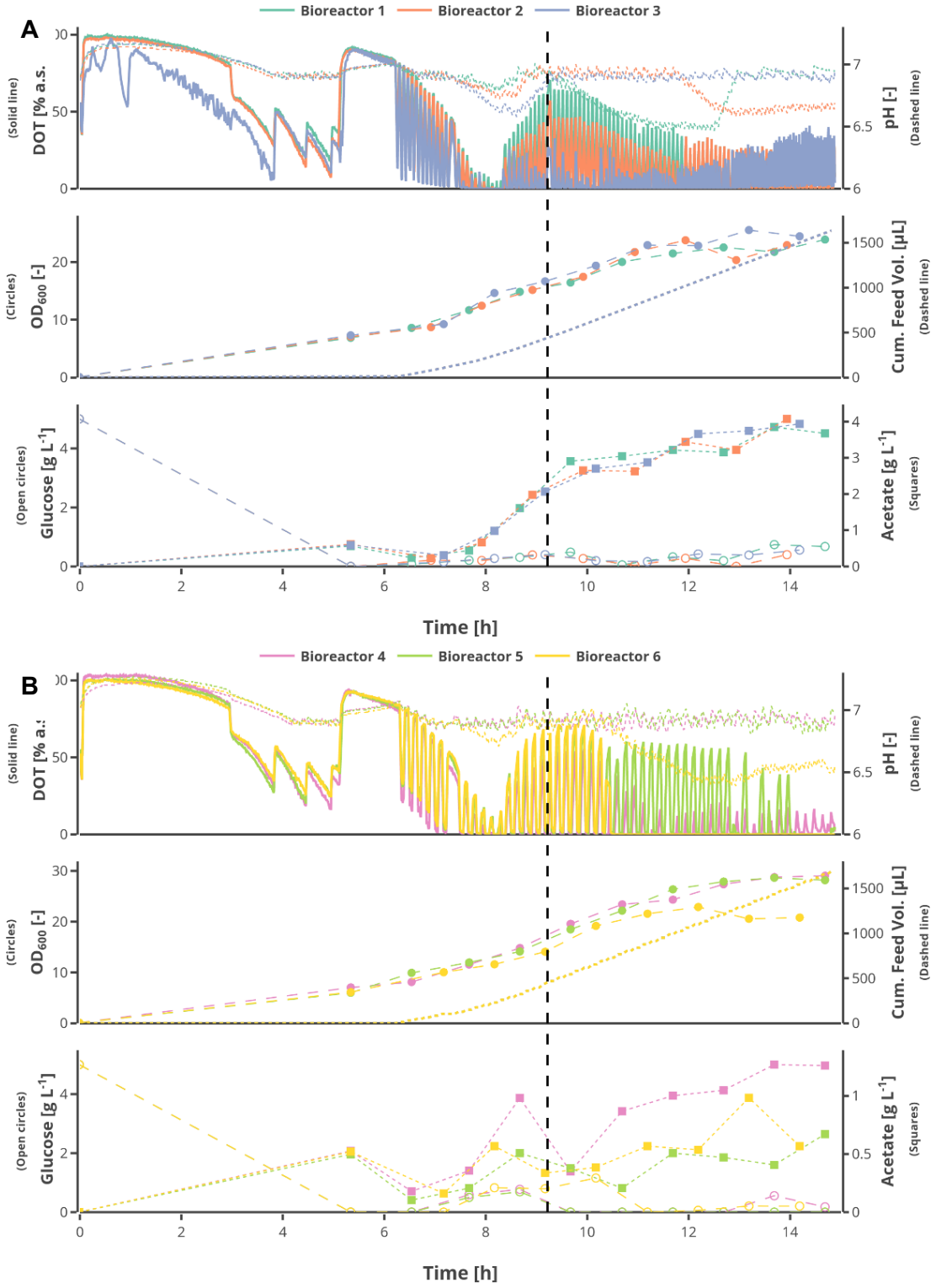

Figure 5 Measured online, at-line and offline values of the *E. coli* BW25113 cultivation with a batch-phase, an exponential fed-batch phase and a production phase with constant feed are shown for six of the used 24 MBRs. The feed additions are performed in a pulse-based form with different interval length in between. The vertical dashed black line indicates the induction with 0.5 mM (Bioreactor 4-6) and 1 mM IPTG (Bioreactor 1-3). The online measured values are DOT and pH, the at-line measurements are OD600, and glucose and acetic acid concentration were measured offline, analysis was performed with the Cedex Bio HT analyzer. The bioreactors with 5 min (A) and 10 min (B) pulse-based intervals are shown.

the 5 min pulses were run under substrate limitation, as can be seen by the glucose measurements; nevertheless, acetate accumulated until a plateau of 3-4 g L^{-1} was reached. Within the cultivations with the 10 min pulses the glucose varied up to 1.16 g L^{-1}, but went back to limitation towards the end of the cultivations. Acetate concentration increased from 0 up to 1.26 g L^{-1} towards the end of the concentration. This is around three times lower compared to the five minute pulses and can be explained by the longer time for glucose and acetate consumption between the pulses.

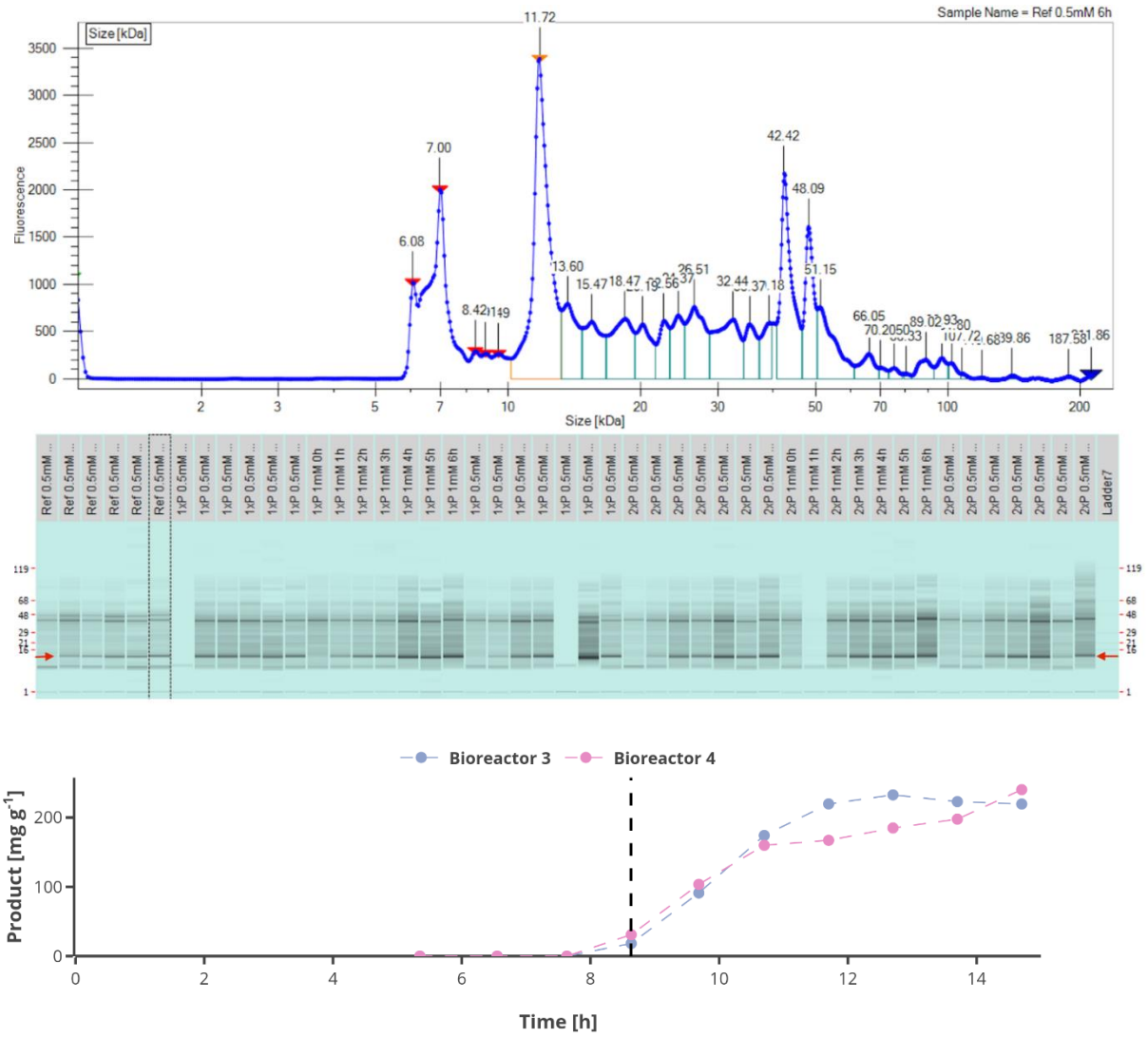

Figure 6 Results of HT protein analysis using the technique of capillary gel electrophoresis. Figure 6A depicts an electropherogram of a sample from an *E. coli* cultivation 6 h after induction, showing the peak of the recombinant protein of interest (orange arrow) at 11.72 kDa. Figure 6B shows a virtual gel of 48 samples, which were taken over the course of the cultivation. They were quantified in HT manner with the LabChip GXII, the band of the recombinant protein of interest is indicated by red arrows. In figure 6C Product formation before and after induction with isopropyl β-D-1-thiogalactopyranoside for two mini bioreactors is exemplarily depicted.

Exemplarily the electropherogram of the HT protein analysis with the LabChip GXII Touch HT from one MBR 6 h after induction is shown (Figure 6A), whereby the protein of interest is indicated by an orange arrow. The area under each peak is integrated and compared to the peak area of protein standards, by which the quantity of each protein can be determined. For each measurement, a virtual gel sample is created, which allows for visual comparison between different samples induced with different IPTG concentrations (Figure 6B). It can be seen that the proteins are separated and the protein of interest can be clearly detected in all samples. By this analysis, the dynamics of the product formation could be followed over the cultivation time as exemplarily shown for two MBRs (Figure 6C). Apart from the analysis, the production of the recombinant protein can be seen in the microscopic pictures taken alongside the cultivation (Figure 7).

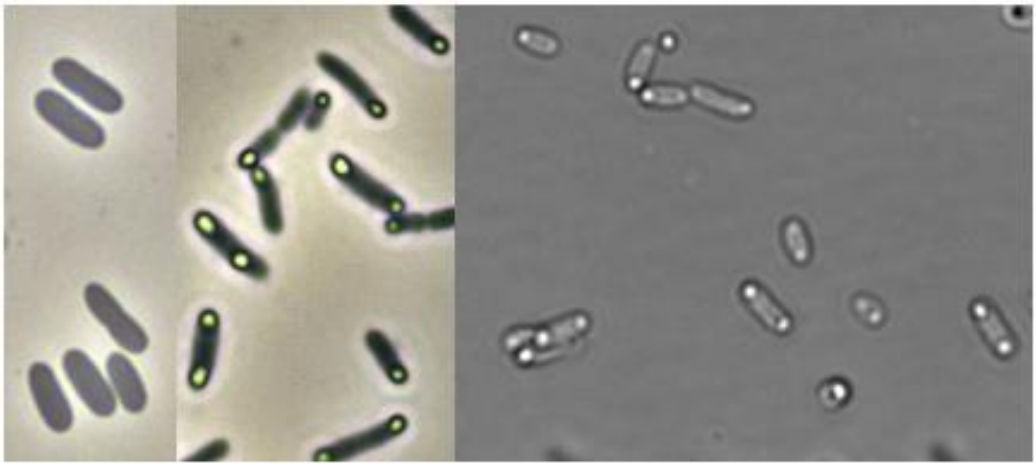

Figure 7 Depicted are microscopic pictures of the *E. coli* BW25113 fed-batch cultivation before induction (left) and three hours after induction (middle and right). The produced recombinant protein after induction can be seen by the inclusion body formation at each site of the microorganism.

4.2.4 Discussion

To our knowledge, this is the first MBR platform that enables fully automated process handling and at-line sample analysis in combination with online model-based control options. The parallel cultivations are performed in milliliter scale under well controlled conditions generating high density data. The experimental setup of the cultivations is designed to mimic oscillating process conditions as present in large scale. The fast at-line analysis of the samples allows for a good monitoring of the process, adaptive input design strategies and progressive characterization of strains phenotype during the experiment. Adaptation and implementation of the operating strategy is possible due to a fast data processing and device communication through the central data storage. The combination of two liquid handlers with modular, well-structured scripts and bidirectional communication drastically increases the sophistication,

capacities, and throughput of the robotic facility. By this, a well-defined culture handling with fast at line analysis is possible tackling the bottleneck of sample analytics from parallel microliter scale cultivations [14].

The modular setup of the developed robotic MBR platform promotes the extension of the at line capabilities by a facilitated integration of new HT at-line analytical devices into the existing software framework.

The development of similar platforms requires an interdisciplinary work between engineering, laboratory automation, liquid handling, software development and mathematical modeling.

The feasibility of 48 microbial cultivations in parallel under well-defined conditions with different feeding strategies was demonstrated over a course of 48 h of cultivation [49]. The application of the platform for the cultivation of different microorganisms, such as *E. coli* and Saccharomyces cerevisiae (*S.cerevisiae*) [49] showed the versatility and flexibility of the developed platform. In the combination with model-based operations complex bioprocess engineering applications, such as scale-down studies [35] and fast strain phenotyping for optimal screening designs were also performed [36].

The platform creates an interface between HT small-scale screenings with low-controlled conditions and the labor intensive well-controlled laboratory scale bioreactors with a low throughput. Through the application of industrially relevant process conditions, failures during development, due to unrepresentative experimental conditions for further scale-up can be avoided.

The use of a central database to connect the devices in the integrated framework requires standard communication protocols (e.g. OPC) which are missing in biotech laboratories. Efforts in that direction are made by the SiLA consortium with the SiLA2 interfaces allowing for multi-user access to devices on simplified, easy to understand commands and with unified data formats [50]. Further applications, as laboratory information management systems (LIMS) and electronic laboratory notebook (ELN) systems, should also be integrated into automated laboratories devices for a holistic data management.

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Conflicts of Interest

The authors declare no conflict of interest.

4.2.5 References

1. Siurkus, J.; Panula-Perälä, J.; Horn, U.; et al. 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. 2010, 9, 1–17.
2. Neubauer, P.; Cruz Bournazou, M. N.; Glauche, F.; et al. Consistent Development of Bioprocesses from Microliter Cultures to the Industrial Scale. Eng. Life Sci. 2013, 13, 224–238.
3. Hemmerich, J.; Noack, S.; Wiechert, W.; et al. Microbioreactor Systems for Accelerated Bioprocess Development. Biotechnol. Bioeng. 2018, 13, 1–9.
4. Islam, R. S.; Tisi, D.; Levy, M. S.; et al. Scale-Up of Escherichia Coli Growth and Recombinant Protein Expression Conditions From Microwell to Laboratory and Pilot Scale Based on Matched k L A. Biotechnol. Bioeng. 2008, 99, 1128–1139.
5. Junker, B. H.; Wang, H. Y. Bioprocess Monitoring and Computer Control : Key Roots of the Current PAT Initiative. 2006.
6. Scheidle, M.; Jeude, M.; Dittrich, B.; et al. High-Throughput Screening of Hansenula Polymorpha Clones in the Batch Compared with the Controlled-Release Fed-Batch Mode on a Small Scale. FEMS Yeast Res. 2010, 10, 83–92.
7. Schäpper, D.; Alam, M. N. H. Z.; Szita, N.; et al. Application of Microbioreactors in Fermentation Process Development: A Review. Anal. Bioanal. Chem. 2009, 395, 679–695.
8. Funke, M.; Diederichs, S.; Kensy, F.; et al. The Baffled Microtiter Plate : Increased Oxygen Transfer and Improved Online Monitoring in Small Scale Fermentations. Biotechnol. Bioeng. 2009, 103, 1118–1128.
9. Duetz, W. A. Microtiter Plates as Mini-Bioreactors : Miniaturization of Fermentation Methods. TRENDS Microbiol. 2007, 15, 469–475.

10. Buchenauer, A.; Hofmann, M. C.; Funke, M.; et al. Micro-Bioreactors for Fed-Batch Fermentations with Integrated Online Monitoring and Microfluidic Devices. Biosens. Bioelectron. 2009, 24, 1411–1416.

11. Neubauer, P.; Junne, S. Scale-down Simulators for Metabolic Analysis of Large-Scale Bioprocesses. Curr. Opin. Biotechnol. 2010, 21, 114–121.

12. Dörr, M.; Fibinger, M. P. C.; Last, D.; et al. Fully Automatized High-Throughput Enzyme Library Screening Using a Robotic Platform. Biotechnol. Bioeng. 2016, 113, 1421–1432.

13. Rohe, P.; Venkanna, D.; Kleine, B.; et al. An Automated Workflow for Enhancing Microbial Bioprocess Optimization on a Novel Microbioreactor Platform. Microb. Cell Fact. 2012, 11.

14. Unthan, S.; Radek, A.; Wiechert, W.; et al. Bioprocess Automation on a Mini Pilot Plant Enables Fast Quantitative Microbial Phenotyping. Microb. Cell Fact. 2015, 14, 1–11.

15. Blesken, C.; Olfers, T.; Grimm, A.; et al. The Microfluidic Bioreactor for a New Era of Bioprocess Development. Eng. Life Sci. 2016, 16, 190–193.

16. Horinouchi, T.; Minamoto, T.; Suzuki, S.; et al. Development of an Automated Culture System for Laboratory Evolution. J. Lab. Autom. 2014, 19, 478–482.

17. Lemoine, A.; Delvigne, F.; Bockisch, A.; et al. Tools for the Determination of Population Heterogeneity Caused by Inhomogeneous Cultivation Conditions. J. Biotechnol. 2017, 251, 84–93.

18. Anane, E.; Sawatzki, A.; Neubauer, P.; et al. Modelling Concentration Gradients in Fed-Batch Cultivations of E. Coli -towards the Flexible Design of Scale-down Experiments. J. Chem. Technol. Biotechnol. 2018, 94, 516–526.

19. Grünberger, A.; Wiechert, W.; Kohlheyer, D. Single-Cell Microfluidics: Opportunity for Bioprocess Development. Curr. Opin. Biotechnol. 2014, 29, 15–23.

20. Neubauer, P.; Junne, S. Scale-up and Scale-down Methodologies for Bioreactors. In: “Bioreactors: Design, Operation and Novel Applications”; CF., M., Ed.; Wiley-VCH, 2016.

21. Neubauer, P.; Cruz Burnazou, M. N. Continuous Bioprocess Development: Methods for Control and Characterization of the Biological System. In Continuous Biomanufacturing: Innovative Technologies and Methods; G., S., Ed.; Wiley-VCH, 2017.

22. Hertzberg, R. P.; Pope, A. J. High-Throughput Screening: New Technology for the 21st Century. Curr. Opin. Chem. Biol. 2000, 4, 445–451.

23. Agresti, J. J.; Antipov, E.; Abate, A. R.; et al. Ultrahigh-Throughput Screening in Drop-Based Microfluidics for Directed Evolution. Proc. Natl. Acad. Sci. 2010, 107, 1–6.

24. Becker, S.; Schmoldt, H. U.; Adams, T. M.; et al. Ultra-High-Throughput Screening Based on Cell-Surface Display and Fluorescence-Activated Cell Sorting for the Identification of Novel Biocatalysts. Curr. Opin. Biotechnol. 2004, 15, 323–329.

25. Ostafe, R.; Prodanovic, R.; Nazor, J.; et al. Ultra-High-Throughput Screening Method for the Directed Evolution of Glucose Oxidase. Chem. Biol. 2014, 21, 414–421.

26. TWB and Hamilton develop a unique and custom made microbial culture robot , utilizing cutting-edge technology for the field of industrial biotechnology High throughput culture platform.

27. Betts, J. I.; Doig, S. D.; Baganz, F. Characterization and Application of a Miniature 10 ML Stirred-Tank Bioreactor, Showing Scale-down Equivalence with a Conventional 7 L Reactor. Biotechnol. Prog. 2006, 22, 681–688.

28. Bareither, R.; Pollard, D. A Review of Advanced Small-Scale Parallel Bioreactor Technology for Accelerated Process Development : Current State and Future Need. 2011.

29. Wu, T.; Zhou, Y. An Intelligent Automation Platform for Rapid Bioprocess Design. J. Lab. Autom. 2014, 19, 381–393.

30. Freier, L.; Hemmerich, J.; Schöler, K.; et al. Framework for Kriging-Based Iterative Experimental Analysis and Design: Optimization of Secretory Protein Production in Corynebacterium Glutamicum. Eng. Life Sci. 2016, 16, 538–549.

31. Haby, B.; Glauche, F.; Hans, S.; et al. Stammcharakterisierung Mittels On-Line-Redesign von Experimenten. BIOspektrum 2018, 24, 39–42.

32. Nickel, D. B.; Cruz Bournazou, M. N.; Wilms, T.; et al. Online Bioprocess Data Generation, Analysis and Optimization for Parallel Fed-Batch Fermentations at ML Scale. Eng. Life Sci. 2016, 1–7.

33. Cruz Bournazou, M. N.; Barz, T.; Nickel, D. B.; et al. Online Optimal Experimental Re-Design in Robotic Parallel Fed-Batch Cultivation Facilities. Biotechnol. Bioeng. 2017, 114, 610–619.

34. Barz, T.; Sommer, A.; Wilms, T.; et al. Adaptive Optimal Operation of a Parallel Robotic Liquid Handling Station. IFAC-PapersOnLine 2018, 51, 765–770.

35. Anane, E.; Haby, B.; Hans, S.; et al. Scaling down Further: Model-Based Scale-down Studies in Minibioreactors. New Biotechnol. 2018, 44, 60–61.

36. Hans, S.; Anane, E.; Glauche, F.; et al. Optimal Conditional Screening in High-Throughput Bioprocess Development. Chemie Ing. Tech. 2018, 90, 1224–1224.

37. Schmid, I.; Aschoff, J. A Scalable Software Framework for Data Integration in Bioprocess Development. Eng. Life Sci. 2017, 17, 1159–1165.

38. BioPhorum Operations Group Ltd. Biomanufacturing Technology Roadmap. https://www.biophorum.com/wp-con- tent/uploads/2017/06/Executive-Summary.pdf

39. Puskeiler, R.; Kaufmann, K.; Weuster-Botz, D. Development, Parallelization, and Automation of a Gas-Inducing Milliliter-Scale Bioreactor for High-Throughput Bioprocess Design (HTBD). Biotechnol. Bioeng. 2005, 89, 512–523.

40. Weuster-Botz, D.; Puskeiler, R.; Kusterer, A.; et al. Methods and Milliliter Scale Devices for High-Throughput Bioprocess Design. Bioprocess Biosyst. Eng. 2005, 28, 109–119.

41. Kusterer, A.; Krause, C.; Kaufmann, K.; et al. Fully Automated Single-Use Stirred-Tank Bioreactors for Parallel Microbial Cultivations. Bioprocess Biosyst. Eng. 2008, 31, 207–215.

42. Knepper, A.; Heiser, M.; Glauche, F.; et al. Robotic Platform for Parallelized Cultivation and Monitoring of Microbial Growth Parameters in Microwell Plates. J. Lab. Autom. 2014, 19, 593–601.

43. Glauche, F.; Pilarek, M.; Bournazou, M. N. C.; et al. Design of Experiments-Based High-Throughput Strategy for Development and Optimization of Efficient Cell Disruption Protocols. Eng. Life Sci. 2017, 17, 1166–1172.

44. Krause, M.; Neubauer, A.; Neubauer, P. The Fed-Batch Principle for the Molecular Biology Lab: Controlled Nutrient Diets in Ready-Made Media Improve Production of Recombinant Proteins in Escherichia Coli. Microb. Cell Fact. 2016, 15, 110.

45. Krause, M.; Ukkonen, K.; Haataja, T.; et al. A Novel Fed-Batch Based Cultivation Method Provides High Cell-Density and Improves Yield of Soluble Recombinant Proteins in Shaken Cultures. Microb. Cell Fact. 2010, 9, 1–11.

46. Anane, E.; López C, D. C.; Neubauer, P.; et al. Modelling Overflow Metabolism in Escherichia Coli by Acetate Cycling. Biochem. Eng. J. 2017, 125, 23–30.

47. Koltunov, V.; Greenblatt, C. L.; Goncharenko, A. V.; et al. Structural Changes and Cellular Localization of Resuscitation-Promoting Factor in Environmental Isolates of Micrococcus Luteus. Microb. Ecol. 2010, 59, 296–310.

48. Deng, X.; Petitjean, M.; Teste, M. A.; et al. Similarities and Differences in the Biochemical and Enzymological Properties of the Four Isomaltases from Saccharomyces Cerevisiae. FEBS Open Bio 2014, 4, 200–212.

49. Sawatzki, A.; Hans, S.; Narayanan, H.; et al. Accelerated Bioprocess Development of Endopolygalacturonase-Production with Saccharomyces Cerevisiae Using Multivariate Prediction in a 48 Mini-Bioreactor Automated Platform. Bioengineering 2018, 5, 1–20.

50. Bär, H.; Hochstrasser, R.; Papenfuß, B. SiLA: Basic Standards for Rapid Integration in Laboratory Automation. J. Lab. Autom. 2012, 17, 86–95.

4.3 Automated Conditional Screening of Multiple *Escherichia coli* Strains in Parallel Adaptive Fed-Batch Cultivations

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Abstract: In bioprocess development, the host and the genetic construct for a new biomanufacturing process are selected in the early developmental stages. This decision, made at the screening scale with very limited information about the performance in larger reactors, has a major influence on the efficiency of the final process. To overcome this, scale-down approaches during screenings that show the real cell factory performance at industrial-like conditions are essential. We present a fully automated robotic facility with 24 parallel mini-bioreactors that is operated by a model-based adaptive input design framework for the characterization of clone libraries under scale-down conditions. The cultivation operation strategies are computed and continuously refined based on a macro-kinetic growth model that is continuously re-fitted to the available experimental data. The added value of the approach is demonstrated with 24 parallel fed-batch cultivations in a mini-bioreactor system with eight different *Escherichia coli* strains in triplicate. The 24 fed-batch cultivations were run under the desired conditions, generating sufficient information to define the fastest-growing strain in an environment with oscillating glucose concentrations similar to industrial-scale bioreactors.

Own Contributions: conceptualization, methodology, software, validation, formal analysis, investigation, resources, data curation, writing—original draft, visualization, project administration, SH

Author Contributions: Conceptualization, MNCB and SH; methodology, SH; software, SH and BH; validation, SH and MNCB.; formal analysis, SH; investigation, SH and MNCB; resources, SH, NK, BH and MNCB; data curation, SH, BH, NK; writing—original draft preparation, SH; writing—review and editing, MNCB, TB, PN, NK; visualization, SH; supervision, MNCB and PN; project administration, SH and MNCB; funding acquisition, PN

4.3.1 Introduction

Emerging technologies in robotic biolaboratories open new opportunities for both high-throughput (HT) screening and HT bioprocess development. On the screening side, significant progress has been made in terms of cultivation scale (down to femtoliter), parallelization and non-invasive observation, which have been widely reviewed [1,2,3]. The focus of this work is conditional screening, where a reduced number of candidate clones are tested under different conditions with the aim to significantly improve the performance at an industrial scale (e.g., media, pH and temperature profiles, bioreactor heterogeneities, induction and feeding strategies). These factors are known to affect the underlying nonlinear dynamics of the bioprocess and are part of the very complex time-dependent interaction between the bioreactor environment and the cell. This highly nonlinear behavior makes it difficult to predict the effect of changes in the cultivating conditions and is responsible for the high failure rate in scale-up [4,5,6]. In order to overcome these challenges, experiments in conditional screening require highly advanced experimental setups able to: (i) operate as similar as possible to the industrial strategy (e.g., fed-batch or continuous cultivations), (ii) mimic the harsh conditions of industrial-scale bioreactors as closely as possible (e.g., growth limitation; bioreactor heterogeneities) and (iii) generate the maximal amount of information possible about the strain's phenotype and its complex dynamic interaction with the process. Many experimental strategies in all scales as well as kinetic and computational fluid dynamics (CFD) modeling approaches focus on this challenge [7,8,9].

The technology to perform parallel experiments with advanced operation in fed-batch or continuous mode has recently become available [10,11,12,13]. Mini-bioreactors (MBR) integrated in liquid handling stations (LHS) allow a large number of parallel cultivations while maintaining the properties of benchtop bioreactors [9]. With working volumes of 2–250 mL, geometric similarities to large-scale reactors [14] and high-frequency measurements and analytics, MBRs have been used for process characterizations [10,12,15] and scale-down studies [16] for up to 48 cultivations in parallel. Such robotic facilities with automated cultivation control, sampling and at-line analytic operations are very powerful systems that can accelerate bioprocess development [11,17,18,19], especially in combination with digital solutions for experiment (re-)planning [20,21,22], data acquisition [11,17] and real-time dynamic analysis [23]. The bottleneck is currently the lack of advanced computer-aided tools to plan the experiments, operate the robots and build the necessary models and digital twins for scale-up and advanced process control. Because of limitations of the planning and operation capacity of humans, much too often, robots are on hold, waiting for the next experiment to be planned, experimental campaigns need to be repeated because of failures that were not detected on

time and the same feeding strategy is used for clones with different characteristics. These are the main issues we address in the present work.

Initial attempts to solve these challenges have demonstrated the added value of model-based tools in terms of accelerating the development process and increasing robustness during scale-up [24]. Nevertheless, the existing solutions are mostly limited to single-strain applications due to the complexity of the used mechanistic models and the difficulty of identifying the parameters for a large number of strains at the same time [25]. Therefore, screening approaches often use simple black-box models for the microorganisms, which do not allow a detailed comparison of their phenotypes.

This contribution proposes an advanced conditional screening design framework and its integration into an autonomously working robotic facility. To achieve this, (i) a macro-kinetic model of the central carbon flux of *Escherichia coli* is defined that can describe the phenotypes of all clones, (ii) a parameter estimation is carried out to cover a characteristic parameter set that describes the individual phenotype and, (iii) based on the unique models, the process is (re)defined in a dynamic process redesign approach as an adapted modelling framework. By this, we not only gain a robust and accurate prediction of the characteristics of each clone but can also quantify and confidently compare their performances. Finally, the method is applied in an online model calibration framework to adaptively define individual optimal feed start and feeding strategies.

During the parallel cultivation of this study, the adaptive framework for conditional screening experiments recursively executes the following steps: (i) collection of cultivation data, (ii) selection of an identifiable parameter (sub)set (sensitivity analysis) for each clone, (iii) estimation of kinetic parameters for each clone, (iv) updating of the optimal feeding profiles and (v) transfer of the new feeding profiles to the database (Figure 1). As a proof of concept, parallel screening experiments with eight different clones, including six knockout mutants of *E. coli* K-12, are conducted in 24 mini-bioreactors. At the start of the experiment, virtually no information on the growth behavior of all these strains was available, as it is common in early conditional screening. From the generated data (of all 24 parallel experiments), it was possible to identify 13 model parameters for all clones, with sufficient accuracy to discriminate the

performance between the clones.

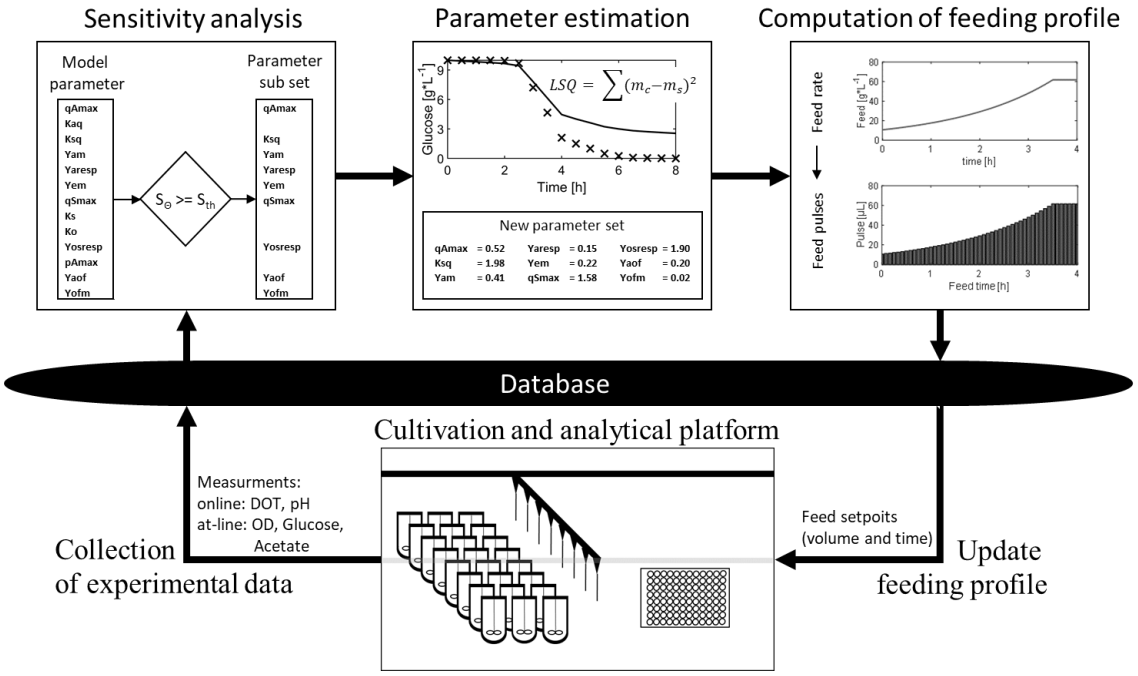

Figure 1: Illustration of the model calibration cycle in the adaptive framework for conditional screening experiments. The cultivation of the clones is performed on the *cultivation and analysis platform* (consisting of two liquid handling stations and a mini-bioreactor system); samples are collected and autonomously analyzed. The generated online and at-line measurements are sent to the central data storage (*database*). The model calibration cycle starts with the collection of all available data. Based on the measurements, the *sensitivity analysis* is performed; based on the results, the identifiable parameters are selected, and non-identifiable parameters are not considered/fixed in the subsequent parameter estimation. In the *parameter estimation*, the identifiable parameter subset is adjusted to fit the model to the measurements. Based on the calibrated model, the feed is calculated during the *feed calculation* step, according to previously defined criteria. The feed is further converted into corresponding pulses with individual times. These time/pulse setpoints are stored in the *database* and executed directly by the *cultivation and analysis platform*.

4.3.2 Materials and Methods

4.3.2.1 HTBD facility

The high-throughput bioprocess development facility is composed of two liquid handling stations (LHS, Freedom Evo 200, Tecan, Switzerland; Microlab Star, Hamilton, Switzerland) and a mini-bioreactor system (48 BioReactor, 2mag AG, Munich, Germany), which is mounted on the Tecan LHS. Both LHSs are connected at the hardware and software level to exchange samples, process and measurement information (Figure 2). The process control (e.g., feed, pH control and volume balance) is carried out by the LHSs in a pulsed based manner. A detailed description of the used hardware and software framework is given in Haby et al. 2019 [17].

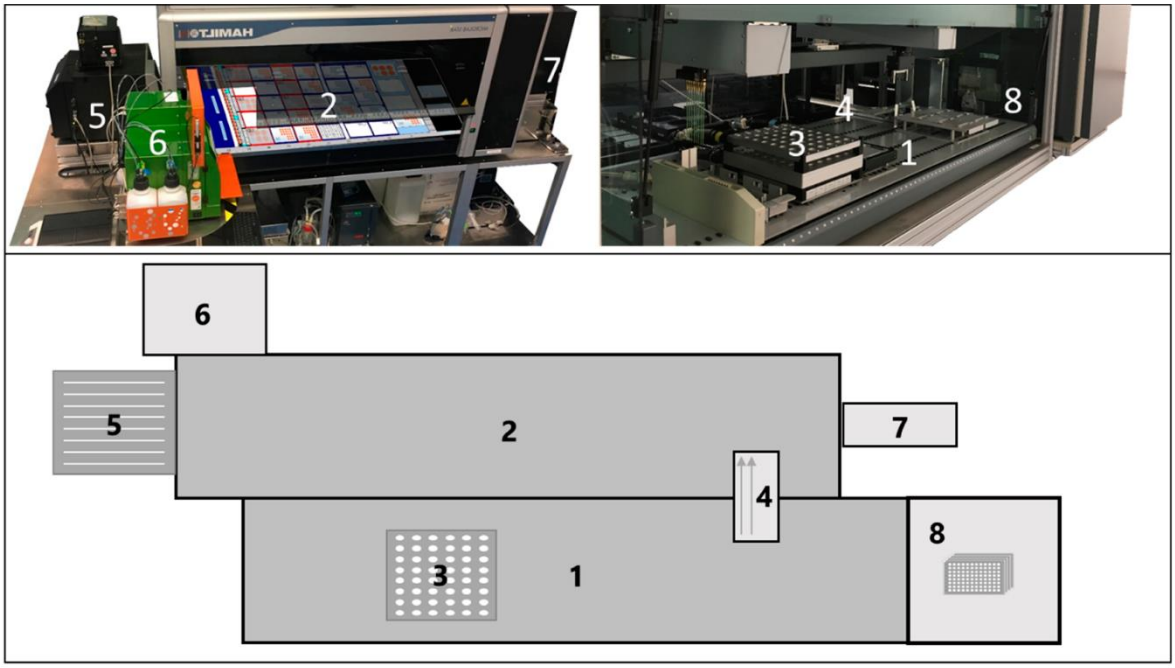

Figure 2: Schematic description of the high-throughput bioprocess development facility used in this study. Composition of the two liquid handling stations (LHS, 1 and 2) and a mini-bioreactor system (3). The liquid handling stations are connected by a linear transfer unit (4) for automatic sample exchange. The facility is surrounded by supporting laboratory equipment (5–8), all accessible by one of the liquid handling stations.

4.3.2.2 Cultivation

Precultures were performed with EnPresso B (Enpresso GmbH, Berlin, Germany) medium with 9 U L−1 Reagent A at 37 °C in a 24-multi-well Oxodish plate to keep the cells in the growth phase (PreSens GmbH, Regensburg Germany). The main culture was started as a batch culture at 37 °C with 5 g L−1 glucose. The initial batch phase was prolonged after 1 h by an additional feed pulse to a final concentration of 5 g L−1 glucose. The stirrer speed was kept constant at 3000 rpm. After the end of the batch phase, a fed-batch was started with a pulse-based glucose feeding every 5 min of feed solution 400 g L−1 glucose dissolved in deionized water. The feeding rate was increased exponentially and switched to a constant feed when the maximum pulse volume of 22 µL was reached. In total, the cultivations were carried out over 8 h with fed-batch phases of 5.4 to 6.1 h, depending on the length of the clone-specific batch phases. The µset for the exponential feed was chosen to be 50% of the model-predicted µmax value and was adapted in every modelling cycle for each clone. The volume of the feed pulses was determined on the basis of the calculated feed rate. All experiments were carried out as biological triplicates.

4.3.2.3 Sampling and Analytic

During the cultivations, pH and dissolved oxygen tension (DOT) were measured online in the mini-bioreactor system. Each column of the bioreactor system was sampled every 45 min in a sequential mode with a sampling interval of 15 min. Samples were inactivated directly with NaOH and stored in 96-well plates at 4 °C on the deck of the LHS until further processing [20]. After 5 samplings, the sampling plates were automatically transferred to the Hamilton LHS for OD_{600}, glucose and acetate measurements in 96-well plates [17]. For the OD_{600} measurements, the samples were diluted to remain in the linear range. The dilution factor was adjusted between 20 and 100 over the course of the cultivation process. All OD_{600} values were multiplied by a correction factor of 2.62 to convert the values to a liquid height of 1 cm. Based on the OD_{600} measurements, the dry cell weight of the biomass was calculated by multiplying the OD_{600} by 0.33 [26]. Due to the time-consuming sampling and analysis procedure, the values for biomass, glucose and acetate were written to the database with a delay of 0.25–1.35 h for the biomass and 0.66–2 h for glucose and acetate, depending on the column of the bioreactor system where the sample was taken.

During the eight hours of cultivation, for each reactor, 1440 values for DOT and pH were collected, as well as 23 samples for biomass (OD_{600}), 20 for glucose and 20 for acetate measurements. This yields, in total, for each reactor, 1503 data points. Considering three replicates, the size of the parameter sensitivity matrix is $(1503\ x\ 18) * 3$

(measurements x parameter) * replicates).

4.3.2.4 Strains

The used strains in this study were *E. coli* K 12 W3110 (F- lambda- IN(*rrnD-rrnE*)1 rph-1), *E. coli* K 12 BW25113 (F-, DE(*araD-araB*)567, lacZ4787(del)::*rrnB-3*, LAM-, *rph-1*, DE(*rhaD-rhaB*)568, *hsdR514*) and six knockout strains obtained from the NBRP at the National Institute of Genetics, Shizuoka, Japan (Keio collection [35]), namely *E. coli* BW25113-JW0554-KC (*ΔompT*), *E. coli* BW25113-JW3975-KC (*ΔaceA*), *E. coli* BW25133-JW1907-KC (*ΔfliA*), *E. coli* BW25133-JW2076-KC (*ΔgatC*), *E. coli* BW25113-JW2082-KC (*ΔgatZ*), *E. coli* BW25133-JW2943-KC (*ΔglcB*).

4.3.2.5 Computational methods

The *E. coli* macro-kinetic growth model [25] consists of 5 ordinary differential equations, describing biomass, glucose, acetate, oxygen and enzymatic glucose release, and represents the major extracellular dynamics of *E. coli*, including the acetic acid overflow. The model contains 18 parameters, from which 13 have been shown to vary with clones and cultivation conditions. All computational methods related to the model calibration and feed calculation

were carried out in MATLAB (The MathWorks, Inc., Natick, Massachusetts, USA), available at https://gitlab.tu-berlin.de/hts_modelling/ModellingFramework. The commit used for this study is efaee5eba813237860264fc33ba79315eef5bbca. Cultivation time and data for the different sequential tasks are summarized in Table 1. All measurements used for the parameter estimation are available in Table S1.

Table 1: Underlying data, i.e. number of analysis of sensor data, for the parameter estimates of one biological triplicate.

Sequential task #	*Cultivation time [h]*	*Available measurements*			
		DOT	*Biomass*	*Glucose*	*Acetate*
1	*1.38*	*321*	*6*	*0*	*0*
2	*1.88*	*411*	*16*	*0*	*0*
3	*2.55*	*531*	*16*	*10*	*10*
4	*3.52*	*705*	*26*	*10*	*10*
5	*3.93*	*780*	*26*	*20*	*20*
6	*5.17*	*999*	*36*	*20*	*20*
7	*5.94*	*1137*	*36*	*30*	*30*
8	*6.91*	*1311*	*46*	*30*	*30*
9	*7.66*	*1440*	*46*	*40*	*40*

4.3.2.6 Parameter estimation

The parameter estimation problem is solved for a reduced (identifiable) parameter subset. This subset is updated in each model calibration cycle in Table 1 and is selected based on the local sensitivity matrix [28]. In doing so, the model calibration updates both the identifiable parameter subset and corresponding parameter values. This approach is useful as the information content in the data increases with each cycle. The algorithm implements a stepwise forward selection of parameters to be included in the estimation problem based on the dynamical parameter sensitivities. Identifiable parameters are selected by a ranking of all parameters according to linear independence and an analysis of the matrix rank condition of the sensitivity matrix.

The parameter estimation is formulated as the following optimization problem:

$$\hat{\theta} \coloneqq \underset{\theta}{\operatorname{argmin}}\, \Phi(U, \theta) \tag{1}$$

where the objective function reads:

$$\Phi(U, \theta) \coloneqq \sum_{i=1}^{5} \frac{1}{N_i} \sum_{j=1}^{N_i} (y_{i,j}(U, \theta) - y_{i,j}^{m})^2 \tag{2}$$

where $y_{i,j}(U, \theta)$ are the simulated, and $y_{i,j}^{m}$ are the corresponding measured states. The index $i = 1, \dots, 5$ indicates the measured variables and the index $j = 1, \dots, N_i$ indicates individual data points. The CVODES solver in SUNDIALS [29] is used to solve the ODE system and the interior-point algorithm is used for optimisation (MATLAB fmincon). Initial values and

lower and upper bounds of the parameter estimation (PE) are based on experts' knowledge and summarized in Table S2.

4.3.2.7 Monte Carlo Simulation

Parameter distribution and pairwise correlations are determined by Monte Carlo simulations based on the last dataset (n = 500) and with the identifiable parameter set based on the subset selection. Monte Carlo simulations were carried out with σ = 0.15 for biomass, glucose and acetate and with σ = 0.05 for DOT.

4.3.2.8 2.5.3. Feed Calculation

The exponential feed was calculated using the standard fed-batch equation [30], which was adapted to consider a pulse-based profile. Since the feed in a fed-batch process is the only major volume-changing factor, volume changes due to sampling are neglected at this point and the volume change could be described as

$$\int_{V_0}^{V} dV = F_0 \int_{t_0=0}^{t} e^{\mu_{set} \cdot t} dt = \frac{F_0}{\mu_{set}} \, e^{\mu_{set} \cdot t}|_0^t \tag{3}$$

With μ_{set} $[h^{-1}]$as the predefined specific growth rate, F_0 $[g\ L^{-1}]$ is the initial feed rate and time t $[h]$. The pulse volume is calculated as

$$V = V_0 + \frac{F_0}{\mu_{set}} (e^{\mu_{set} \cdot t} - 1) \tag{4}$$

with

$$F_0 = \frac{\mu_{set}}{Y_{X/S} * S_i} \, X_0 \, V_0 \tag{5}$$

where $Y_{X/S}$ $[g\ g_x^{-1}]$ is the yield coefficient of glucose per biomass, S_i $[g\ L^{-1}]$ is the glucose concentration in the feed solution, X_0 $[g]$ the biomass concentration and V_0 the volume at the feed start. Volume manipulations by the pipetting robot (e.g. volume balancing, sampling, base addition for pH control) are considered in the feed calculation apart of the equations above.

Biomass and volume for the calculation of F_0 (Equation 5) were estimated by simulations based on the last calibrated model. The end of the batch phase was defined as the time point where the predicted glucose and acetate concentrations were below 0.02 g L^{-1}, but not later than 45 min after the depletion of glucose.

4.3.3 Results

Eight different *E. coli* K-12 clones were cultivated in parallel with an industrial process-relevant feeding design consisting of batch, exponential fed-batch and constant feed phases. The feed was applied as pulses to expose the cells to inhomogeneities similar to those in large-scale bioreactors.

4.3.3.1 Parallel cultivation

The length of the batch phase varied among the clones and lasted 1.65 h for *E. coli* W3110 (the fastest growing clone) and 1.86 h for *E. coli* BW25113 ΔglcB (the slowest growing clone). After the end of the batch phase, the feed was automatically started. Due to the pulse nature of the feed procedure, the feed start is visible through the oscillating DOT values (Figure 3a). These oscillations, as well as the glucose at-line data, prove that glucose limitation was maintained during the fed-batch phase in all cultivations. Furthermore, no significant acetate accumulation was observed (Figure 3b). The cultivations show a low variance between triplicates, which is obvious from the online DOT and pH profiles as well as from the automatically analyzed biomass, glucose and acetate values. As expected, the pH decreased during the batch phase and started to increase after glucose depletion (typically caused by acetate consumption). During each glucose pulse cycle, a perturbation of pH is observed, which is caused by the transient production of acetic acid (Figure 3c). Finally, a small increase in pH was observed after the switch to constant pulse-based feed.

4.3.3.2 Prediction of Batch and Feed start

After inoculation, samples for biomass were taken and, together with the initial parameter set, served as the basis for the first prediction of the batch phase, feed start and feed rate (Figure 4a, black dashed line). The difference in the initial biomass between the clones led to different simulation results and different predicted batch phases prior to the first model calibration. After the second biomass measurement, the first model calibration cycle was initiated after 1.4 h of batch cultivation and the feed start and rate were re-computed using the updated models. From that time onwards, the model calibration cycle was started after each entry of new at-line data (biomass or glucose/acetate). The end of batch was defined as the time point at which glucose as well as acetate (produced during overflow growth) were depleted (Table 2). Therefore, the fed-batch phase in our cultivations started purposely later compared to typical fed-batch processes, which are mostly started when glucose is depleted and the DO signal increases. Note that feeding was started only when acetate had been metabolized. This prevent possible overfeeding with glucose by co-metabolism of the remaining acetate and thus allowed a higher process stability.

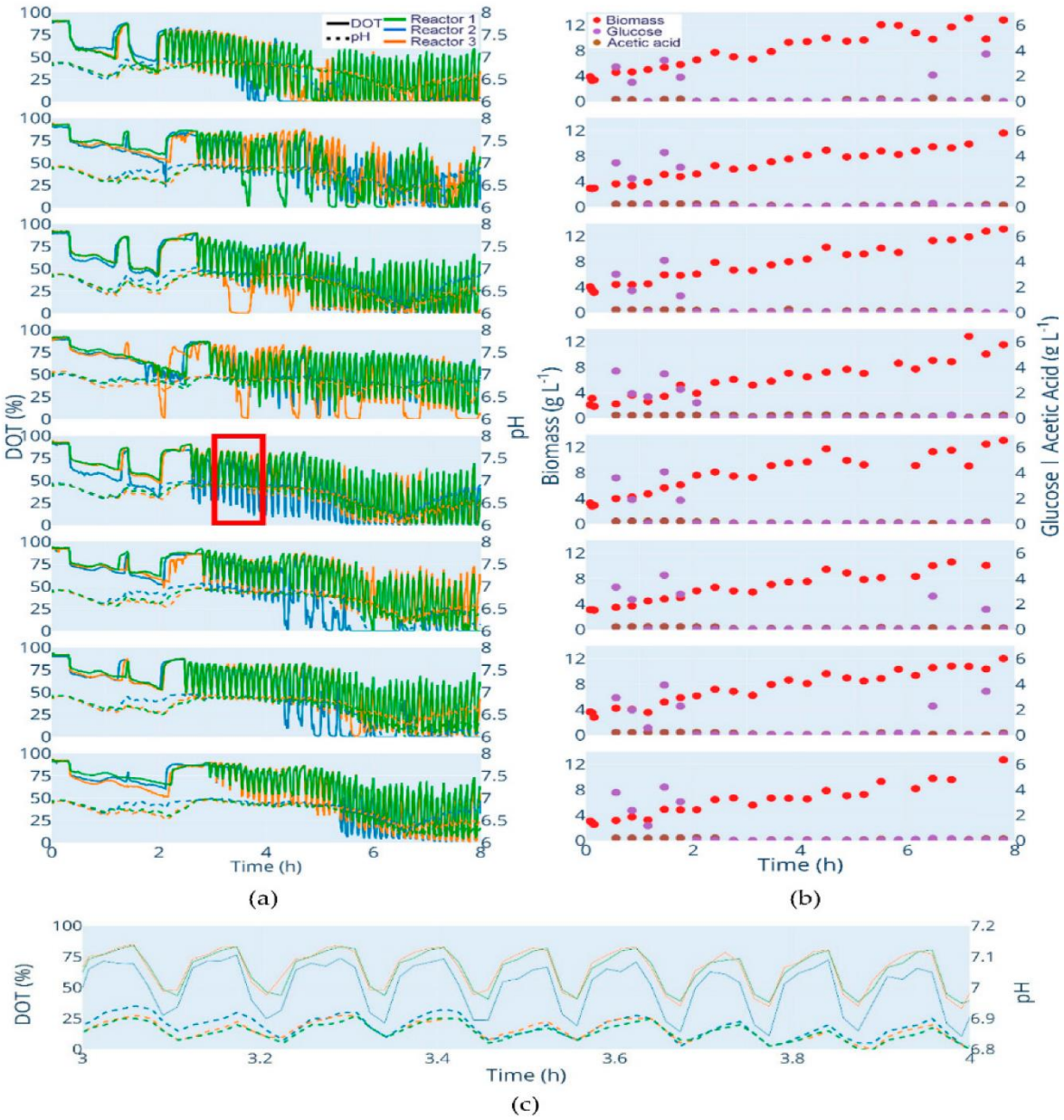

Figure 3. Cultivation data of all strains. Clones from top to bottom: *E. coli* W3110; *E. coli* BW25113; *E. coli* BW25113 Δ*ompT*; *E. coli* BW25113 Δ*aceA*; *E. coli* BW25113 Δ*fliA*; *E. coli* BW25113 Δ*gatC*; *E. coli* BW25113 Δ*gatZ*; *E. coli* BW25113 Δ*glcB*. (**a**) DOT (%): solid lines, pH: dotted lines. (**b**) Biomass (g L^{-1}): red dots; glucose (g L^{-1}): purple dots; acetic acid (g L^{-1}): brown dot. (**c**) Illustration of the oscillating pH values with each glucose pulse. The figure shows the section marked in (**a**) red. An interactive version of (**a**,**b**) is available at http://www.bioprocess.tu-berlin.de/fileadmin/fg187/Publications/Hans_2020/fig2.html.

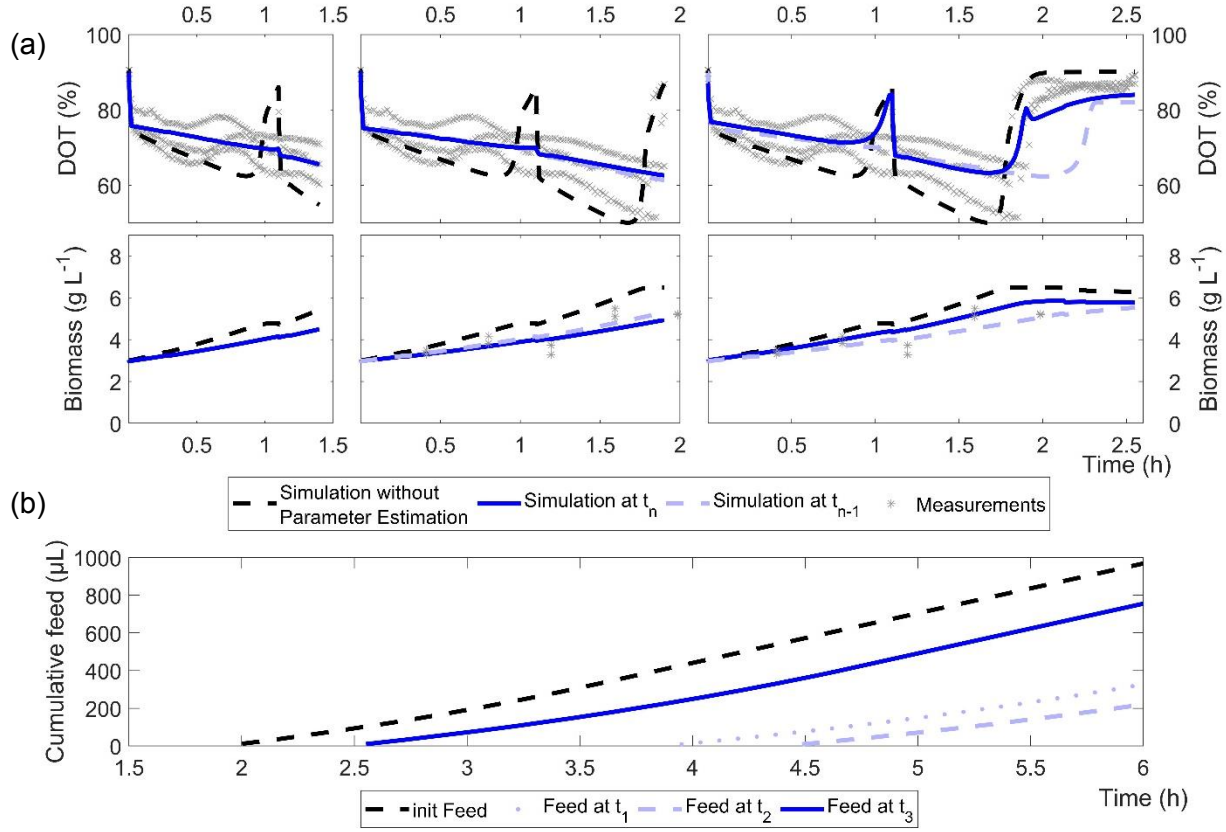

Figure 4: Illustration of the results of the sequential tasks 1 to 3 for the cultures of *E. coli* BW25113 Δ*glcB*. (a): Comparison of parameter estimation results at different times during the cultivation, the initial parameter set and the measurements for strain *E. coli* BW25113 Δ*glcB*; (b): Results for computed feeding profiles after the sequential task 1-3 as cumulative volume.

Table 2: *Batch end prediction overview: initial, adjusted (parameter estimation after 1:52 hour) and observed times for consumption of glucose and acetate and the actual feed start based on the first executed glucose pulse.*

Strain	*Glucose consumption (hh:mm)*			*Acetate consumption (hh:mm)*			*Feed start (hh:mm)*
	initial	*adjusted*	*Observed*	*initial*	*adjusted*	*observed*	
E. coli W3110	*01:46*	*01:40*	*01:39 ± 00:01*	*02:03*	*01:48*	*01:48*	*01:55*
E. coli BW25113	*01:52*	*01:38*	*01:49 ± 00:03*	*02:00*	*03:02*	*> 02:23*	*02:23*
E. coli BW25113 ΔompT	*01:46*	*01:40*	*01:40 ± 00:01*	*01:53*	*03:05*	*02:10*	*02:23*
E. coli BW25113 ΔaceA	*02:13*	*02:11*	*01:48 ± 00:21*	*02:22*	*03:06*	*> 02:37*	*02:37*
E. coli BW25113 ΔfliA	*01:51*	*01:36*	*01:42 ± 00:01*	*01:59*	*02:13*	*02:07*	*02:16*
E. coli BW25113 ΔgatC	*01:49*	*01:39*	*01:46 ± 00:05*	*01:57*	*02:50*	*02:25*	*02:30*
E. coli BW25113 ΔgatZ	*01:46*	*01:43*	*01:43 ± 00:01*	*01:55*	*03:03*	*> 02:09*	*02:09*
E. coli BW25113 ΔglcB	*01:55*	*01:56*	*01:51 ± 00:03*	*02:03*	*02:24*	*02:30*	*02:37*

Figure 4a illustrates the outcome of the model calibration cycle during the batch phase, with the example of the *E. coli* BW25113 *ΔglcB* cultivation data (grey cross) and simulations after model calibration (blue line). It is obvious that the first parameter estimate indicates, for this strain, a slower growth compared to the initial parameter set. However, with each model

calibration cycle, the computed growth rate (μ_{max}) increased from 0.36 h^{-1} at t_1 to 0.58 h^{-1} at t_2 and up to 0.82 h^{-1} at the third shown model calibration cycle. The fit to the cultivation data is improved with each modelling cycle and the trend of the cultivation is well represented, at least after the third modelling cycle.

In addition, due to the underestimated μ_{max}, the first model calibration cycle failed to propose the end of the batch phase properly. An accurate estimation of the specific glucose uptake rate is only reached after the first glucose measurements are available, but then the estimation is very precise. Although the glucose depletion is equally estimated in the third model calibration cycle (1.94 h) and in the initial, unadjusted model (1.92 h; Figure 4a, black dashed line), the feed (Figure 4b) started 21 min later (calibrated model: 2.40 h; initial model: 2.01 h). This is due to differences in the production and consumption rates of acetate, resulting in different starting times of the fed-batch phase. Based on the DOT profiles, acetate is consumed after 2.5 h; this also corresponds well with the at-line measurements of *E. coli* BW25113 *ΔglcB* in Figure 3.

The predicted end of the batch phase is very close to the observed one in all cultivations, even after the second model calibration and 1.5 h of cultivation (Table 2). For some cultivations, the time of glucose depletion was predicted with an accuracy of less than one minute (*E. coli* BW25113 *ΔgatZ*). In the worst case, the glucose depletion was predicted 22.8 min too late (*E. coli* BW25113 *ΔaceA*). A missed batch end and even a short starvation phase could lead to unwanted metabolic reactions by the clone and influence the process and product quality. However, with this triplicate, the variance of glucose uptake is very high, because with one triplicate, the glucose consumption is clearly leading (Figure 3a). Without the leading one, the gap between predicted and observed glucose depletion is significant lower. Nevertheless, a difference of 22 min is still in an operational range for conditional screening. Due to operational reasons, the model calibration with all clones was maintained. The overall mean difference between the observed and predicted glucose depletion is 6.9 min for the calibrated model after 1.5 h and, thus, better, compared to the initial model with a mean prediction error of 7.3 min.

Complete consumption of acetate was only observed for five of the eight clones. For all these clones, the adjusted model predicted the acetic acid consumption to better compare to the initial model, with the exception of *E. coli* BW25113 *Δomp*. Complete consumption of acetic acid was not observed for three clones because of the time-dependent restrictions in the feed start (maximum tolerance between end of glucose depletion and feed start; see Section 2.5.3). Nevertheless, for these three clones, the initial model predicted a faster, and the adjusted model, a slower, acetic acid consumption rate. The times of the first feed pulse are summarized in Table 2 (feed start); the predicted end of batch and the first pulse may differ due to technical

reasons (delay in computation or first pulses are calculated with 0 µL due to the minimal pipetting volume restrictions).

4.3.3.3 Feed and fed-batch

During the fed-batch phase, the size of the feed pulses is re-computed during each model calibration cycle. The maximal glucose uptake rate was determined as basis for the new feed rates. However, the biomass concentration and the substrate yield coefficient ($Y_{X/S}$) have a major impact on the initial feed rate (F_0) and influence the feed as well. With the exception of *E. coli* BW25113 *ΔglcB* (Figure 5h), the first feed rate (grey bars) was higher than the following calculated feed pulses. However, the second applied feed rates for *E. coli* BW25113 *ΔompT*, *E. coli* BW25113 *ΔfliA* and *E. coli* BW25113 *ΔgatZ* (Figure 5c,d,f) were close to the initial feed rates, indicating only a minor parameter drift, but were reduced in the later model calibration cycles. In the case of *E. coli* BW25113 *ΔglcB,* the second feed was somewhat higher than the later one, which is reflected in both the initial feed rate and in the slope of the feed (all feed pulses are summarized in Figure 5). Feed pulses were calculated by the optimization algorithm for each clone and applied to all biological triplicates. In this way, eight different feeding rates were calculated and 24 cultivations were carried out in parallel.

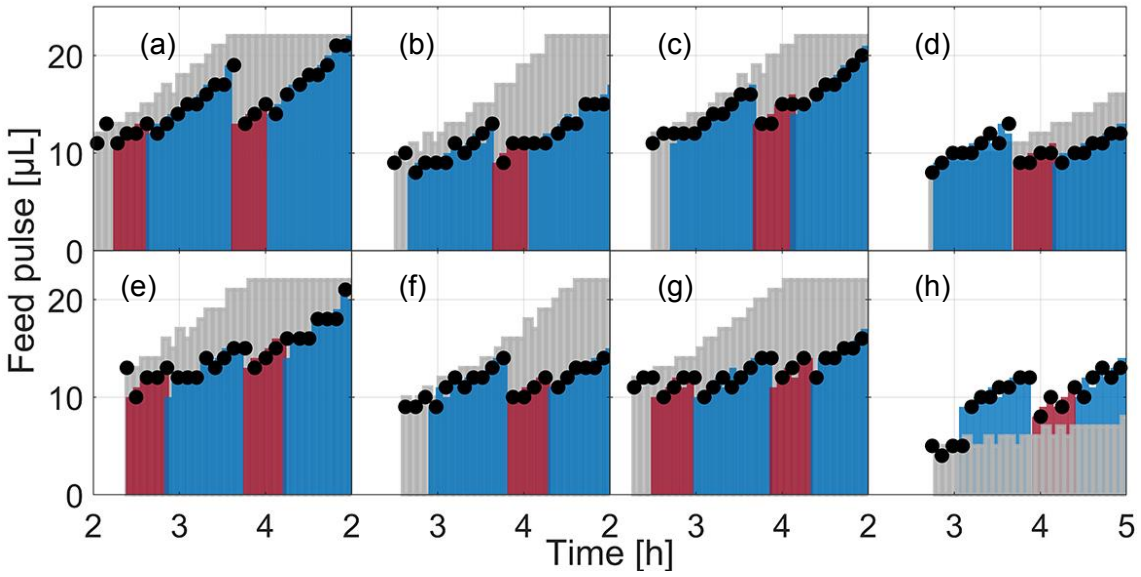

Figure 5. The applied feed and scheduled feeds for each clone between cultivation hours 2 and 5. Black dots: the applied feed based on the real executed feed, logged by the LHS. Light grey bars: scheduled feed at feed start. Colored bars: scheduled feeds in the following modelling cycles; the color change indicates the next modelling cycle. (**a**) *E. coli* W3110; (**b**) *E. coli* BW25113; (**c**) *E. coli* BW25113 Δ*ompT*; (**d**) *E. coli* BW25113 Δ*aceA*; (**e**) *E. coli* BW25113 Δ*fliA*; (**f**) *E. coli* BW25113 Δ*gatC*; (**g**) *E. coli* BW25113 Δ*gatZ*; (**h**) *E. coli* BW25113 Δ*glcB*.

4.3.3.4 Parameter estimation

During all model calibration cycles, the model parameters are estimated on the basis of all available data (Table S1), i.e., all data which were collected from the start of the cultivations to

the actual time point. For all clones, the measurements and dynamics of cultivation are well represented in the simulation of the calibrated model, as illustrated in Figure 6 for the strain *E. coli* BW25113 *ΔglcB* (last modelling cycle; for the other strains, see supplementary Figures S1–S7). In contrast to the calibrated model, the initial model overestimated the biomass formation. This trend could be observed for all strains, with the exception for *E. coli* BW25113 (Figure S2). The DOT measurements indicate a slower glucose uptake rate than predicted in the last modelling cycle. A lower maximal specific glucose uptake rate (qS_{max}) was calculated in the first two modelling calibration cycles compared to the later ones (Figure 7). In the first two model calibration cycles, no glucose measurements were available due to the time delay in the at-line analytics. The prediction error of acetic acid was decreased in the batch and fed-batch phase after model calibration. The cultivation dynamics of all cultivation measurements are well represented by the calibrated model. The parameters to be adjusted in each model calibration cycle are selected by the included subset selection (sensitivity analysis, Figure 1). The parameters $K_{ap,}$ k_La and q_m are not adjusted in one model calibration cycle. This means that the underlying measurement data are not sufficient to determine these model parameters with sufficient certainty, not even in the last model calibration cycle. The model parameters K_o, K_{sq}, Y_{ofm} and Y_{oresp} are only partly selected for parameter estimation (Figure 7, filed dots). Within the subset selection model parameters can influence other's parameter sensitivity if they have dependencies to each other. Accordingly, the selection of one parameter may lead to a lower sensitivity of another one and the latter may be excluded from the subset selection although it was active in previous cycles. All parameter subsets and parameter values are summarized in the Supplementary Tables S3–S10. Regularization of parameter estimation using a subsets selection method (López et al., 2013) was used to ensure a meaningful parameter set and to avoid non-physiological model calibrations..

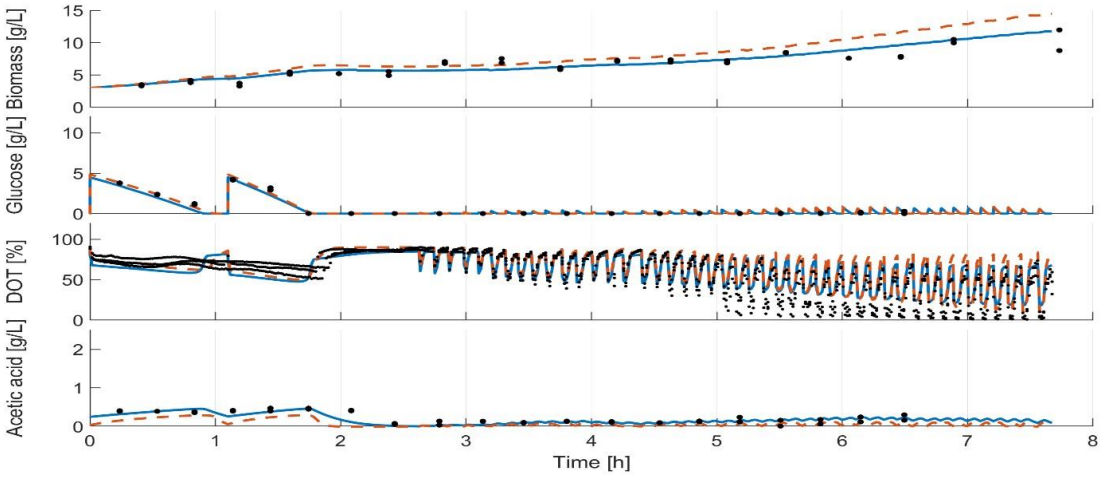

Figure 6. Model for *E. coli* BW25113 *ΔglcB* after the last model calibration cycle and at the beginning of the experiment. Solid line: calibrated model; dashed line: initial model; dots: measurements.

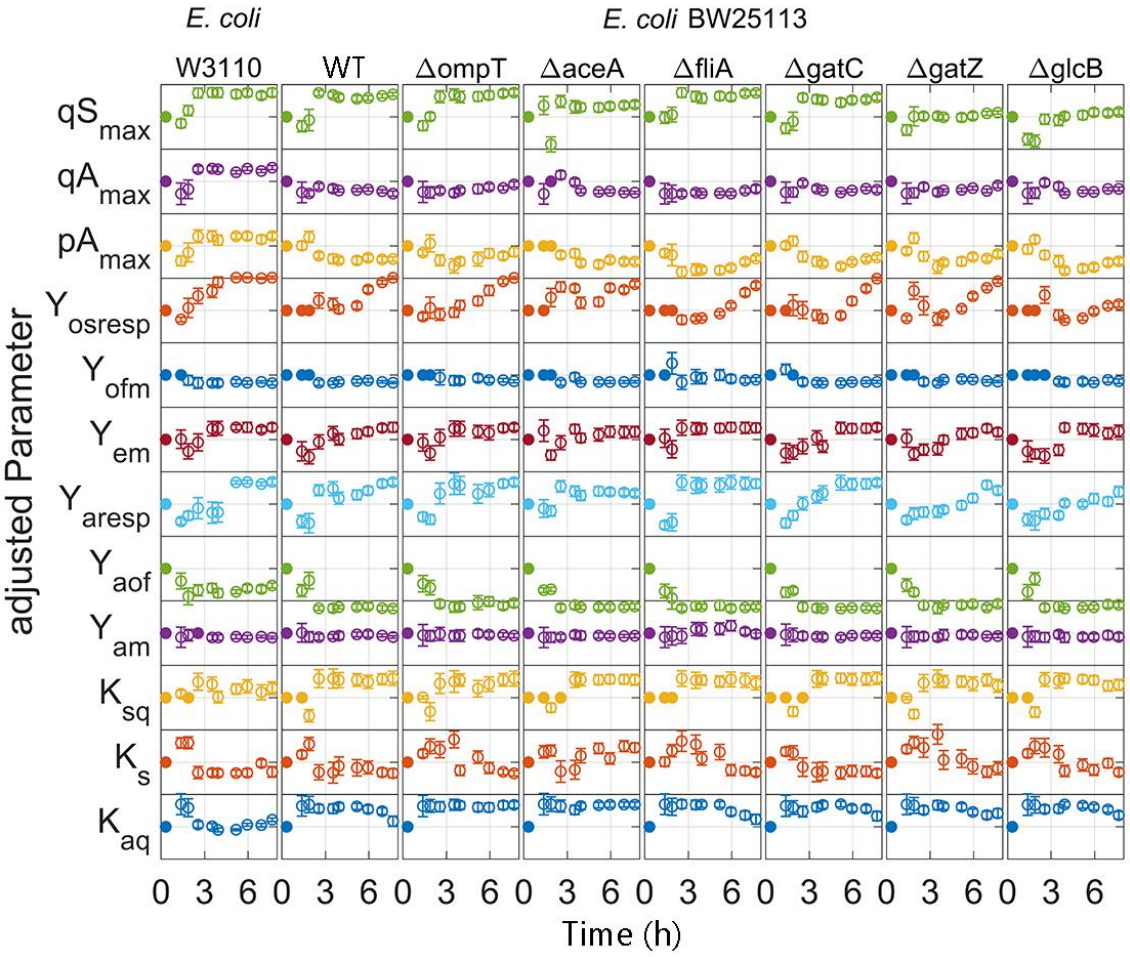

Figure 7: Adjusted parameters of the time for each *E. coli* strain: Parameters are normalized to the initial value and scaled to three times the standard derivation. Filled dots: parameter fixed by the subset selection; open dots: estimated parameter.

Monte Carlo simulations have been shown to give a good insight into actual, non-linear parameter distribution (Krausch et al., 2019) and were therefore performed to gain a better understanding of the parameter correlation and its variances. In Figure 8, the parameter distributions for *E. coli* BW25113 *ΔglcB* (last model calibration cycle) are shown based on Monte Carlo simulations. The correlation between all parameters is very weak. Only K_{aq} and K_{sq} showed a correlation with qA_{max}. The model parameters K_{aq} and K_{sq} are the affinity constants for acetate and glucose uptake, respectively, and a dependence to the maximal acetate uptake rate (qA_{max}) cannot be avoided in the model. The high significance of each parameter is indicated by a narrow distribution in Figure 8 as well as low variation for the most important model parameters (Table 3), especially for the model parameters Y_{em}, qS_{max} and $Y_{osresp.}$ Normal distribution is given for all parameters except for Y_{am}. This parameter is quite close to the lower bound of the previously defined solution space (lower and upper parameter

bound). It is noted that this situation should be avoided as it might reduce the accuracy of the parameter estimates.

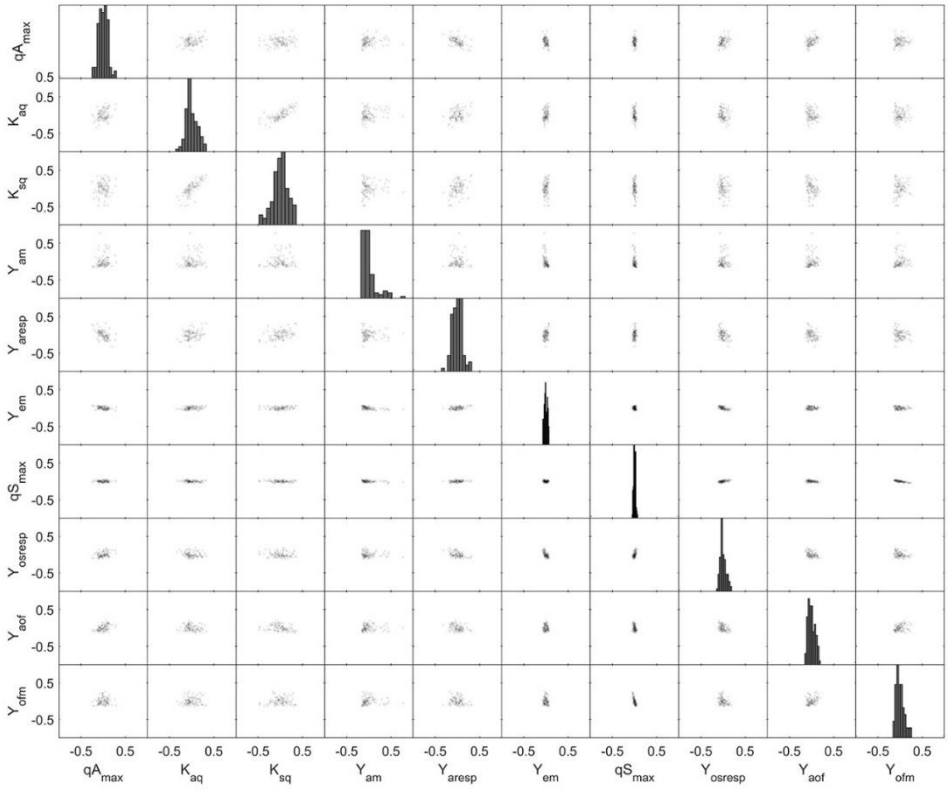

Figure 8. Monte Carlo parameter estimation: pair plots of the 500 best Monte Carlo parameter estimation results and with the identifiable parameter set based on the dataset for *E. coli* BW25113 *ΔglcB* during the last modelling cycle. Monte Carlo simulations were carried out with σ = 0.15 for biomass, glucose and acetate and with σ = 0.05 for DOT.

Table 3: Values, variance and relative variance of the adjusted parameters for all clones after the final model calibration cycle.

Parameter	Unit	inital guess	*E.coli* W3110			*E. coli* BW215113																				
						WT			ΔompT			ΔaceA			ΔfliA			ΔgatC			ΔgatZ			ΔglcB		
			θ	σ_θ	$\%\sigma_\theta$	θ	σ_θ	$\%\sigma_\theta$	θ	σ_θ	$\%\sigma_\theta$	θ	σ_θ	$\%\sigma_\theta$	θ	σ_θ	$\%\sigma_\theta$	θ	σ_θ	$\%\sigma_\theta$	θ	σ_θ	$\%\sigma_\theta$	θ	σ_θ	$\%\sigma_\theta$
qAmax	$g\,g^{-1}\,h^{-1}$	1,0252	1,59	0,11	6,78	0,52	0,11	21,74	0,90	0,05	5,92	0,56	0,15	26,05	0,72	0,11	15,59	0,68	0,08	12,19	0,86	0,10	11,54	0,71	0,07	9,54
Kaq	$g\,L^{-1}$	0,2133	0,59	0,14	23,11	0,55	0,09	16,28	0,98	0,07	7,11	0,98	0,14	13,82	0,60	0,12	20,70	0,68	0,10	14,16	0,75	0,12	15,97	0,70	0,04	6,24
Ksq	$g\,L^{-1}$	1,0667	1,52	0,33	21,71	1,98	0,34	16,98	1,97	0,26	13,17	1,91	0,32	16,95	1,77	0,29	16,26	1,99	0,25	12,35	1,63	0,31	18,80	1,68	0,34	20,14
Yam	$g\,g^{-1}$	0,1955	0,40	0,03	7,18	0,41	0,04	10,79	0,44	0,05	10,16	0,44	0,08	18,79	0,48	0,04	8,04	0,44	0,08	17,80	0,44	0,04	8,89	0,42	0,03	6,82
Yaresp	$g\,g^{-1}$	0,1672	0,15	0,01	4,94	0,15	0,01	5,09	0,15	0,01	4,55	0,12	0,01	8,36	0,15	0,01	4,79	0,15	0,01	8,02	0,13	0,01	6,65	0,13	0,00	3,44
Yem	$g\,g^{-1}$	0,56	0,60	0,01	2,48	0,60	0,01	1,82	0,60	0,02	2,89	0,58	0,02	2,70	0,60	0,01	1,94	0,60	0,02	3,27	0,58	0,01	2,19	0,59	0,01	1,53
qSmax	$g\,g^{-1}\,h^{-1}$	1,3431	1,60	0,02	1,20	1,58	0,03	2,09	1,60	0,03	2,02	1,47	0,03	1,79	1,59	0,03	1,83	1,55	0,03	2,08	1,39	0,02	1,16	1,40	0,04	2,54
Ks	$g\,L^{-1}$	0,05	0,03	0,01	22,73	0,03	0,00	15,72	0,03	0,01	27,93	0,08	0,01	8,44	0,03	0,01	19,59	0,03	0,01	21,88	0,04	0,01	29,35	0,03	0,01	18,79
Ko	$g\,L^{-1}$	1	19,87	1,52	7,64	18,13	1,87	10,32	14,51	1,27	8,76	18,64	1,93	10,38	16,29	1,66	10,17	16,00	2,22	13,88	14,13	0,95	6,69	9,57	1,27	13,28
Yosresp	$g\,g^{-1}$	1	2,00	0,05	2,54	2,00	0,04	1,90	1,99	0,09	4,46	1,80	0,09	4,78	1,76	0,05	3,03	1,97	0,08	4,31	1,89	0,06	3,04	1,19	0,02	2,03
pAmax	$g\,g^{-1}\,h^{-1}$	1,3091	1,60	0,07	4,18	0,93	0,12	13,29	1,13	0,08	7,31	0,86	0,07	8,64	0,95	0,07	7,45	0,98	0,09	8,96	1,08	0,09	8,37	0,87	0,09	10,77
Yaof	$g\,g^{-1}$	0,4607	0,35	0,01	3,88	0,20	0,02	12,23	0,24	0,02	7,52	0,21	0,02	6,99	0,21	0,02	7,05	0,20	0,02	8,88	0,23	0,02	8,21	0,23	0,02	7,91
Yofm	$g\,g^{-1}$	0,2795	0,20	0,01	4,74	0,20	0,02	7,53	0,22	0,01	5,46	0,21	0,02	9,87	0,23	0,01	5,75	0,21	0,02	10,21	0,22	0,01	5,20	0,22	0,01	5,08

In the present work, eight strains were examined in 24 successful cultivations. The end of glucose uptake was, in part, predicted with small errors of less than one minute, thanks to the iterative model calibration cycle. The feed start was automatic and in an operable acceptable time window using the dynamic process redesign as defined in the model calibration cycle.

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The model parameter sets estimated are always unique and with a physiological meaning, even with very little data in the initial phase of this study, e.g., the first 3 h of cultivation. This is ensured by the built-in subset selection and is proven by the Monte Carlo simulations made afterwards.

4.3.4 Discussion

In this study, we presented a computational framework able to design and operate parallel *E. coli* cultivations without human supervision. The results demonstrate that a robust operation tailored to each specific clone is possible through an adaptive input design. Undesired experimental conditions (e.g., overfeeding and starvation) are avoided while sufficient information to allow a confident discrimination of the clones is generated. Both start time and feed rate were accurately predicted for each one of the eight clones, using feedback information from online and at-line cultivation measurements. This is essential in an experimental facility aimed to perform screening cultivations for clones whose phenotype is not known beforehand. The relevance of an adaptive and specific experimental design can be seen in this case study. As illustrated in Figure 9, despite the fact that the clone characteristics differ only minimally from each other, an experiment with a fixed start time and feeding rate would have violated important experimental constrains (here, overfeeding).

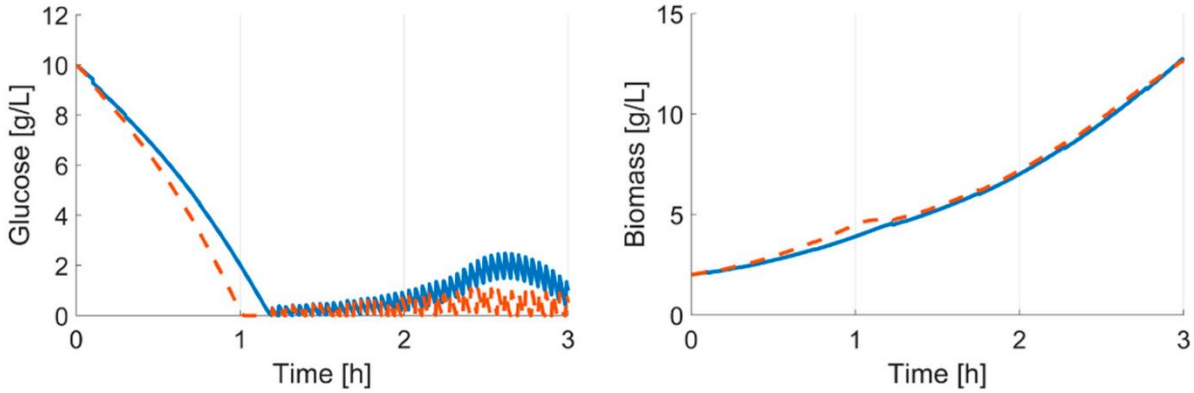

Figure 9. In-silico comparison of different clones; simulation based on last modelling cycle parameter set. Initial values: glucose 10 g L^{-1}; biomass 2 g L^{-1}. Solid line: *E. coli* BW25113; dashed line: *E. coli* BW25113 *ΔgatZ*. Feed start is simulated at 2.3 h; μ_{set} is fixed at 0.5 h^{-1}. If the feed start and rate are only adjusted to one strain, the cultivation of the *ΔgatZ* mutant would lead to overfeeding.

Additionally, the use of a macro-kinetic growth model that describes the main extracellular dynamics of *E. coli* was shown to be sufficient, even though it is insufficient to describe the complex nonlinear dynamics of the bioprocess and the different genotypes of the clones. The adaptive nature of the framework ensures a proper prediction within the current horizon and is sufficient to assure a robust operation of the cultivations. On average, the predicted feed start differed by less than 10 min for the optimal one, which is in an acceptable range and is mainly caused by unobserved disturbances in the system. If necessary, the mismatch can be further

reduced by increasing the frequency of model calibration. Furthermore, the framework provides all necessary parameters and actions to define a wide range of alternative feet start triggers (e.g., glucose reduction or acetate consumption).

As expected, the parameter variance in general decreases with every model calibration cycle. After the cultivation, the parameter distribution is generally very narrow. This is also reflected in the small deviations of the simulation results of the Monte Carlo studies (Figure 10) and demonstrates the value of the parameters for further in-silico studies. Important parameters, such as maximum glucose uptake (qS_{max}), can be distinguished with statistical significance and used for clone discrimination (Figure 7, Table 3). In this study, *E. coli* BW25113 Δ*ompT* had the largest qS_{max} value and *E. coli* BW25113 Δ*gatZ* the lowest one. However, some parameters could not, or only with insufficient confidence, be identified. This hampered a distinction of some essential parameters, such as the maximal acetate uptake rate (qA_{max}). Furthermore, parameter identifiability can be increased in future applications using methods for optimal experimental design (OED) [20,32,33] or enhanced parameter identifiability analysis [34–36].

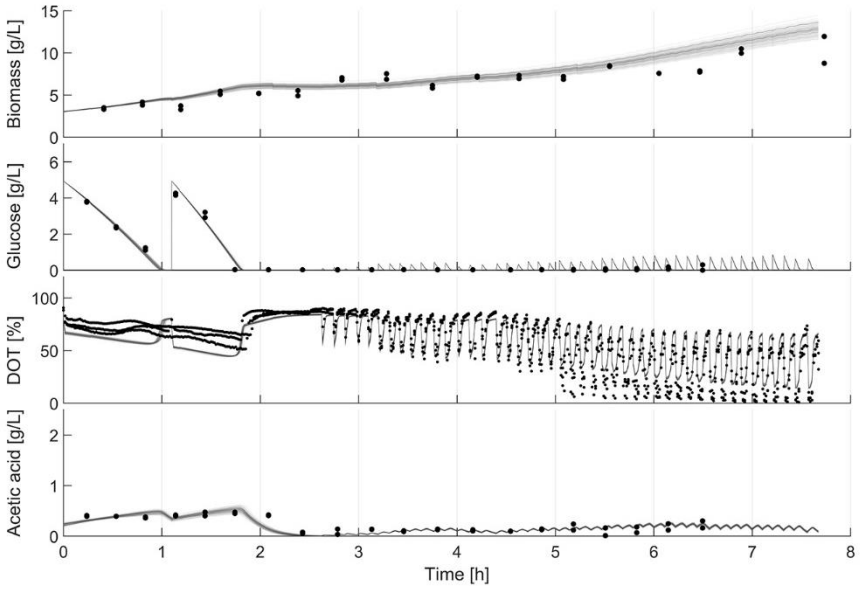

Figure 10: . Model uncertainty based on parameter standard deviation. Monte Carlo simulation: results of 1000 parameter estimates based on in-silico data. In-silico data were generated based on the last data set for *E. coli* BW25113 *ΔglcB* and by a random σ of 0.15 for biomass, glucose and acetate and a σ of 0.05 for DOT.

The frequency of the parameter estimation was defined based on the availability of at-line data (biomass, glucose and acetate), and as expected, the at-line data are decisive to achieve model identifiability. Still, the results show that especially parameters related to glucose consumption can be identified using only the online DOT signal. This shows that, even though in a significantly limited manner, the framework can also be used to increase the robustness of robotic facilities that do not have embedded at-line analytics. This significantly reduces the operative effort of the experimental setup. The glucose consumption rate seems to be observable from the DOT signal, by which a reduced version

of the macro-kinetic model could be used to build an observer-based feeding control. Finally, we also demonstrated that the length of the batch phase is essential to assure sufficient data before the start of the feeding so as to allow a reliable operation of the following phases.

Some parameters drift over time or change rapidly between batch and fed-batch (Figure 7). This could be related to the increasing information content of the growing data set and the resulting addition of previously neglected model parameters to the subset selection. However, the variations in the parameters caused by intracellular changes in the metabolic machinery together with heterogeneous mutations in the population [37] are not represented in the model and could also cause such parameter changes. Such uncovered intracellular changes may also explain the apparently poor representation of the batch phase in the last model calibration cycle compared to earlier ones. Therefore, an iterative recalculation of the feed is necessary to cope with disturbances in the experiments and inaccuracies in the model prediction. Moving horizon approaches can also increase the model prediction accuracy by allowing different parameter sets in different cultivation phases for a single clone [38].

4.3.5 Conclusions

The operation of robotic experiments with multiple fed-batch cultivations in parallel is very challenging even for skilled operators, since many decisions and tasks are needed at the same time. In this work, we present an adaptive framework for conditional screening for parallel fed-batch experiments, aiming to identify the best candidate strain for industrial scale biomanufacturing. We demonstrate that the use of a macro-kinetic growth model in an adaptive framework using online and at-line data information in a feedback loop is necessary to:

1.) Design a specific strategy for each different clone of the conditional screening experiment.
2.) Increase the robustness of the robotic operation against experimental disturbance.
3.) Give an approximation of the reliability of the simulation results with respect to production scale performance.

To our knowledge, this is the first successful model-based operation of 24 fed-batch cultivations with as many as eight different clones in parallel including its characterization, sufficient for clone discrimination. The results clearly demonstrate the capabilities of the framework to increase the efficiency of combined mini-bioreactor systems with liquid handling stations to drastically reduce the experimental time, efforts and failure rate in high-throughput bioprocess development.

Supplementary Materials:

The following are available online at www.mdpi.com/xxx/s1, Table S1: all experimental measurements used for the modelling cycles; Table S2: initial parameter values, upper and lower bounds and units; Table S3: parameter subsets and values for *E. coli* W3110; Table S4: parameter subsets and values for *E. coli* BW25113; Table S5: parameter subsets and values

for BW25113 *ΔompT*; Table S6: parameter subsets and values for BW25113 *ΔaceA*; Table S7: parameter subsets and values for BW25113 *ΔfliA*; Table S8: parameter subsets and values for BW25113 *ΔgatC*; Table S9: parameter subsets and values for BW25113 *ΔgatZ*; Table S10: parameter subsets and values for BW25113 *ΔglcB*; Figure S1: Comparison of measurement and simulation for *E. coli* W3110; Figure S2: Comparison of measurement and simulation for *E. coli* BW25113; Figure S3: Comparison of measurement and simulation for *E. coli* BW25113 *ΔompT*; Figure S4: Comparison of measurement and simulation for *E. coli* BW25113 *ΔaceA*; Figure S5: Comparison of measurement and simulation for *E. coli* BW25133 *ΔfliA*; Figure S6: Comparison of measurement and simulation for *E. coli* BW25133 *ΔgatC*; Figure S7: Comparison of measurement and simulation for *E. coli* BW25113 *ΔgatZ*.

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Conflicts of Interest

The authors declare no conflict of interest.

4.3.6 References

1. Dusny, C.; Grünberger, A. Microfluidic single-cell analysis in biotechnology: from monitoring towards understanding. Curr. Opin. Biotechnol. 2020, 63, 26–33, doi:10.1016/j.copbio.2019.11.001.
2. Leavell, M.D.; Singh, A.H.; Kaufmann-Malaga, B.B. High-throughput screening for improved microbial cell factories, perspective and promise. Curr. Opin. Biotechnol. 2020, 62, 22–28, doi:10.1016/j.copbio.2019.07.002.
3. Bunzel, H.A.; Pi, X.G.; Pott, M.; Hilvert, D. Speeding up enzyme discovery and engineering with ultrahigh-throughput methods. Curr. Opin. Struct. Biol. 2018, 48, 149–156, doi:10.1016/j.sbi.2017.12.010.
4. Nicolaou, S.A.; Gaida, S.M.; Papoutsakis, E.T. A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: From biofuels and chemicals, to biocatalysis and bioremediation. Metab. Eng. 2010, 12, 307–331, doi:10.1016/j.ymben.2010.03.004.

5. Crater, J.S.; Lievense, J.C. Scale-up of industrial microbial processes. FEMS Microbiol. Lett. 2018, 365, 1–5, doi:10.1093/femsle/fny138.

6. Delvigne, F.; Takors, R.; Mudde, R.; Van Gulik, W.; Noorman, H. Bioprocess scale-up/down as integrative enabling technology: from fluid mechanics to systems biology and beyond. Microb. Biotechnol. 2017, 10, 1267–1274, doi:10.1111/1751-7915.12803.

7. Ho, P.; Westerwalbesloh, C.; Kaganovitch, E.; Grünberger, A.; Neubauer, P.; Kohlheyer, D.; Von Lieres, E. Reproduction of Large-Scale Bioreactor Conditions on Microfluidic Chips. Microorganisms 2019, 7, 105, doi:10.3390/microorganisms7040105.

8. Anane, E.; García, Ángel C.; Haby, B.; Hans, S.; Krausch, N.; Krewinkel, M.; Hauptmann, P.; Neubauer, P.; Bournazou, M.N.C. A model-based framework for parallel scale-down fed-batch cultivations in mini-bioreactors for accelerated phenotyping. Biotechnol. Bioeng. 2019, 116, 2906–2918, doi:10.1002/bit.27116.

9. Tajsoleiman, T.; Mears, L.; Krühne, U.; Gernaey, K.V.; Cornelissen, S. An Industrial Perspective on Scale-Down Challenges Using Miniaturized Bioreactors. Trends Biotechnol. 2019, 37, 697–706, doi:10.1016/j.tibtech.2019.01.002.

10. Hemmerich, J.; Noack, S.; Wiechert, W.; Oldiges, M. Microbioreactor Systems for Accelerated Bioprocess Development. Biotechnol. J. 2018, 13, 1–25, doi:10.1002/biot.201700141.

11. Janzen, N.H.; Striedner, G.; Jarmer, J.; Voigtmann, M.; Abad, S.; Reinisch, D. Implementation of a Fully Automated Microbial Cultivation Platform for Strain and Process Screening. Biotechnol. J. 2019, 14, 1800625, doi:10.1002/biot.201800625.

12. Kusterer, A.; Krause, C.; Kaufmann, K.; Arnold, M.; Weuster-Botz, D. Fully automated single-use stirred-tank bioreactors for parallel microbial cultivations. Bioprocess Biosyst. Eng. 2008, 31, 207–215, doi:10.1007/s00449-007-0195-z.

13. Morschett, H.; Freier, L.; Rohde, J.; Wiechert, W.; Von Lieres, E.; Oldiges, M. A framework for accelerated phototrophic bioprocess development: integration of parallelized microscale cultivation, laboratory automation and Kriging-assisted experimental design. Biotechnol. Biofuels 2017, 10, 26, doi:10.1186/s13068-017-0711-6.

14. Bareither, R.; Pollard, D. A review of advanced small-scale parallel bioreactor technology for accelerated process development: Current state and future need. Biotechnol. Prog. 2010, 27, 2–14, doi:10.1002/btpr.522.

15. Tai, M.; Ly, A.; Leung, I.; Nayar, G. Efficient high-throughput biological process characterization: Definitive screening design with the Ambr250 bioreactor system. Biotechnol. Prog. 2015, 31, 1388–1395, doi:10.1002/btpr.2142.

16. Knorr, B.; Schlieker, H.; Hohmann, H.-P.; Weuster-Botz, D. Scale-down and parallel operation of the riboflavin production process with Bacillus subtilis. Biochem. Eng. J. 2007, 33, 263–274, doi:10.1016/j.bej.2006.10.023.

17. Haby, B.; Hans, S.; Anane, E.; Sawatzki, A.; Krausch, N.; Neubauer, P.; Bournazou, M.N.C. Integrated Robotic Mini Bioreactor Platform for Automated, Parallel Microbial Cultivation with Online Data Handling and Process Control. SLAS Technol. Transl. Life Sci. Innov. 2019, 24, 569–582, doi:10.1177/2472630319860775.

18. Newton, J.; Oeggl, R.; Janzen, N.H.; Abad, S.; Reinisch, D. Process adapted calibration improves fluorometric pH sensor precision in sophisticated fermentation processes. Eng. Life Sci. 2020, 20, 331–337, doi:10.1002/elsc.201900156.

19. Hemmerich, J.; Tenhaef, N.; Steffens, C.; Kappelmann, J.; Weiske, M.; Reich, S.J.; Wiechert, W.; Oldiges, M.; Noack, S. Less Sacrifice, More Insight: Repeated Low-Volume Sampling of Microbioreactor Cultivations Enables Accelerated Deep Phenotyping of Microbial Strain Libraries. Biotechnol. J. 2018, 14, 1–10, doi:10.1002/biot.201800428.

20. Bournazou, M.C.; Barz, T.; Nickel, D.; Cárdenas, D.L.; Glauche, F.; Knepper, A.; Neubauer, P. Online optimal experimental re-design in robotic parallel fed-batch cultivation facilities. Biotechnol. Bioeng. 2017, 114, 610–619, doi:10.1002/bit.26192.

21. Von Stosch, M.; Willis, M.J. Intensified design of experiments for upstream bioreactors. Eng. Life Sci. 2016, 17, 1173–1184, doi:10.1002/elsc.201600037.

22. Bayer, B.; Von Stosch, M.; Striedner, G.; Duerkop, M. Comparison of Modeling Methods for DoE-Based Holistic Upstream Process Characterization. Biotechnol. J. 2020, 15, e1900551, doi:10.1002/biot.201900551.

23. Hans, S.; Ulmer, C.; Narayanan, H.; Brautaset, T.; Krausch, N.; Neubauer, P.; Schäffl, I.; Sokolov, M.; Bournazou, M.N.C. Monitoring Parallel Robotic Cultivations with Online Multivariate Analysis. Processes 2020, 8, 582, doi:10.3390/pr8050582.

24. Delvigne, F.; Goffin, P. Microbial heterogeneity affects bioprocess robustness: Dynamic single-cell analysis contributes to understanding of microbial populations. Biotechnol. J. 2013, 9, 61–72, doi:10.1002/biot.201300119.

25. Anane, E.; López C, D.C.; Neubauer, P.; Bournazou, M.N.C. Modelling overflow metabolism in Escherichia coli by acetate cycling. Biochem. Eng. J. 2017, 125, 23–30, doi:10.1016/j.bej.2017.05.013.

26. Sauer, U.; Lasko, D.R.; Fiaux, J.; Hochuli, M.; Glaser, R.; Szyperski, T.; WüthrichK.; Bailey, J.E. Metabolic Flux Ratio Analysis of Genetic and Environmental Modulations of Escherichia coli Central Carbon Metabolism. J. Bacteriol. 1999, 181, 6679–6688, doi:10.1128/jb.181.21.6679-6688.1999.

27. Baba, T.; Ara, T.; Hasegawa, M.; Takai, Y.; Okumura, Y.; Baba, M.; A Datsenko, K.; Tomita, M.; Wanner, B.L.; Mori, H. Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. 2006, 2, 2006.0008, doi:10.1038/msb4100050.

28. López C, D.C.; Barz, T.; Peñuela, M.; Villegas, A.; Ochoa, S.; Wozny, G. Model-based identifiable parameter determination applied to a simultaneous saccharification and fermentation process model for bio-ethanol production. Biotechnol. Prog. 2013, 29, 1064–1082, doi:10.1002/btpr.1753.

29. Hindmarsh, A.C.; Brown, P.N.; Grant, K.E.; Lee, S.L.; Serban, R.; Shumaker, D.E.; Woodward, C.S. SUNDIALS. ACM Trans. Math. Softw. 2005, 31, 363–396, doi:10.1145/1089014.1089020.

30. Moser, A.; Moser, P.D.A. Haggstrom, Bioprocess Technology: Fundamentals and Applications; Springer Science & Business Media: New York, NY, USA, 1988, doi:10.1007/978-1-4613-8748-0.

31. Krausch, N.; Barz, T.; Sawatzki, A.; Gruber, M.; Kamel, S.; Neubauer, P.; Bournazou, M.N.C. Monte Carlo Simulations for the Analysis of Non-linear Parameter Confidence Intervals in Optimal Experimental Design. Front. Bioeng. Biotechnol. 2019, 7, 122, doi:10.3389/fbioe.2019.00122.

32. Barz, T.; Sommer, A.; Wilms, T.; Neubauer, P.; Bournazou, M.N.C. Adaptive optimal operation of a parallel robotic liquid handling station. IFAC-PapersOnLine 2018, 51, 765–770, doi:10.1016/j.ifacol.2018.04.006.

33. Franceschini, G.; Macchietto, S. Model-based design of experiments for parameter precision: State of the art. Chem. Eng. Sci. 2008, 63, 4846–4872, doi:10.1016/j.ces.2007.11.034.

34. Anane, E.; López C, D.C.; Barz, T.; Sin, G.; Gernaey, K.V.; Neubauer, P.; Bournazou, M.N.C. Output uncertainty of dynamic growth models: Effect of uncertain parameter estimates on model reliability. Biochem. Eng. J. 2019, 150, 107247, doi:10.1016/j.bej.2019.107247.

35. Dynamics of Mathematical Models in Biology. Dyn. Math. Models Biol. 2016, 31–41, doi:10.1007/978-3-319-45723-9.

36. Villaverde, A.F.; Banga, J.R. Structural Properties of Dynamic Systems Biology Models: Identifiability, Reachability, and Initial Conditions. Processes 2017, 5, 29, doi:10.3390/pr5020029.

37. Neubauer, P.; Lin, H.Y.; Mathiszik, B. Metabolic load of recombinant protein production: Inhibition of cellular capacities for glucose uptake and respiration after induction of a heterologous gene in Escherichia coli. Biotechnol. Bioeng. 2003, 83, 53–64, doi:10.1002/bit.10645.

38. Jabarivelisdeh, B.; Carius, L.; Findeisen, R.; Waldherr, S. Adaptive predictive control of bioprocesses with constraint-based modeling and estimation. Comput. Chem. Eng. 2020, 135, 106744, doi:10.1016/j.compchemeng.2020.106744.

4.4 Monitoring Parallel Robotic Cultivations with Online Multivariate Analysis

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Abstract: In conditional microbial screening, a limited number of candidate strains are tested at different conditions searching for the optimal operation strategy in production (e.g., temperature and pH shifts, media composition as well as feeding and induction strategies). To achieve this, cultivation volumes of >10 mL and advanced control schemes are required to allow appropriate sampling and analyses. Operations become even more complex when the analytical methods are integrated into the robot facility. Among other multivariate data analysis methods, principal component analysis (PCA) techniques have especially gained popularity in high throughput screening. However, an important issue specific to high throughput bioprocess development is the lack of so-called golden batches that could be used as a basis for multivariate analysis. In this study, we establish and present a program to monitor dynamic parallel cultivations in a high throughput facility. PCA was used for process monitoring and automated fault detection of 24 parallel running experiments using recombinant *E. coli* cells expressing three different fluorescence proteins as the model organism. This approach allowed for capturing events like stirrer failures and blockage of the aeration system and provided a good signal to noise ratio. The developed application can be easily integrated in existing data- and device-infrastructures, allowing automated and remote monitoring of parallel bioreactor systems.

Own Contribution: Conceptualization, Software writing, Validation, Writing original draft, Visualization

Author Contributions: Conceptualization, CU, SH and MNCB; methodology, CU; software, CU and SH; validation, CU, SH, HN, MNCB and MS; formal analysis, CU; resources, TB, NJK and IS; investigation, CU; data curation, CU; writing—original draft preparation, CU, HN and SH; writing—review and editing, MS, TB, MNCB and PN; visualization, CU, NJK and SH; supervision, MS, MNCB and PN; project administration, PN; funding acquisition, PN

4.4.1 Introduction

Microbial cell factories are widely used for biotechnological production processes. The development of effective bioprocesses requires screening of many microbial strains under various cultivation conditions. State-of-the-art bioprocess development is known to be time consuming, laborious, and for having a low success rate [1]. Process parameters, such as microbial host, vector size and copy-number, feeding strategy, and media composition have a significant impact on the profitability, reliability, and sustainability of the final manufacturing process. Considering all these factors and evaluating them in relation to each other often calls for a high number of cultivations. To ensure reliability of data and results, parallel cultivations providing a number of replicates to compensate for the variance of biological systems are additionally needed. Many bioprocess conditions are difficult to study due to the lack of suitable high throughput (HT) facilities to perform all these cultivations in a short time. Consequently, current process improvement strategies in high throughput bioprocess development (HTBD) are based on expert knowledge [2] and static design of experiments (DoE) [3,4,5]. In order to reduce the number of cultivations to an appropriate level, many factors are only partially and incompletely weighed against each other. On the other hand, emerging technologies such as automation and digitization enable a faster product development and shorter cycles from construction of a microbial clone to an optimal bioprocess [6,7,8] by increasing the possible number of parallel cultivations. Although model-based tools (e.g., for process control or computer aided design) are the standard in other industries [9,10,11,12], they are rarely used in bioprocess development despite their big potential [13,14]. A major challenge is the lack of suitable tractable mathematical models that are required but difficult to develop due to the complexity of biological systems [15]. This is especially the case for process development, where knowledge of the new microbial strains is limited and an exhaustive investigation of mutants likely to be discarded is considered unnecessary.

The difference between process control (batch vs. fed-batch), cultivation system (e.g., shake flask, multi well plate or lab scale bioreactor vs. production scale bioreactor) and the resulting different metabolic and stress conditions between screening and manufacturing makes scale-up between these stages challenging. An essential factor for scale up is a detailed knowledge about the process dynamics [16]. Changes in pH, oxygen limitation or the availability of media components should be considered during the conditional screening phase. The technical requirements are already met by modern HT robots with developments at the µL scale. Significant advantages are offered by mini-bioreactors (MBR) with working volumes between 10 and 250 mL [17,18,19], integrated online sensors for pH and DOT, integrated control of pH and substrate feeding [20,21,22] and automated at-line sampling and analysis [8,21,23]. Additionally, computer aided tools that enable advanced process control and

feedback operation of the robots and the MBRs have been developed, but are rarely used [17,18,20,22,24,25,26].

However, the number of parallel cultivations made possible in this way is now hardly achievable in the present way, with manual corrections and interventions. Therefore, process monitoring of parallel cultivations is a major challenge in HTBD, especially if no models to describe the bioprocess are available to guide the operator. Fully automated solutions that include process and feeding control, online and at-line monitoring are very challenging in parallel MBR cultivations [20,21]. The main difficulties are the analysis of multi-dimensional and highly correlated data sets, monitoring and operating a large number of bioreactors and the intrinsic need to solve an optimal experimental design problem considering all MBRs simultaneously [24,27] in a period of time. Additional challenges arise when industrial conditions are investigated at the milliliter scale [22,25].

In production processes, the operation strategy is well defined, historical data is at hand, and the goal is typically to run the current cultivations within predefined critical quality attributes (CQAs) or the “golden batch” conditions. Under these conditions, multivariate analysis (MVA) tools have been widely applied to supervise the process and assure its reproducibility [28]. MVA tools such as principal component analysis (PCA) have become increasingly popular in the field of bioprocesses due to their capability to represent highly correlated multi-dimensional datasets in a reduced space, separating process noise while retaining maximum information. Some of the early applications of PCA include process monitoring, detection of failures or anomalies, and statistical process control [19,28,29,30,31,32,33]. However, for all these applications, a PCA model is usually trained based on an “in-control”, i.e., “golden batch”, basis to detect deviations from the targeted production run characteristics [34].

During screening, where the goal is to find new strains best suited for the bioprocess at hand, this training data is of course unavailable. The lack of a “golden batch” makes it very challenging to diagnose or even identify faults or disturbances in cultivations with no historical data. Although in principle historical data of similar processes can be used as reference points for development, one cannot rely on pre-defined process behavior and constraints, as is typically the case in production. Due to various factors, the physiology and phenotype of the cells is known to vary during cultivation time [35] (e.g., population heterogeneities [36]). This makes choosing a setup and tuning of control strategies in advance very difficult. For this reason, we need to develop tools that exploit the information generated in parallel MBRs online to rapidly develop models for process monitoring and to project the large data sets into a low dimensional visual representation.

Our previous work showed that PCA can be used in parallel MBR experiments to identify and improve feeding strategies with a low number of experiments [27]. In this work we developed a program that enables the monitoring of parallel dynamic cultivations in real-time,

supported with visual representations as well as automated event triggers (Figure 1). The added value of this method is enhanced process monitoring and automated fault detection. This is demonstrated in an experimental campaign with 24 parallel MBR operated by a fully automated robotic system. This program allows a compressed representation and visualization of the ongoing experiments, enabling a comparison between the states of parallel cultivations. The PCA method is applied in a moving horizon framework to allow a rapid detection of specific events and to track the dynamic evolution of the reactors. The two approaches together provide an informative overview of the bioreactor's performance and state. Thus, they enable the operator to determine whether all parallel cultivations are running within critical parameter limits and display a warning in case of deviations. Critical bioreactors can be easily identified and tended to.

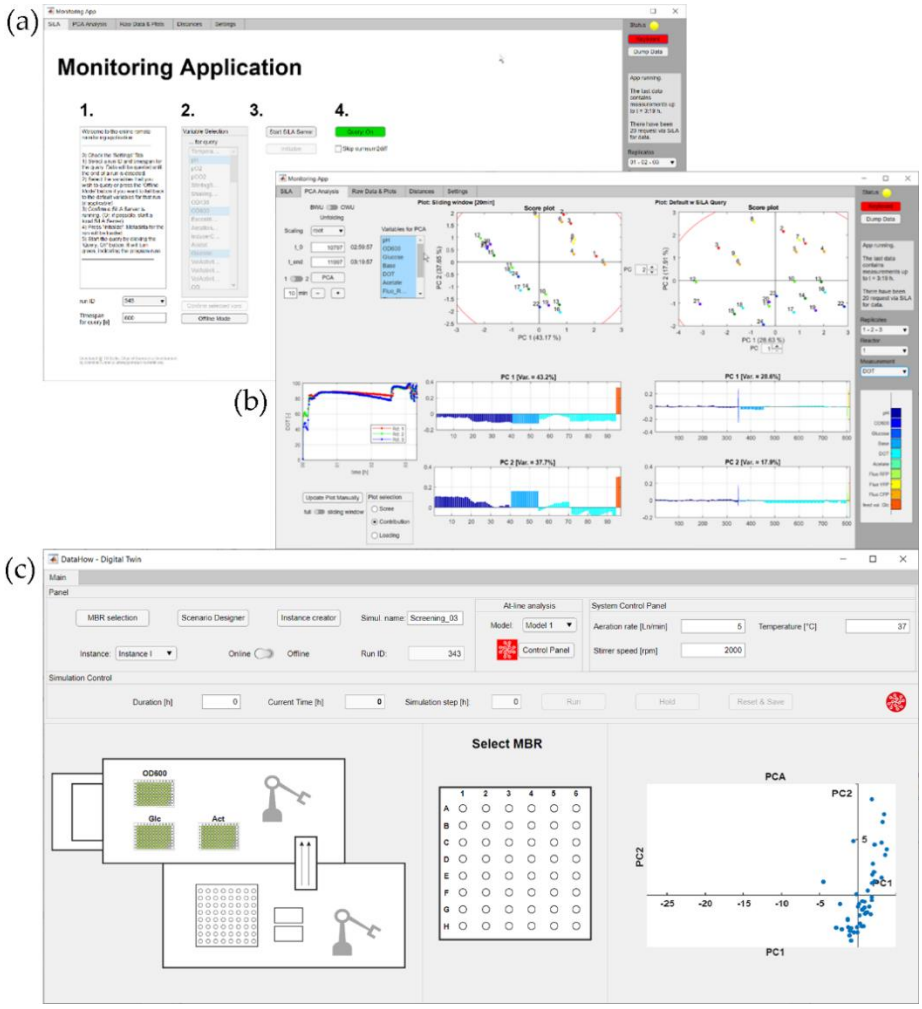


Figure 1. Screenshots of the monitoring application. Screenshot (a) depicts the landing page where the user selects an experiment, adapts settings, and starts the data query and subsequent online data analysis; (b) shows the main panel with plots for process data and results from the PCA; and (c) depicts the central control app of the digital twin (DTW) of the process. It allows for fast process monitoring via direct control and observation of single MBRs as well as fast identification of process failures based on PCA.

In addition, they enable an automated and secure transfer of the cultivation data during the runtime of the experiment. This allows the often computationally intensive online data evaluation to be performed in specialized laboratories, as a service or by project partners. Here we used an efficient protocol for communication, enabling the monitoring of the robotic experiments remotely, reducing the physical barrier separating theoretical work and practical wet lab. The implementation is based on a SILA2 protocol (preliminary standard as of January 2019).

4.4.2 Materials and Methods

4.4.2.1 Experimental Facility

The wet experiments were performed in a high throughput bioprocess development facility composed of two liquid handling stations (LHS) (Freedom EVO 200, Tecan Group Ltd., Männedorf, Switzerland; Microlab Star, Hamilton Company, Bonaduz, Switzerland) and one mini bioreactor system (bioREACTOR 48, 2mag AG, Munich, Germany) which is mounted on the first LHS (Tecan). The entire facility and its functionality is described in [21]. To trigger non-optimal microbial cultivation conditions the volume balance control described by Haby et al. [21] was switched off.

4.4.2.2 Microbial Model Strain and Cultures

All cultivations were performed with *E. coli* K-12 BW25113 (F-, DE(araD-araB)567, lacZ4787(del)::rrnB-3, LAM-, rph-1, DE(rhaD-rhaB)568, hsdR514) carrying plasmid pAG032. As described by Gawin et al. 2018 [37] the plasmid pAG032 contains three fluorescent protein (CFP, YFP, RFP) encoding genes, each under transcriptional control of different promotors. The CFP fluorescent signal gene is coupled to an σ32 related promoter and is constitutively expressed. The rpsJ constitutive promoter is responsible for the YFP expression and serves as an indicator for the number of ribosomes in the cell. As placeholder for a recombinant product RFP expression is under control of the XylS/Pm promoter system. Frozen cells (stored at −80 °C) were transferred into TY medium and kept for 5 h at 37 °C. Afterwards an aliquot was added to EnPresso B medium (Enpresso GmbH, Berlin, Germany) with 6 U L^{-1} Reagent A and stored at 37 °C overnight. The main culture was adjusted to an OD600 of 1 in MS medium [38] with 5 g L^{-1} glucose and distributed to the MBR culture vessels as 10 mL aliquots. Ampicillin (0.1 g L^{-1}) was added to all cultures for plasmid maintenance. The main cultivation was started with 2000 rpm at 37 °C. The stirrer speed was increased stepwise by 200 rpm every 5 min to 3000 rpm. The maximum specific growth rate of *E. coli* BW25113 (pAG032) was calculated based on previous experiments without induction at 0.72 h−1. The applied feed rates are summarized in Table 1, the calculations are based on the equations by Enfors 2019

[39]. After six hours of cultivation the cultures were induced by the addition of 50 µL 0.1 M m-toluic acid to a final concentration 0.5 mM.

Table 1. Summary of applied feed rates for cultivation of *E. coli* BW25113 (pAG032). The listing of the reactors in columns and rows represents the reactor allocation at the two liquid handling stations (LHS).

Reactor			μ_{set}	% of μ_{max}
1	2	3	0.65	90
4	5	6	0.58	80
7	8	9	0.50	70
10	11	12	0.43	60
13	14	15	0.36	50
16	17	18	0.29	40
19	20	21	0.22	30
22	23	24	0.14	20

4.4.2.3 Sampling and Analytics

On-line measurements for pH and DOT were taken every 30 s. For at-line analysis, the MBRs were sampled column-wise every 15 min during the cultivation, and samples were directly transferred into V-shaped 96-microwell plates. The sampling plates contained 15 µL dried 2M NaOH to ensure direct metabolic inactivation of the samples [8]. The samples were stored for five cycles on the LHS deck at 4 °C before being transferred to the second analytic LHS. The sample storage time on the LHS deck was between 2 and 75 min. For OD600 and fluorescence measurements the samples were diluted by the LHS [21] and measured in a SynergyTM MX microwell plate reader (BioTek Instruments GmbH, Bad Friedrichshall, Germany). The undiluted samples were filtrated to isolate the cells, and glucose and acetic acid concentrations in the supernatant were measured. The detailed procedure of the automated workflow is described in Haby et al. 2019 [21]. Outliers based on traceable technical issues are marked as invalid and not included in the data analysis.

4.4.2.4 Software Framework

Online data transfer was enabled by a server–client architecture based on the SiLA 2 (Association Consortium Standardization in Lab Automation, Rapperswil-Jona, Switzerland, sila-standard.org) standard. The server is located at the Chair of Bioprocess Engineering at Technische Universität Berlin, while the Client is distributed with the application. The server–client framework is written in Java 8 (Oracle Corporation, Santa Clara, CA, USA). The client requests information about an (running or completed) experiment to which the server replies. The server is equipped with a driver that connects to the centralized MySQL (Oracle

Corporation, Santa Clara, CA, USA) database, allowing access to all process and meta data of an experiment. Upon request by a client, the server pulls the data from the database via SQL queries and returns the information to the client. The client formats the information into a string complying to the XML standard and saves it on the local machine.

4.4.2.5 Monitoring Application

The monitoring application serves as graphical user interface (Supplementary Code S1). The application itself is written in MATLAB (2018a, MathWorks, Natick, MA, USA, 2019) using the MATLAB App Designer environment. A MATLAB script initiates the client and parses the information of the XML file into a data structure compatible to MATLAB. The parsing is based on a modified version of the xml2struct function from MATLAB File Exchange [40].

4.4.2.6 Data Processing for PCA

The input variables for the PCA consisted of on-line (pH, DOT) and at-line (OD600, glucose and acetate concentrations, fluorescence for red, yellow, and cyan fluorescent proteins) measurements as well as the logged volumes for base and glucose addition. The time differences between the sampling of at-line measurements were interpolated to a reference time using piecewise cubic Hermite interpolating polynomials.

The PCA of the three-dimensional dataset (reactor x variable x time) is unfolded in a batch-wise manner [41]. This approach essentially converts time to a distinguishing factor of each variable, i.e., defining one variable per time instance where it was quantified. Following the unfolding the dataset was mean-centered and scaled to unit-variance. Additionally, to account for the different frequency of measurements, the data was block-scaled: the trajectories of one variable among all was scaled by dividing each column by the square root of available number of data points [42].

4.4.2.7 Principal Component Analysis

The PCA is computed by the built-in MATLAB function pca. Detailed mathematical representation of the algorithm can be found in [43]. The optimal number of principal components (PCs) are selected automatically based on the improvement in % variance explained. An empirical threshold was set at 5%.

Score plots were used to visually represent the replicates' run behavior. Given the unfolding choice, the traditional loading plots become too complex with many lines for direct interpretation. Thus, contribution plots were employed to aid the operator in relating patterns in the score plots to actual occurrences in the process [44].

PCA was applied to series of dataset collected in time that are augmented in two ways. First, a moving horizon or sliding window mode of data augmentation is used to detect failures of sensors or faulty measurements rapidly. In this approach a window length is chosen (x mins)

and an update time is chosen (y mins). All data available in the window length is used to build the PCA model and a new model is built by sliding the window by y minutes. Secondly, a full horizon mode of data augmentation is used to track and compare the dynamic evolution of the different MBRs. In this approach, all data available is used to build the PCA model. In this work we chose to build a full horizon PCA model for each time in the reference time set.

The Hotelling's T² distance measure was used to detect unexpected drifts of an MBR compared to its replicates. Automated triggers were set for reactors outside the 90% confidence ellipse. The variable causing the behavior was automatically detected using key properties of PCA [43] to have a preliminary diagnosis of the event. The latent variable contributing most to the run was identified using the formula stated below:

$$cos^2_{i,l} = \frac{f^2_{i,l}}{\sum_l f^2_{i,l}} \quad (1)$$

where $f^2_{i,l}$ is the squared score of observation *i* on latent variable *l*. Subsequently, squared loadings of all the variables on this principal component were analyzed to identify the key driver of the failure.

4.4.2.8 Automated Warnings

Robotic experiments typically run without automated supervision systems. Failures besides arm movements or device malfunction cannot be detected unless an operator is monitoring the process. To tackle this issue, a simple method to trigger alarms was developed. For this, the Euclidean distance of each point to the center of its cluster of replicates was calculated in the sliding window triggering a message if user defined constraints were violated. This information allows the operator to quickly grasp the current status of the overall cultivation and assess the similarity of the replicates. Additionally, this first step towards online automatic classification of outliers enables more robust data selection for online optimal experimental re-design [45], a process that requires fast and thorough data selection that can hardly be done manually.

4.4.2.9 Pipetting Accuracy

To assess the pipetting accuracy of the cultivation LHS, weighed 96 well plates are filled with coloured demineralised water. Absorption maximum of the used liquid was determined via a spectral scan by the plate reader at 445 nm. The pipetting scheme was set up so each needle pipettes three columns in one row on one aspiration cycle to mimic the experimental setup in the cultivation. The factor for absorption mL^{-1} was calculated as shown below and applied to the plate.

$$\text{Factor}\left[\frac{\text{absortion}}{\text{mL}}\right] = \frac{\text{avrg. absortion of all wells} * \text{no. of wells}}{\frac{\text{weight differnce}}{\text{density water}}} = \frac{\text{total absortion}}{\text{total volume in plate}} \quad (2)$$

4.4.3 Results

The aim of the study was to demonstrate the functionality of the application and its capability to remotely monitor parallel cultivations, detect failures and guide the operator through the experiment. To this end, 24 microbial cultivations were carried out with eight different feeding strategies in triplicate. The aim of the study was to force several failures to test the performance of the program under critical conditions.

4.4.3.1 Microbial Cultivation

As model system, we use previously constructed recombinant strain *E. coli* BW25113 (pAG032) that expresses three different fluorescence proteins under individual control of three different promoters [37]. The batch phase of the 24 parallel cultivations lasted 2.6 h ± 2.04 min. The feed for the first nine reactors was started at 3.2 h of cultivation after consumption of acetic acid. The feed for all other reactors was started at 4 h. Different feed rates were set for each reactor triplicate. The feed rates varied between 20% and 90% of the maximum growth rate (see Table 1). Due to the pulse-based nature of the feeding, DOT oscillations started together with the feed additions. Cultivation data are shown in Figure 2 and available in Supplementary Table S1. Depending on the feeding rate, the increase of reactor volumes over time differs. For reactors 1–3 (with the highest feed rate of 90% of μ_{max}) the critical volume was reached after 5.5 h, reactors 10–12 (60% of μ_{max}) reached the critical volume after 8.8 h. Reactors with a low feed rate never reached critical volume levels. The DOT profile of reactor 17 decreased between 8.7 h and 9.8 h, however, this was due a technical issue and was solved during the running cultivation. When a critical volume level was reached, the DOT dropped to zero and the glucose consumption rate decreased.

In all cultivations a decrease of CFP activity (σ^{32} related promoter) was observed during the batch phase. Furthermore, the CFP activity is on the same level and stays constant during the feeding phase for low and moderate feed rates. For higher feed rates (60–90% of μ_{max}) the CFP activity is lower during the feeding phase and increases with the beginning of the oxygen limitation. The specific CFP signal increases during the batch phase and decreased during the feed phase at higher feed rates. In cultivations with a lower feed rate (20–40% of μ_{max}) the YFP signal (indicator for ribosomes per cell) stays constant during the feed rate. Between the cultivation time from 3–5 h, some YFP measurements were marked invalid because the detector limit of the used plate reader was exceeded. Later samples are analyzed in a higher dilution. For the two highest feed rates nearly no specific increase of RFP (inducible product) was observed. The highest specific RFP activity was reached with a feed rate of μ_{set} of 0.22 h^{-1} (30% of μ_{max}).

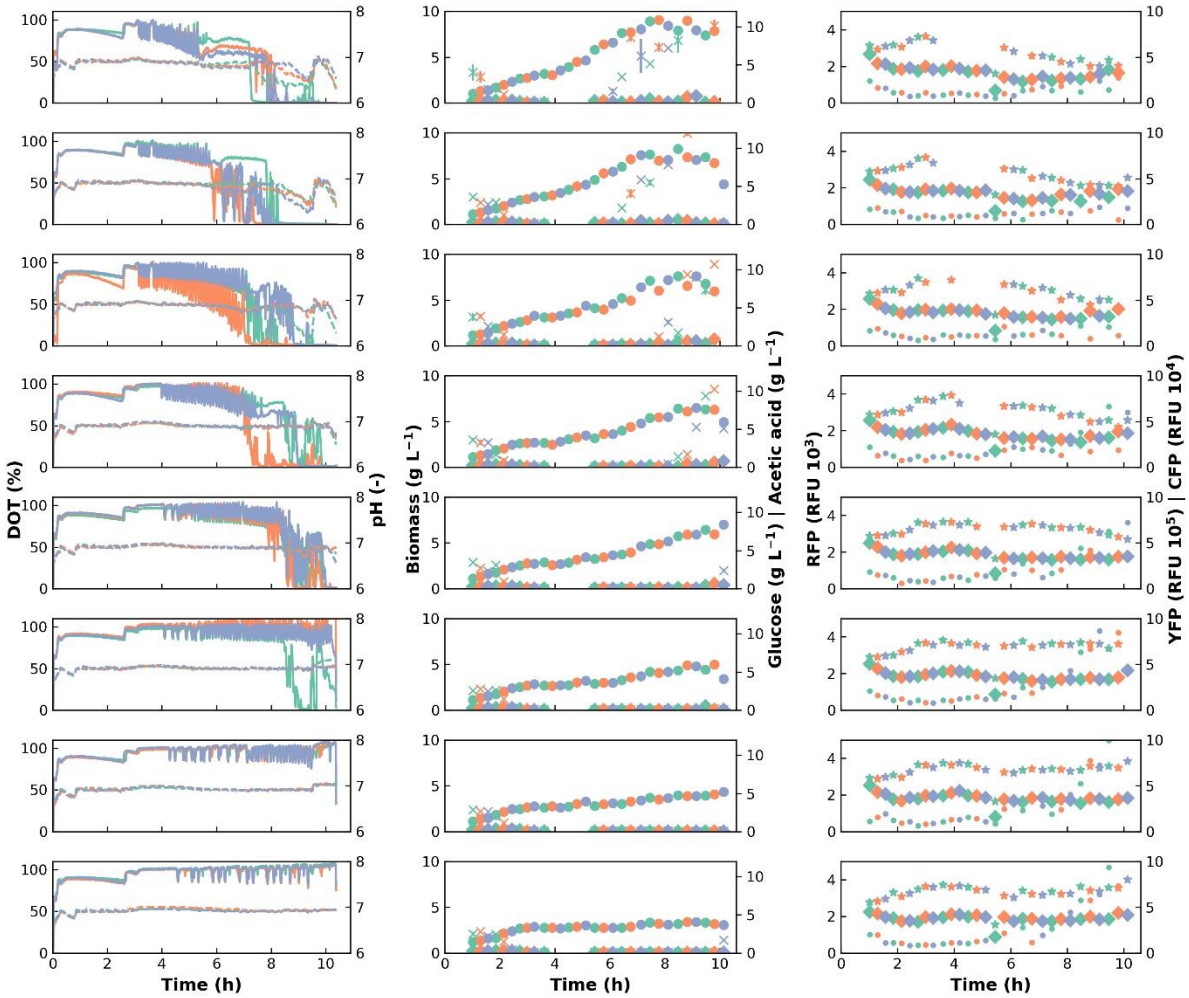


Figure 2. Cultivation data of the experiment. The rows represent a group of replicates with three mini-bioreactors (MBR). From top to bottom row, the applied feed decreased by 10% from 90% to 20% of μmax. (a) Solid lines: DOT (%); dashed lines: pH (-); (b) dots: biomass (g L−1); stars: glucose (g L−1); diamonds: acetic acid (g L−1); (c) dots: RFP (RFU × 103) (rpsJ constitutive promoter); stars: YFP (RFU × 105) (inducible XylS/Pm promoter); diamonds: CFP (RFU × 104) (σ32 related constitutive promoter).

4.4.3.2 User Interface

The main features of the program developed here are (i) the operator support with a visual compression of the large number of bioreactors and variables that need to be supervised, (ii) the secure and reliable remote access via the framework, and (iii) developing an automated event trigger and fault detection tool. Additionally, a user-friendly interface was developed to demonstrate the added value of the tool and allow its test in real experiments with experienced operators.

The central program developed in MATLAB covers all Server-Client connections, data management and -analyses and offers a graphical user interface. The user may choose from different plots commonly used in PCA such as score, scree, contribution, and loading plots. Input variables for data analysis can be varied to explore different aspects. To monitor the

cultivation, the application separates data for a full horizon and a moving window approach (see Figure 3).

4.4.3.3 Moving and Full Horizon Setup

In the moving horizon setup, the window's timeframe was set to 20 min, a duration empirically determined based on experience and trials with historical data. Thus, the input variables for

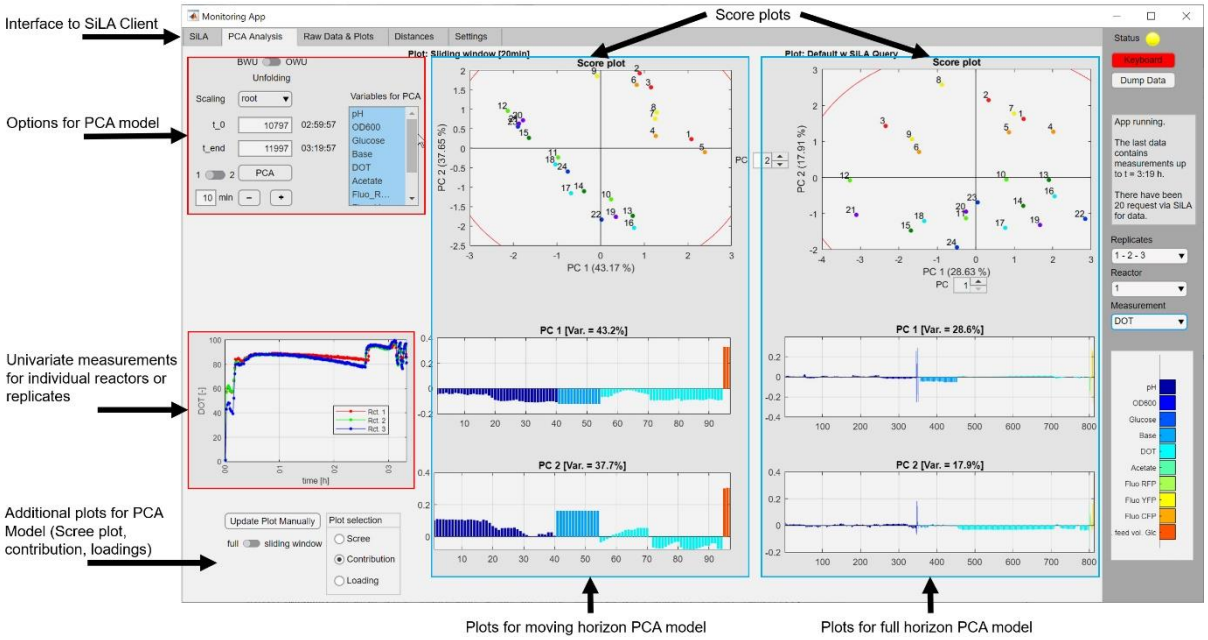


Figure 3. A screenshot of the graphical user interface of the application. The data shown is from the described experiment at 3:19 h. The left blue shows plots for the moving horizon PCA model, whereas the right blue rectangle depicts the same plots for a full horizon PCA model.

the sliding window PCA are the set points for pipetting volumes (base + feed) and the online measurements (pH + DOT).

Analysis of the loading vectors in the full horizon setup showed that the variables cumulated glucose feed and biomass correlate positively and are strongly pronounced on the first principal component. As the feed was set differently for each group of replicates, this finding is sound. However, from 03:00 h onwards, a trend can be observed on the second PC where the scores for the reactors of the replicates have monotonous decreasing values on the y-axes (see Figure 4). The posteriori analysis of the pipetting system showed that the feedings were indeed following this trend.

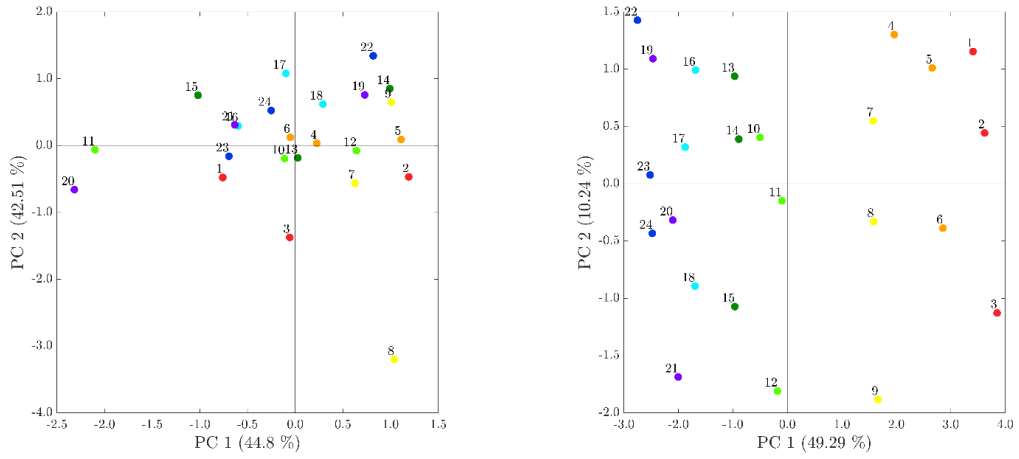
4.4.3.4 Event Monitoring Based on PCA

During this study, the program continuously observed the cultivations, updating its data every 10 min. Several incidents observed during the cultivation were detected properly by the program. We discuss three of these events: (1) stirrer failure in one bioreactor, caused by

problems in the magnetic system, (2) overfilling of a bioreactor, caused by deactivation of the volume control, and (3) disturbance of air supply in a bioreactor, caused by, e.g., droplets in the inlet.

4.4.3.4.1 Stirring Failure

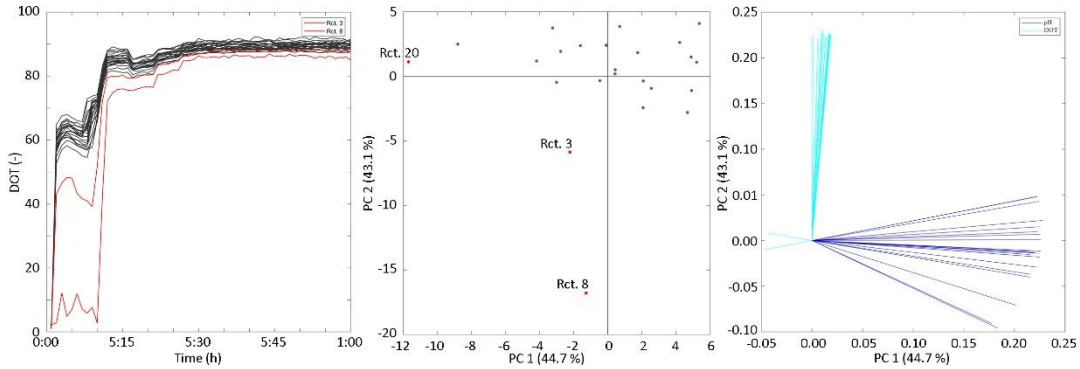
The moving horizon PCA model with eleven pH and DOT measurements revealed at least three reactors behaving differently after 20 min of batch phase. Reactors 3, 8, and 20 were identified as outliers by the automated program (see Figure 5b). DOT was identified as the causal variable for reactor 3, while pH was identified the causal variable for reactor 20. The



(**a**) (**b**)

Figure 4. Full horizon PCA approach at different times. (a) Score plot for the full horizon principal component analysis (PCA) model at time point t = 30 min. (b) Score plot for the full horizon PCA model at t = 10:22 h (entire cultivation). The eight groups of replicates are indicated by color and the reactors are numbered consecutively. The variance explained by the PC is indicated in percent in parenthesis.

first and second PC explain 44.4% and 43.1% variance, respectively. In the loading plots the orthogonal relation of the input variable pH and DOT is clearly visible, hence allowing to trace back the deviation in the score plot to the raw measurements (Figure 5c). While the variable from the DOT trajectory impacts the second PC almost exclusively, the pH trajectory has an impact on the first PC. Corresponding deviations were also observed in the on-line measurements. For reactors 3 and 8 lower DOT values were measured during the first 10 min of the cultivation (Figure 5a). In both reactors this was caused by a technical issue. The magnetic stirrer of reactor 8 did not start properly (this issue was detected by the operator and solved promptly). Reactor 20 is located the furthest away from the center point in respect to the first PC, indicating a lower pH but usual DOT. This finding is supported by the physiological state of the reactor.



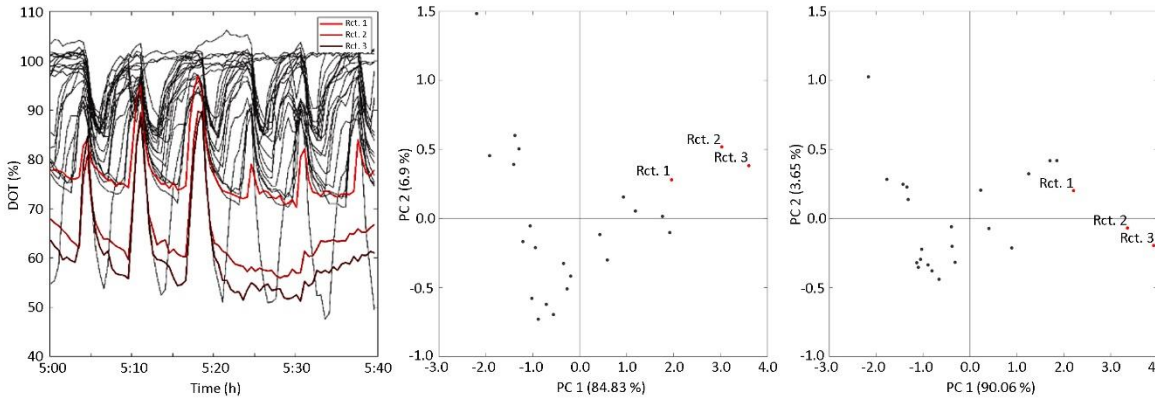
(**a**) (**b**) (**c**)

Figure 5. Detection of stirrer failures. (a) Trajectories for DOT and pH for all 24 reactors in the first hour of the cultivation. (b) Score plot for the sliding window PCA model with DOT and pH trajectories as input variables. The timeframe is t0 = 0 to tend = 20 min. Reactors 8 and 20 are distinctly separated from the main cluster. The variance explained by the PC is indicated in percent in parenthesis. (c) Loadings of the first two principal components for the same PCA model.

4.4.3.4.2 Reactor Overfill

At 05:40 h, the program detected reactor 3 to be an outlier. The contributing variable was identified to be the pH. Analyzing the score plot of the sliding window PCA at 05:40 h showed that reactors 2 and 3 did separate from their cluster of replicates (Figure 6c). Compared to the score plots at 05:20 h (Figure 6b), these two reactors where the only ones that did not move uniformly in one direction. Rather, the scores of reactors 2 and 3 shifted from the first to the fourth quadrant. Inspection of the first two PC shows that they explain more than 90% of the variance. The weights for the loading vector of the second component show negative correlation of pH and base addition to DOT (not shown). The DOT trajectory for these reactors did not feature the expecting oscillating pattern, indicating that the cultivation stopped reacting to the pulse-based feeding (Figure 6a).

While all three MBR were fed the same volume of glucose, the added volume of base differed. The total volume added decreases in reverse order of the reactors (3–1). Due to the missing volume control, this caused the reactors to exceed their upper volume limit, causing a blockage of the aeration system. The at-line analysis of the glucose and acetate media concentration showed a drastic increase of glucose and slight increase of acetate (Figure 2).

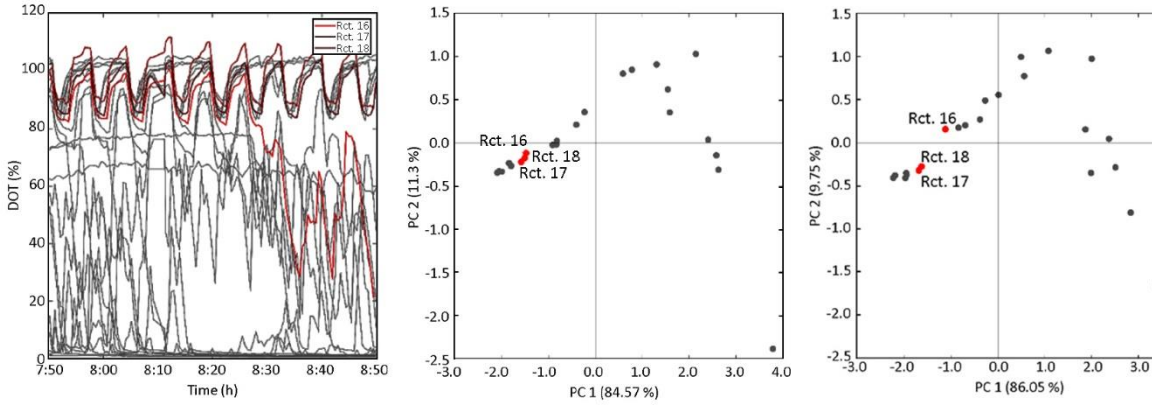


(**a**) (**b**) (**c**)

Figure 6. Reactor overfill. (a) Trajectories of the DOT profile from 05:00–05:40 h with highlighted reactors (1,2,3; highest feed rate). The pattern of peaks corresponds to the pulse-based feeding. (b,c) Score plot of the first two PC. The sliding window PCA model was built with data from 05:00–05:20 h (b) and 05:20–05:40 h (c) into the cultivation, respectively. The variance explained by the PC is indicated in percent in parenthesis.

4.4.3.4.3 Blockage of the Aeration System

Another instance causing an automated trigger was at 08:50 h when reactor 16 was identified to be an outlier with DOT as the causal variable. The score plots of the sliding window PCA models showed that the replicates (reactors 16, 17 and 18) behaved very similarly up to the time point 08:50 h (Figure 7b). At this point the score corresponding to reactor 16 abruptly digressed from the cluster (Figure 7c). The DOT profile of reactor 16 decreased unexpectedly in respect to its two replicates. This difference in sensor data was detected in the score plot for the first two PCs. The loadings for the DOT trajectories are negative in both sliding window PCA models (loading plot not shown). Comparison with the actual data implies a move of the score towards the positive axes, a trend that can be seen in the score plot (Figure 7c). The variance is explained to more than 95% by the first two principal components, indicating that the PCA model is well suited to describe the data and that the depicted score plot is sufficient for online monitoring. On the loading vector for the 1st PC, DOT and pH both correlates negatively to the base addition. Moreover, the weights of the input variable trajectories in the loadings do not differ significantly throughout one variable for the first loading vector. For the loading vector of the second PC, the weights for the most recent DOT measurement values increase (data not shown). Despite the fact that reactor 16 did not exceed its critical volume level, droplets of reactor medium may have covered the aeration hole and temporarily hinder oxygen transport into the reactor.



(**a**) (**b**) (**c**)

Figure 7. Aeration system blockage. (a) Trajectories of the DOT profile from 07:50–08:50 h into the cultivation with highlighted reactors (16,17,18; feed rate: μset = 40% of μmax).The DOT profiles of Reactor 16 deviates from its group of replicates, indicating the system stops reacting to the pulse-based feeding. (b,c) Score plot based on the sliding window PCA model with input variables pH, DOT, base volume addition, and feed volume addition in the time range t0 = 08:10 h to tend = 08:30 h (b) and t0 = 08:30 h to tend 08:50 h (c).

4.4.3.4.4 Pipetting Volume Inaccuracy

Based on the information of the scalar representation of abnormality, an overall trend stood out. The score plots of the PCA after 03:00 h showed that the value of the second PC usually monotonously de- or increased (see Figure 4b), corresponding to the arrangement of the MBR in the 2mag system. The accuracy of pipetting volume on the LHS for the cultivation was investigated after conclusion of the experiment. For this the LHS was given an identical setpoint for pipetting a small volume of colored water. The absorption differed from the actual pipetting volume by 2% for each column of MBR compared to the setpoint (data not shown). This pipetting volume inaccuracy accumulated throughout the cultivation and caused the pattern of score scattering on the second PC, as their order corresponds to increasing column indices of the MBR setup.

4.4.4 Discussion

Here we present a program to monitor parallel bioreactor systems in high-throughput microbial cultivations. With this program, we can supervise if replicates follow a coherent pattern throughout the experiment or, in case of irregularities, identify the failure automatically. Furthermore, the most common handling errors like stirrer failure, overfilling, and blockage of the aeration system were properly identified during the cultivation by the program.

The major benefits of this tool are manifold: Firstly, a compression of the information originally in multivariate form into a few two-dimensional plots facilitates supervision of the process. Secondly, a basic analysis of the data using standard PCA is sufficient to detect a number of undesired events during cultivation as well as to monitor the reproducibility of the

process and the operating robots. Finally, the automated event detection methods in the program are a step forward to a better and more intensive use of robotic experimental facilities, allowing longer campaigns and experiments with low supervision.

4.4.4.1 MVA for Monitoring Parallel Cultivations

With PCA as the mathematical method that is applied to the data, it is worth reflecting on its applicability in the herein described case. The PCA was used for data visualization and modest triggers such as outliers, but not for building a model of the process. This can only be done to a limited extent by the monitoring platform.

In future work, sophistication of the mathematical methods that are utilized for the tasks of data pre-processing, data analysis and model generation would improve the insights gained with the program. Some possibilities are PLS models for prediction, or the integration of existing mechanistic models [45–47] for the use of semi-parametric hybrid models [48,49]. In addition, alternative methods to the Euclidean distance that account for the correlation nature of the variable should be explored to ensure broader applicability of the classification of “unwanted” reactor behavior based on the scores. Possible methods would be a variation of the Mahalanobis distance with the benefit of considering the explained variance in the projected space [50].

Regarding the time delay of the at-line analytics, which on average accumulated to about 45 min in this cultivation, possible solutions would be to use different measurement methods or re-designing the workflow of the LHS that carried out the at-line analytics to free resources for an adapted sampling scheme. Alternative measurement methods additionally improve the timely availability of process data. As replacement of at-line measurements with online sensors, Raman spectroscopy [51], non-invasive biomass sensors [52] or fluorescence measurements of intracellular reporter systems [37] can further improve the approach. Another benefit of the presented program is the support in terms of calibration, operation, and selection of analytical devices and measurement frequency, i.e., the sampling and analytics workflow of the LHS could be adjusted dynamically based on the importance of input variables to the PCA as indicated in the loadings of the PCs [53].

4.4.4.2 Common Failure Events

The stirrer failure event demonstrated that quick reaction is crucial for a successful cultivation. A supervision of the cultivations’ status by simple visual inspection of each critical process variable in each reactor is already difficult for 24 reactors. The undesired reactor disturbances and the variables with the largest contribution to it are automatically detected using modest approach such as the inherent properties of PCA. Additionally, the trends are visualized in a concise two-dimensional score and contribution plots which allows the operator

to examine the status of reactors of interest in more depth. However, as with the stirrer failure, the timely availability of data remains a key issue.

Still, the results also show that the abstraction of PCA is not sufficient to derive concrete measures to steer the process back to the experimental constraints. However, in the case of the events that caused the sudden drop of DOT, PCA proved to be useful in the reduction of the data dimension. As the feeding strategy in high throughput experiments usually varies for each group of replicates and the DOT trajectory is oscillating due to the pulse-based feeding, visually inspecting the profiles for all reactors throughout a 10 h cultivation would become very cumbersome or even impossible. Thus, as demonstrated in this paper, score plots are a great tool to help identify irregularities.

4.4.5 Conclusions

We here develop and demonstrate the benefits of a remote tool for online multivariate analysis of parallel cultivations and its applicability to monitor dynamic HT experiments—a task that has become hardly manageable by lab operators. The black box nature of the MVA makes it independent of the type of experiments or cultivation vessels used. This feature makes it particularly useful for early-stage bioprocess development with little process understanding and highly varying experimental systems. We have shown that the program is able to provide data-based insight to guide operator decisions in a large multidimensional data set at the example of complex HT fed-batch cultivations. Despite high process variability and the large amount of datapoints, the tool simplified the task to derive decisions and allows conclusion to be drawn about the similarity of replicates and causes for deviations, improving the reproducibility of a bioprocess.

The freely available program (see below) presented in this work is a useful tool for the monitoring and operation of high throughput bioprocess development facilities. Due to the modular program structure and the fact that the software relies on an open server–client interface, only small code adjustments on the backend are necessary if the app is deployed in a different environment, such as a different working group.

This approach is in the scope of the different model variants [13]: A descriptive model allows for visualization and interpretation in a reduced dimension space along the central process information. Moreover, it also supports diagnosis of abnormalities and deviations. For the future, it would be important to further develop the tool towards predictive and prescriptive capabilities, i.e., to proactively foresee deviations and suggest on possible process control actions, so to most efficiently utilize the experimental data and potential of each ongoing experiment.

Supplementary Materials

The following are available online at https://www.mdpi.com/2227-9717/8/5/582/s1, Table S1: Cultivation Data, Code S1: MonitoringApp, available at: https://gitlab.tubit.tu-berlin.de/publication/2019_Ulmer.

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Conflicts of Interest

The authors declare no conflict of interest.

4.4.6 References

1. Neubauer, P.; Glauche, F.; Bournazou, M.N.C. Editorial: Bioprocess Development in the era of digitalization. Eng. Life Sci. **2017**, 17, 1140–1141.
2. Neubauer, P. Editorial: Towards faster bioprocess development. Biotechnol. J. **2011**, 6, 902–903.
3. Islam, R.S.; Tisi, D.; Levy, M.S.; Lye, G.J. Framework for the Rapid Optimization of Soluble Protein Expression in Escherichia coli Combining Microscale Experiments and Statistical Experimental Design. Biotechnol. Prog. **2007**, 23, 785–793.
4. Glauche, F.; Pilarek, M.; Bournazou, M.N.C.; Grunzel, P.; Neubauer, P. Design of experiments-based high-throughput strategy for development and optimization of efficient cell disruption protocols. Eng. Life Sci. **2016**, 17, 1166–1172.
5. Küppers, T.; Steffen, V.; Hellmuth, H.; O'Connell, T.; Bongaerts, J.; Maurer, K.-H.; Wiechert, W. Developing a new production host from a blueprint: Bacillus pumilus as an industrial enzyme producer. Microb. Cell Factories **2014**, 13, 46.
6. Jorgensen, J.T. A challenging drug development process in the era of personalized medicine. Drug Discov. Today **2011**, 16, 891–897.
7. Paritala, P.K.; Manchikatla, S.; Yarlagadda, P.K.D.V. Digital Manufacturing-Applications Past, Current, and Future Trends. Procedia Eng. **2017**, 174, 982–991.

8. Nickel, D.B.; Bournazou, M.N.C.; Wilms, T.; Neubauer, P.; Knepper, A. Online bioprocess data generation, analysis, and optimization for parallel fed-batch fermentations in milliliter scale. Eng. Life Sci. **2016**, 17, 1195–1201.
9. Kim, J.H. A Review of Cyber-Physical System Research Relevant to the Emerging IT Trends: Industry 4.0, IoT, Big Data, and Cloud Computing. J. Ind. Integr. Manag. **2017**, 2, 1750011.
10. Ghobakhloo, M. The future of manufacturing industry: A strategic roadmap toward Industry 4.0. J. Manuf. Technol. Manag. **2018**, 29, 910–936.
11. Gani, R. Computer Aided Process and Product Engineering, Comput; WILEY-VCH Verlag GmbH & Co. KGaA: Weinheim, Germany, 2006; pp. 647–666.
12. Stephanopoulos, G.; Reklaitis, G.V. Process systems engineering: From Solvay to modern bio- and nanotechnology. Chem. Eng. Sci. **2011**, 66, 4272–4306.
13. Narayanan, H.; Luna, M.F.; Von Stosch, M.; Bournazou, M.N.C.; Polotti, G.; Morbidelli, M.; Butté, A.; Sokolov, M. Bioprocessing in the Digital Age: The Role of Process Models. Biotechnol. J. **2019**, 15, e1900172.
14. Abt, V.; Barz, T.; Cruz-Bournazou, M.N.; Herwig, C.; Kroll, P.; Möller, J.; Pörtner, R.; Schenkendorf, R.; Cruz, N. Model-based tools for optimal experiments in bioprocess engineering. Curr. Opin. Chem. Eng. **2018**, 22, 244–252.
15. Löffler, M.; Simen, J.D.; Jäger, G.; Schäferhoff, K.; Freund, A.; Takors, R. Engineering E. coli for large-scale production – Strategies considering ATP expenses and transcriptional responses. Metab. Eng. **2016**, 38, 73–85.
16. Sonnleitner, B. Bioprocess automation and bioprocess design. J. Biotechnol. **1997**, 52, 175–179.
17. Wilson, L.J.; Lewis, W.; Kucia-Tran, R.; Bracewell, D.G. Identification of upstream culture conditions and harvest time parameters that affect host cell protein clearance. Biotechnol. Prog. **2019**, 35, e2805.
18. Randek, J.; Mandenius, C.-F. On-line soft sensing in upstream bioprocessing. Crit. Rev. Biotechnol. **2017**, 38, 106–121.
19. Kourti, T. Process Analytical Technology Beyond Real-Time Analyzers: The Role of Multivariate Analysis. Crit. Rev. Anal. Chem. **2006**, 36, 257–278.
20. Hemmerich, J.; Noack, S.; Wiechert, W.; Oldiges, M. Microbioreactor Systems for Accelerated Bioprocess Development. Biotechnol. J. **2018**, 13, 1700141.
21. Haby, B.; Hans, S.; Anane, E.; Sawatzki, A.; Krausch, N.; Neubauer, P.; Bournazou, M.N.C. Integrated Robotic Mini Bioreactor Platform for Automated, Parallel Microbial Cultivation With Online Data Handling and Process Control. SLAS Technol. Transl. Life Sci. Innov. **2019**, 24, 569–582.

22. Anane, E.; García, Á.C.; Haby, B.; Hans, S.; Krausch, N.; Krewinkel, M.; Hauptmann, P.; Neubauer, P.; Bournazou, M.N.C. A model-based framework for parallel scale-down fed-batch cultivations in mini-bioreactors for accelerated phenotyping. Biotechnol. Bioeng. **2019**, 116, 2906–2918.
23. Janzen, N.H.; Striedner, G.; Jarmer, J.; Voigtmann, M.; Abad, S.; Reinisch, D. Implementation of a Fully Automated Microbial Cultivation Platform for Strain and Process Screening. Biotechnol. J. **2019**, 14, e201800625.
24. Bournazou, M.N.C.; Barz, T.; Nickel, D.B.; Cárdenas, D.L.; Glauche, F.; Knepper, A.; Neubauer, P. Online optimal experimental re-design in robotic parallel fed-batch cultivation facilities. Biotechnol. Bioeng. **2016**, 114, 610–619.
25. Barz, T.; Sommer, A.; Wilms, T.; Neubauer, P.; Bournazou, M.N.C.; Throughput, A. Adaptive optimal operation of a parallel robotic liquid handling station. In Proceedings of the 9th Vienna Internacional Conference Mathematical Model, Vienna, Austria, 21–23 February 2018; pp. 901–906.
26. Hans, S.; Gimpel, M.; Glauche, F.; Neubauer, P.; Bournazou, M.N.C.; Gimpel, M. Automated Cell Treatment for Competence and Transformation of Escherichia coli in a High-Throughput Quasi-Turbidostat Using Microtiter Plates. Microorganisms **2018**, 6, 60.
27. Sawatzki, A.; Hans, S.; Narayanan, H.; Haby, B.; Krausch, N.; Sokolov, M.; Glauche, F.; Riedel, S.L.; Neubauer, P.; Bournazou, M.N.C. Accelerated Bioprocess Development of Endopolygalacturonase-Production with Saccharomyces cerevisiae Using Multivariate Prediction in a 48 Mini-Bioreactor Automated Platform. Bioengineering **2018**, 5, 101.
28. Nomikos, P.; MacGregor, J.F. Monitoring batch processes using multiway principal component analysis. AIChE J. **1994**, 40, 1361–1375.
29. Sokolov, M.; Ritscher, J.; MacKinnon, N.; Brühlmann, D.; Rothenhäusler, D.; Thanei, G.; Soos, M.; Stettler, M.; Souquet, J.; Broly, H.; et al. Robust factor selection in early cell culture process development for the production of a biosimilar monoclonal antibody. Biotechnol. Prog. **2016**, 33, 181–191.
30. Undey, C.; Ertunç, S.; Mistretta, T.; Looze, B. Applied advanced process analytics in biopharmaceutical manufacturing: Challenges and prospects in real-time monitoring and control. J. Process. Control. **2010**, 20, 1009–1018.
31. Gunther, J.; Baclaski, J.; Seborg, D.; Conner, J. Pattern matching in batch bioprocesses—Comparisons across multiple products and operating conditions. Comput. Chem. Eng. **2009**, 33, 88–96.

32. Thomassen, Y.E.; Van Sprang, E.N.; Van Der Pol, L.A.; Bakker, W.A. Multivariate data analysis on historical IPV production data for better process understanding and future improvements. Biotechnol. Bioeng. **2010**, 107, 96–104.
33. Kirdar, A.; Conner, J.; Baclaski, J.; Rathore, A.S. Application of Multivariate Analysis toward Biotech Processes: Case Study of a Cell-Culture Unit Operation. Biotechnol. Prog. **2007**, 23, 61–67.
34. Wang, X.; Kruger, U.; Irwin, G.W. Process Monitoring Approach Using Fast Moving Window PCA. Ind. Eng. Chem. Res. **2005**, 44, 5691–5702.
35. Brand, E.; Junne, S.; Anane, E.; Bournazou, M.N.C.; Neubauer, P. Importance of the cultivation history for the response of Escherichia coli to oscillations in scale-down experiments. Bioprocess Biosyst. Eng. **2018**, 41, 1305–1313
36. Delvigne, F.; Goffin, P. Microbial heterogeneity affects bioprocess robustness: Dynamic single-cell analysis contributes to understanding of microbial populations. Biotechnol. J. **2013**, 9, 61–72.
37. Gawin, A.; Peebo, K.; Hans, S.; Ertesvåg, H.; Irla, M.; Neubauer, P.; Brautaset, T. Construction and characterization of broad-host-range reporter plasmid suitable for on-line analysis of bacterial host responses related to recombinant protein production. Microb. Cell Factories **2019**, 18, 80.
38. Anane, E.; Sawatzki, A.; Neubauer, P.; Bournazou, M.N.C. Modelling concentration gradients in fed-batch cultivations of E. coli - towards the flexible design of scale-down experiments. J. Chem. Technol. Biotechnol. **2018**, 94, 516–526.
39. Enfors, S.-O. Fermentation Process Technology; Technische Universität Berlin: Sven-Olof Enfors, Stockholm, Sweden, 2019.
40. Wouter Falkena, xml2struct, MATLAB Central File Exchange. Available online: https://www.mathworks.com/matlabcentral/fileexchange/28518-xml2struct (accessed on 1 January 2019).
41. MacGregor, J.; Kourti, T. Statistical process control of multivariate processes. Control. Eng. Pr. **1995**, 3, 403–414.
42. Jolliffe, I.T. Principal Component Analysis. Springer Series in Statistics **2002**, 98, 487.
43. Abdi, H.; Williams, L. Principal component analysis. Wiley Interdiscip. Rev. Comput. Stat. **2010**, 2, 433–459.
44. Anane, E.; Neubauer, P.; Bournazou, M.N.C. Modelling overflow metabolism in Escherichia coli by acetate cycling. Biochem. Eng. J. **2017**, 125, 23–30.
45. Sokolov, M.; Ritscher, J.; MacKinnon, N.; Souquet, J.; Broly, H.; Morbidelli, M.; Butté, A. Enhanced process understanding and multivariate prediction of the relationship between cell culture process and monoclonal antibody quality. Biotechnol. Prog. **2017**, 33, 1368–1380.

46. Duan, Z.; Wilms, T.; Neubauer, P.; Kravaris, C.; Bournazou, M.N.C. Model reduction of aerobic bioprocess models for efficient simulation. Chem. Eng. Sci. **2020**, 217, 115512.
47. Von Stosch, M.; Hamelink, J.-M.; Oliveira, R. Hybrid modeling as a QbD/PAT tool in process development: An industrial E. coli case study. Bioprocess Biosyst. Eng. **2016**, 39, 773–784.
48. Narayanan, H.; Sokolov, M.; Morbidelli, M.; Butté, A. A new generation of predictive models: The added value of hybrid models for manufacturing processes of therapeutic proteins. Biotechnol. Bioeng. **2019**, 116, 2540–2549.
49. Neubauer, P.; Cruz, N.; Glauche, F.; Junne, S.; Knepper, A.; Raven, M. Consistent development of bioprocesses from microliter cultures to the industrial scale. Eng. Life Sci. **2013**, 13, 224–238.
50. Mercier, S.M.; Diepenbroek, B.; Wijffels, R.H.; Streefland, M. Multivariate PAT solutions for biopharmaceutical cultivation: Current progress and limitations. Trends Biotechnol. **2014**, 32, 329–336.
51. Lee, H.L.; Boccazzi, P.; Gorret, N.; Ram, R.J.; Sinskey, A.J. In situ bioprocess monitoring of Escherichia coli bioreactions using Raman spectroscopy. Vib. Spectrosc. **2004**, 35, 131–137.
52. Buchenauer, A.; Hofmann, M.; Funke, M.; Büchs, J.; Mokwa, W.; Schnakenberg, U. Micro-bioreactors for fed-batch fermentations with integrated online monitoring and microfluidic devices. Biosens. Bioelectron. **2009**, 24, 1411–1416.
53. Kourti, T.; Nomikos, P.; MacGregor, J.F. Analysis, monitoring and fault diagnosis of batch processes using multiblock and multiway PLS. J. Process. Control. **1995**, 5, 277–284.

5 Discussion

The acceleration of two essential parts of bioprocess engineering, library screening and conditional screening, is the focus of this work. For this purpose, it was necessary to develop novel tools to combine automation, modelling and data processing. An automated workflow for the bioprocess, sampling, analysis, data storage in a central location and data treatment was realized. This was the basis to run model-based algorithms for enhanced bioprocess designs. The workflow is further enhanced to allow automatic execution of the model-based bioprocess design by the LHS. This thesis closes the loop of process inputs, execution, and monitoring to enable autonomously acting bioprocess facilities.

RQ 1: How can dynamic data input within a statically working LHS be achieved?

In all four studies, methods are performed that are not fully defined prior to process start-up by the LHS. On the Tecan LHS, which is hosting the MBR, these include amongst others feed, pH control and volume balancing (Paper 2-4), on the Hamilton LHS side these include the turbidostat culture dilution rate (Paper 1) and the OD_{600} sample dilution rate (Paper 2-4).

For the turbidostat, the cells are incubated, the OD_{600} is measured by the plate reader, the data are stored in a file, the file is read in by the LHS software and the data are written into the relational database. The modelling application is then started by the LHS software, retrieves the OD_{600} readings of the current and the last measurement from the database, determines the respective growth rate, and calculates the corresponding dilutions. These dilutions are saved to another table in the same database. The LHS software pauses for the duration of the modelling application execution and then retrieves the dilution volumes from the database. This represents a complex process flow with several software from different vendors involved. However, on the LHS side the process can be mapped in simple loops. Using the developed database interface for the Hamilton LHS, it is possible to specify the database interactions before starting the method. No changes in the LHS method depending on the current bioprocess state are necessary and a dynamic operation is possible. Data exchange through the database ensures modularity in that there are no dependencies between the data generating system (LHS) and the data processing algorithm.

The PyHamilton, an open source framework for controlling Hamilton LHS, is introduced with the example of a turbidostat in MWP, too (Chory et al., 2020). Here the whole LHS and plate reader control is transferred into Python commands and the necessary calculations are handled in a single application. Alternatively, the inclusion of the calculations as Hamilton library could be considered. Similar packages to PyHamilton are also available for Tecan LHS as RobotEvo, (Viña Rodríguez, 2018) pyTecan (Townshend, 2013) or pyTecanFluent (Youngblut, 2017). Combining LHS operations and model calculations into one application simplifies the entire process. However, it significantly reduces the transferability of the method

and requires expert knowledge of the LHS as well as the biological / mathematical background. This can be neglected for such simple methods as the turbidostat, but not for more complex models as used in paper 3.

The MBR-hosting LHS performs various work steps that have an impact on the reactor volume (e.g.: feed, pH control, sampling). These methods are integrated as individual modules, store and receive their data from the database and do not require any communication between each other. This makes modification or replacement of these modules extremely easy. The volume balancing in the mini bioreactors is one of the more complex tasks from the data processing view. For this purpose, the information of the modules mentioned need to be merged. The use of the database also decouples this task from the other modules and impressively illustrates the advantages of a central data repository for the modularity of a facility. Porting the methods to other systems is quickly possible. Likewise, the creation of new modules or the expansion of the dataset can be done with only a few adaptations in the SQL queries and would be more time-consuming with a file-based solution.

RQ 2: How can multiple parallel bioprocess be monitored?

Process monitoring is important to ensure a stable process. An undesirable process can only be counteracted if it is detected in time. This applies equally to manual and automated bioprocesses. Here (in Paper 4) PCA is used to build a user-friendly interface that quickly shows the most important process failures in the experiment. The PCA is fed with all available information. Thus, not only on-line and at-line analytics are included in the model, but also pipetting volumes and feeding rates. Dependent or correlated data are weighted accordingly within the PCA and combined into principal components. The application created here was able to identify the most important process irregularities and to present them in an easily understandable way.

Multivariate Data Analysis (MVDA) methods like PCA have been used numerous times for process monitoring in the past (Nomikos and MacGregor, 1994; Kourti, 2006; Kirdar et al., 2007; Gunther et al., 2009; Ündey et al., 2009; Thomassen et al., 2010; Glassey, 2013; Sokolov et al., 2017). In general, the most applications train a PCA model on a "golden batch" basis to detect deviations and are therefore only very limitedly applicable to unknown strains, as is the case here. In this study, the detection of unwanted behavior is only possible with the use of several replicates which are compared to each other. Thus, it is not so much the deviation from an ideal experimental course that is evaluated (the "golden batch" is not available), but the uniformity of the replicates. This requires that the models are created during experiments, a computationally intensive process that can be improved by refined methods (Wang et al., 2005). Hybrid modeling approaches consisting of mechanistic and MVDA models, as previously used in bioprocess evaluation (von Stosch et al., 2014; Narayanan et

al., 2019; 2020), can further improve the online monitoring approach by incorporating an abstraction of a typical bioprocess run.

RQ 3: Can online model adaptation and dynamic redesign be used for continuous growth alignment?

With the implementation of the quasi-turbidostat in the study Automated Cell Treatment for Competence and Transformation of *Escherichia coli* in a High-Throughput Quasi-Turbidostat Using Microtiter Plates (Paper 1) it is possible to align strains with different growth behaviour. By iteratively monitoring the biomass, determining the growth rate, and predicting the growth for the next process interval, the required target biomass value for all strains could be successfully achieved at a predictable time point. This has been successfully used to make strains competent and to transform them in a single automated process.

In this study, the major achievement is not cell competence and subsequent transformation in an automated manner focus, as this has been done by others before (Finley et al., 2004; Hughes et al., 2007). Crucial for an efficient transformation is the initial step of cell harvesting in the exponential phase. A simplified knowledge-based model is used here. The mathematical model represents the growth of microbials like *E. coli* and consists only of one equation ($\frac{dx}{dt} = \mu_{max}\, X$). Only the parameter μ_{max} needs to be adjusted to fit to the respective host. Since the model is very simple, most of the parameter fitting problems are eliminated and the model can even be fitted to a raw data set. Assumptions made in advance , e.g. consideration of only one batch phase and independency of the growth from medium, enable the use of such a simple model. Even though it is known that these conditions do not hold true, as for example diauxic phases happen in complex media (Selvarasu et al., 2009), these influences were neglected in the model.

The turbidostat represents a contiuous cultivation at the maximum growth rate in MWP format and promises a broad application range. Complex evolution experiments, some of which last thousands of generations, can be automated and parallelized (Lenski et al., 1991). In addition to saving material and personnel costs, the higher throughput could also shorten the experimental times until a targeted adaptation is achieved. These experiments can be further supported by adaptive laboratory evolution experiments models and improved in a targeted manner (LaCroix et al., 2017).

RQ 4: How can high-quality parameter fitting with potentially inadequate data be ensured?

In this thesis (paper 3) a mechanistic model with 5 state variables and 18 parameters was individually adapted to eight different strains. The model was able to reproduce the process

data at any time and to make precise predictions for further process behavior with increasing process duration (increasing data basis).

The calibration of mechanistic models to on- and at-line data with the aim to accelerate the design of experiments has been successfully performed several times in the past (McMullen and Jensen, 2011; Barz et al., 2013; Echtermeyer et al., 2017) also for bioprocesses (Cruz Bournazou et al., 2017). However, the experiments in these studies are not ordinary experiments but rather specifically tailored for model fitting, using special hardware or needing several sequential experiments. Here a common experimental protocol should be followed, consisting of batch, exponential fed-batch and constant fed-batch process phases.

The quality of the model parameter estimation varies depending on the amount of data and the process dynamics. To ensure a significant parameter set, parameter subset selection was used to fit only those parameters for which the data set is sufficient. Alternative and complementary methods for parameter regularization are available and can be used for a more robust model fitting (Gábor et al., 2017; Abdulla and Poteau, 2021).

However, parameter regularization is only the second-best way to achieve an optimal model calibration. An improved data set would be much more beneficial. In addition to more frequent sampling, the use of spectral on-line measurements, like RAMAN and NIR, promises particular added value (Claßen et al., 2017).

RQ 5: An autonomously working bioprocess development facility

The main achievement of this thesis is the development of the autonomous bioprocess facility. It is capable of successfully cultivating up to 48 unknown strains in parallel. To be able to carry out high-quality bioprocesses in parallel, the executing facility must be able to operate the process in a -target oriented manner. The facility needs to have contextual knowledge about the strains and the bioreactor which can only be passed into the facility with a mechanistic model. The automatic calibration of this model to the respective uncharacterized strains made an individually tailored bioprocess strategy possible.

The target quality parameters in this work are the accurate feed start and the avoidance of overfeeding or starvation. The feed start was determined based on simulation of the mechanistic model. In the model biomass, glucose, acetate, and oxygen are considered. The simulation results can be used to calculate when glucose and acetate are depleted and thus the time for the feed start can be determined. Using a well-calibrated model, this allows prediction of the batch end to the minute. Alternatives to batch end detection, e.g. using the DOT or pH signal (Janzen et al., 2019), can only be used recursively. The model used here also estimates the volume and biomass concentration, information that are needed to calculate the initial feed rate.

For the feeding, model parameters are combined to determine the maximum growth rate. To ensure a robust process design, 50% of the maximum growth rate is considered optimal in this study. However, the feedrate is one of the most optimised parameters in later bioprocess development and can be controlled by various approaches (Modak et al., 1986; Mears et al., 2017). Because of parameter variations, the strict definition in this study led to a frequent adjustment of the feeding profile The changes to the feed have been greatest in the early stages of the bioprocess when the data bases for model calibration were fewer and the model parameters varied most between model calibrations.

Model based bioprocess design is sufficient for a robust process but does not yet represent an optimal process and leaves out possibilities of the used facility. Using Model Predictive Control (MPC) allows more complex multivariate control and has been applied many times in industry processes (Lee, 2011). However, pure mechanistic models reach their limits in MPC applications, and hybrid models are a promising alternative for enhanced bioprocess design (Narayanan et al., 2020).

6 Conclusions and Outlook

Screening a large number of new strains or vectors in the shortest possible time with meaningful results will become one of the challenges of the coming years. This can only be achieved by integrating modular automation, intelligent data management and self-learning models for bioprocess control. This work is a step in this direction and shows that even with the simplest models, highly complex automation tasks can be properly computed.

The models presented in this work are tailored solutions for the particular issues and available datasets. The knowledge-based modelling approaches used here are extremely useful for bioprocess control, as demonstrated in the two examples shown. The complexity of the models can be sufficiently reduced, the influences that are no longer represented in the model after model reduction are known, and the informative value of the model can be estimated in advance. However, black-box modelling approaches can be a useful addition to existing models, especially in cases where knowledge-based approaches face knowledge gaps, are too complex for the available data, or meaningful model reduction is no longer possible. Hybrid combinations of mechanistic models and neural networks have been developed in many areas and will further strengthen autonomous bioprocessing, as will model discrimination methods, where different models are tested to detect the best candidate for a specific task. Here, a decision algorithm has to determine which model should be used for process control in the respective experimental phase. Such a framework would enable different groups to test their model concepts in real scenarios without the risk of complete failure of the respective experiment.

Further advances in measurements and sensors will also have a positive effect on the modeling and thus on the autonomy of the bioprocess facility. On the one hand, this can mean a compression of the existing measurement data (e.g. OD_{600}, glucose, acetate) as well as the extension of the analytical spectrum. The most obvious extension is the detection and quantification of recombinant products beyond fluorescent proteins. For example, ELISA assays have already been widely implemented and automated on LHS. Advanced spectrometric (RAMAN, NIR) and imaging techniques have also been successfully automated and can provide important information for online bioprocess modelling.

The increase in the measurements goes hand in hand with more devices to be integrated and more complex automation solutions. In this work, monolithic methods were run in various loops. Keeping the individual process steps self-contained and modular has proven successful and led to flexible methods. However, the process flow was fixed and could only be changed in the volumes to be pipetted. The integration of new devices and software is made more difficult in this way since the overall system must always be adapted as well. The use of a superordinate orchestration framework will therefore be unavoidable in the near future. Here,

the process steps are broken down into smallest tasks and subtasks and triggered by a third software. Such a solution would give new freedom to modeling and process optimization, will simplify the integration of new devices, analyses or software packages, but will also increase the overall complexity. The development of a new and comprehensively dynamic workflow orchestration concept is one of the most urgent tasks in order to increase the results' quality with the same effort (in equipment, time and costs).

Furthermore, the use of a central data lake where all data is consistently stored plays a decisive role for rapid expansion and modularity. The relational database used here was one of the success factors for cross-device communication and online model calibration. With the predicted introduction of new measurement methods, the structure of the database needs to be reconsidered. Relational databases are well suited for structured data. Spectral data can also be stored conditionally well in them. Nevertheless, unstructured data such as microscopy images require more extensive data management.

In the fourth study presented, the development of a SiLA-enabled database interface in particular is an invitation to interested groups to participate in the development of new modeling and monitoring techniques. The data stored in the database are easy to interpret for domain competent scientists, but difficult to comprehend for researchers from other domains, such as mathematics or computer science. Making the data available according to FAIR Data principles is therefore a worthy goal and will require even further documentation and explanation of the respective experiments.

In the bioprocess monitoring study, the PCA was able to detect the most important and frequent process faults in the operation with the used facility. Afterwards, a possibility of automatic error exposure was developed. However, its implementation into a real alarm function is still pending. With more data, new training cases can be implemented. In addition, the presented approach for process error exposure should be further refined.

7 References

Abdulla, U.G., Poteau, R., 2021. Identification of parameters for large-scale kinetic models. Journal of Computational Physics 429, 110026. doi:10.1016/j.jcp.2020.110026

Agresti, J.J., Antipov, E., Abate, A.R., Ahn, K., Rowat, A.C., Baret, C., Marquez, M., Klibanov, A.M., Grif, A.D., Weitz, D.A., Aga, G.A.L., 2010. Ultrahigh-throughput screening in drop-based microfluidics for directed evolution. Proceedings of the National Academy of Sciences 107, 1–6. doi:10.1073/pnas.1002891107

Almquist, J., Cvijovic, M., Hatzimanikatis, V., Nielsen, J., Jirstrand, M., 2014. Kinetic models in industrial biotechnology - Improving cell factory performance. Metabolic Engineering 24, 38–60. doi:10.1016/j.ymben.2014.03.007

Anane, E., López C, D.C., Neubauer, P., Cruz Bournazou, M.N., 2017. Modelling overflow metabolism in Escherichia coli by acetate cycling. Biochemical Engineering Journal 125, 23–30. doi:10.1016/j.bej.2017.05.013

Arain, S., John, G.T., Krause, C., Gerlach, J., Wolfbeis, O.S., Klimant, I., 2006. Characterization of microtiterplates with integrated optical sensors for oxygen and pH, and their applications to enzyme activity screening, respirometry, and toxicological assays. Sensors and Actuators, B: Chemical 113, 639–648. doi:10.1016/j.snb.2005.07.056

Argelaguet, R., Velten, B., Arnol, D., Dietrich, S., Zenz, T., Marioni, J.C., Buettner, F., Huber, W., Stegle, O., 2018. Multi-Omics Factor Analysis—a framework for unsupervised integration of multi-omics data sets. Molecular Systems Biology 14, 1–13. doi:10.15252/msb.20178124

Axe, D.D., Bailey, J.E., 1995. Transport of lactate and acetate through the energized cytoplasmic membrane of Escherichia coli. Biotechnology and Bioengineering 47, 8–19. doi:10.1002/bit.260470103

Barz, T., López Cárdenas, D.C., Arellano-Garcia, H., Wozny, G., 2013. Experimental evaluation of an approach to online redesign of experiments for parameter determination. AIChE Journal 59, 1981–1995. doi:10.1002/aic.13957

Basan, M., Hui, S., Okano, H., Zhang, Z., Shen, Y., Williamson, J.R., Hwa, T., 2015. Overflow metabolism in Escherichia coli results from efficient proteome allocation. Nature 528, 99–104. doi:10.1038/nature15765

Bauer, K.A., Ben-Bassat, A., Dawson, M., La Puente, V.T. De, Neway, J.O., 1990. Improved expression of human interleukin-2 in high-cell-density fermentor cultures of Escherichia coli K-12 by a phosphotransacetylase mutant. Applied and Environmental Microbiology 56, 1296–1302. doi:10.1128/aem.56.5.1296-1302.1990

Blesken, C., Olfers, T., Grimm, A., Frische, N., 2016. The microfluidic bioreactor for a new era of bioprocess development. Engineering in Life Sciences 16, 190–193. doi:10.1002/elsc.201500026

BMBF, 2010. Nationale Forschungsstrategie BioÖkonomie 2030 Unser Weg zu einer bio-basierten Wirtschaft. Berlin / Bonn.

Bryson, V., Szybalski, W., 1952. Microbial Selection. Science 116, 45–51. doi:10.1126/science.116.3003.45

Buchenauer, A., Hofmann, M.C., Funke, M., Büchs, J., Mokwa, W., Schnakenberg, U., 2009. Micro-bioreactors for fed-batch fermentations with integrated online monitoring and microfluidic devices. Biosensors and Bioelectronics 24, 1411–1416. doi:10.1016/j.bios.2008.08.043

Calleja, D., Kavanagh, J., Mas, C. de, López-Santín, J., 2016. Simulation and prediction of protein production in fed-batch E. coli cultures: An engineering approach. Biotechnology and Bioengineering 113, 772–782. doi:10.1002/bit.25842

Camacho, D.M., Collins, K.M., Powers, R.K., Costello, J.C., Collins, J.J., 2018. Next-Generation Machine Learning for Biological Networks. Cell 173, 1581–1592. doi:10.1016/j.cell.2018.05.015

Cancer, G.P., Zimmermann, H.F., Trauthwein, H., Dingerdissen, U., 2004. Monitoring aerobic Escherichia coli growth in shaken microplates by measurement of culture fluorescence 36, 36–38.

Chassagnole, C., Noisommit-Rizzi, N., Schmid, J.W., Mauch, K., Reuss, M., 2002. Dynamic modeling of the central carbon metabolism of Escherichia coli. Biotechnology and Bioengineering 79, 53–73. doi:10.1002/bit.10288

Chory, E.J., Gretton, D.W., DeBenedictis, E.A., Esvelt, K.M., 2020. Flexible open-source automation for robotic bioengineering. bioRxiv. doi:10.1101/2020.04.14.041368

Claßen, J., Aupert, F., Reardon, K.F., Solle, D., Scheper, T., 2017. Spectroscopic sensors for in-line bioprocess monitoring in research and pharmaceutical industrial application. Analytical and Bioanalytical Chemistry 409, 651–666. doi:10.1007/s00216-016-0068-x

Costa, R.S., Machado, D., Rocha, I., Ferreira, E.C., 2010. Hybrid dynamic modeling of Escherichia coli central metabolic network combining Michaelis-Menten and approximate kinetic equations. BioSystems 100, 150–157. doi:10.1016/j.biosystems.2010.03.001

Cruz Bournazou, M.N., Barz, T., Nickel, D.B., Lopez Cárdenas, D.C., Glauche, F., Knepper, A., Neubauer, P., 2017. Online optimal experimental re-design in robotic parallel fed-batch cultivation facilities. Biotechnology and Bioengineering 114, 610–619. doi:10.1002/bit.26192

Cui, L., Wang, Z., Xu, T., Song, H., 2017. Prediction of the product formation with the AdaBoost algorithm in bioprocesses. International Journal of System Control and Information

Processing 2, 180–189. doi:10.1504/IJSCIP.2017.089816

Doppler, P., Veiter, L., Spadiut, O., Herwig, C., Rajamanickam, V., 2020. A chemometric tool to monitor and predict cell viability in filamentous fungi bioprocesses using UV chromatogram fingerprints. Processes 8. doi:10.3390/PR8040461

Duetz, W.A., 2007. Microtiter plates as mini-bioreactors: miniaturization of fermentation methods. Trends in Microbiology 15, 469–475. doi:10.1016/j.tim.2007.09.004

Duetz, W.A., Witholt, B., 2004. Oxygen transfer by orbital shaking of square vessels and deepwell microtiter plates of various dimensions. Biochemical Engineering Journal 17, 181–185. doi:10.1016/S1369-703X(03)00177-3

Duetz, W.A., R??edi, L., Hermann, R., O'Connor, K., B??chs, J., Witholt, B., 2000. Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. Applied and Environmental Microbiology 66, 2641–2646. doi:10.1128/AEM.66.6.2641-2646.2000

Dugar, D., Stephanopoulos, G., 2011. Relative potential of biosynthetic pathways for biofuels and bio-based products. Nature biotechnology 29, 1074—1078. doi:10.1038/nbt.2055

Echtermeyer, A., Amar, Y., Zakrzewski, J., Lapkin, A., 2017. Self-optimisation and model-based design of experiments for developing a C-H activation flow process. Food, Pharmaceutical and Bioengineering Division 2017 - Core Programming Area at the 2017 AIChE Annual Meeting 2, 747–760. doi:10.3762/bjoc.13.18

Edwards-Parton, S., Thornhill, N.F., Bracewell, D.G., Liddell, J.M., Titchener-Hooker, N.J., 2008. Principal component score modeling for the rapid description of chromatographic separations. Biotechnology Progress 24, 202–208. doi:10.1021/bp070240j

Edwards, J.S., Palsson, B.O., 2000. The Escherichia coli MG1655 in silico metabolic genotype: Its definition, characteristics, and capabilities. Proceedings of the National Academy of Sciences 97, 5528–5533. doi:10.1073/pnas.97.10.5528

Enfors, S.-O., 2019. Fermentation Process Technology. Stockholm.

EPSO, 2011. THE EUROPEAN BIOECONOMY IN 2030 Delivering Sustainable Growth by addressing the Grand Societal Challenges.

Feist, A.M., Palsson, B.Ø., 2008. The growing scope of applications of genome-scale metabolic reconstructions using Escherichia coli. Nature biotechnology 26, 659—667. doi:10.1038/nbt1401

Fellner, M., Delgado, A., Becker, T., 2003. Functional nodes in dynamic neural networks for bioprocess modelling. Bioprocess and Biosystems Engineering 25, 263–270. doi:10.1007/s00449-002-0297-6

Feng, X., Xu, Y., Chen, Y., Tang, Y.J., 2012. Integrating flux balance analysis into kinetic models to decipher the dynamic metabolism of shewanella oneidensis MR-1. PLoS Computational Biology 8. doi:10.1371/journal.pcbi.1002376

Feyo De Azevedo, S., Dahm, B., Oliveira, F.R., 1997. Hybrid modelling of biochemical processes: A comparison with the conventional approach. Computers and Chemical Engineering 21. doi:10.1016/s0098-1354(97)87593-x

Finley, J.B., Qiu, S.H., Luan, C.H., Luo, M., 2004. Structural genomics for Caenorhabditis elegans: High throughput protein expression analysis. Protein Expression and Purification 34, 49–55. doi:10.1016/j.pep.2003.11.026

Freeman, R.H.M., Yeudall, A.D., 1986. Liquid handling station. WO1988000707A1.

Funke, M., Diederichs, S., Kensy, F., Müller, C., Büchs, J., 2009. The baffled microtiter plate: Increased oxygen transfer and improved online monitoring in small scale fermentations. Biotechnology and Bioengineering 103, 1118–1128. doi:10.1002/bit.22341

Funke, M., Buchenauer, A., Schnakenberg, U., Mokwa, W., Kensy, F., Bu, J., Diederichs, S., Mertens, A., Mu, C., 2010. Microfluidic BioLector — Microfluidic Bioprocess Control in Microtiter Plates 107, 497–505. doi:10.1002/bit.22825

Gábor, A., Villaverde, A.F., Banga, J.R., 2017. Parameter identifiability analysis and visualization in large-scale kinetic models of biosystems. BMC Systems Biology 11, 1–16. doi:10.1186/s12918-017-0428-y

Gebhardt, G., Hortsch, R., Kaufmann, K., Arnold, M., Weuster-Botz, D., 2011. A new microfluidic concept for parallel operated milliliter-scale stirred tank bioreactors. Biotechnology Progress 27, 684–690. doi:10.1002/btpr.570

Glassey, J., 2013. Multivariate Data Analysis for Advancing the Interpretation of Bioprocess Measurement and Monitoring Data. In: Mandenius, C.-F., Titchener-Hooker, N.J. (Eds.), Measurement, Monitoring, Modelling and Control of Bioprocesses. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 167–191. doi:10.1007/10_2012_171

Glauche, F., John, G.T., Arain, S., Knepper, A., Neubauer, A., Goelling, D., Lang, C., Violet, N., King, R., Neubauer, P., 2015. Toward Microbioreactor Arrays: A Slow-Responding Oxygen Sensor for Monitoring of Microbial Cultures in Standard 96-Well Plates. Journal of Laboratory Automation 20, 438–446. doi:10.1177/2211068215573924

Glauche, F., Glazyrina, J., Cruz Bournazou, M.N., Kiesewetter, G., Cuda, F., Goelling, D., Raab, A., Lang, C., Neubauer, P., 2017. Detection of growth rate-dependent product formation in miniaturized parallel fed-batch cultivations. Engineering in Life Sciences 17, 1215–1220. doi:10.1002/elsc.201600029

Gunther, J.C., Baclaski, J., Seborg, D.E., Conner, J.S., 2009. Pattern matching in batch bioprocesses—Comparisons across multiple products and operating conditions. Computers & Chemical Engineering 33, 88–96. doi:10.1016/j.compchemeng.2008.07.001

Haby, B., Hans, S., Anane, E., Sawatzki, A., Krausch, N., Neubauer, P., Cruz Bournazou, M.N., 2019. Integrated Robotic Mini Bioreactor Platform for Automated, Parallel Microbial

Cultivation With Online Data Handling and Process Control. SLAS TECHNOLOGY: Translating Life Sciences Innovation 24. doi:10.1177/2472630319860775

Hannig, G., Makrides, S.C., 1998. Strategies for optimizing heterologous protein expression in Escherichia coli. Trends in biotechnology 16, 54–60. doi:10.1016/S0167-7799(97)01155-4

HEL, 2019. Bioreactor Systems For Rapid Bioprocess Development [WWW Document]. URL https://www.helgroup.com/systems_brochure/biousa.pdf

Henry, C.S., DeJongh, M., Best, A.A., Frybarger, P.M., Linsay, B., Stevens, R.L., 2010. High-throughput generation, optimization and analysis of genome-scale metabolic models. Nature Biotechnology 28, 977–982. doi:10.1038/nbt.1672

Hollinshead, W., He, L., Tang, Y.J., 2014. Biofuel production: an odyssey from metabolic engineering to fermentation scale-up. Frontiers in Microbiology 5, 344. doi:10.3389/fmicb.2014.00344

Hsu, S.-Y., Lin, Y.-S., Li, S.-J., Lee, W.-C., 2014. Co-expression of a heat shock transcription factor to improve conformational quality of recombinant protein in Escherichia coli. Journal of Bioscience and Bioengineering 118, 242–248. doi:10.1016/j.jbiosc.2014.02.012

Huber, R., Ritter, D., Hering, T., Hillmer, A., Kensy, F., Müller, C., Wang, L., Büchs, J., 2009. Robo-Lector – a novel platform for automated high-throughput cultivations in microtiter plates with high information content 15, 1–15. doi:10.1186/1475-2859-8-42

Huber, R., Roth, S., Rahmen, N., Büchs, J., 2011. Utilizing high-throughput experimentation to enhance specific productivity of an E.coli T7 expression system by phosphate limitation. BMC Biotechnology 11, 22. doi:10.1186/1472-6750-11-22

Hughes, S.R., Dowd, P.F., Hector, R.E., Riedmuller, S.B., Bartolett, S., Mertens, J.A., Qureshi, N., Liu, S., Bischoff, K.M., Li, X.-L., John S. Jackson, J., Sterner, D., Panavas, T., Rich, J.O., Farrelly, P.J., Butt, T., Cotta, M.A., 2007. Cost-Effective High-Throughput Fully Automated Construction of a Multiplex Library of Mutagenized Open Reading Frames for an Insecticidal Peptide Using a Plasmid-Based Functional Proteomic Robotic Workcell with Improved Vacuum System. JALA: Journal of the Association for Laboratory Automation 12, 202–212. doi:10.1016/j.jala.2007.04.007

Janzen, N.H., Striedner, G., Jarmer, J., Voigtmann, M., Abad, S., Reinisch, D., 2019. Implementation of a Fully Automated Microbial Cultivation Platform for Strain and Process Screening. Biotechnology Journal 14, 1800625. doi:10.1002/biot.201800625

Jensen, E.B., Carlsen, S., 1990. Production of recombinant human growth hormone in Escherichia coli: Expression of different precursors and physiological effects of glucose, acetate, and salts. Biotechnology and Bioengineering 36, 1–11. doi:10.1002/bit.260360102

Jeude, M., Dittrich, B., Niederschulte, H., Anderlei, T., Knocke, C., Klee, D., Bu, J., 2006. Fed-

Batch Mode in Shake Flasks by Slow-Release Technique. doi:10.1002/bit

John, G.T., Goelling, D., Klimant, I., Schneider, H., Heinzle, E., 2003. pH-sensing 96-well microtitre plates for the characterization of acid production by dairy starter cultures. Journal of Dairy Research 70, 327–333. doi:10.1017/S0022029903006344

Junker, B.H., Wang, H.Y., 2006. Bioprocess Monitoring and Computer Control : Key Roots of the Current PAT Initiative. doi:10.1002/bit.21087

Kaganovitch, E., Steurer, X., Dogan, D., Probst, C., Wiechert, W., Kohlheyer, D., 2018. Microbial single-cell analysis in picoliter-sized batch cultivation chambers. New Biotechnology 47, 50–59. doi:10.1016/j.nbt.2018.01.009

Kensy, F., Zang, E., Faulhammer, C., Tan, R.-K., Büchs, J., 2009. Validation of a high-throughput fermentation system based on online monitoring of biomass and fluorescence in continuously shaken microtiter plates. Microbial Cell Factories 8, 31. doi:10.1186/1475-2859-8-31

Khodayari, A., Maranas, C.D., 2016. A genome-scale Escherichia coli kinetic metabolic model k-ecoli457 satisfying flux data for multiple mutant strains. Nature communications 7, 13806. doi:10.1038/ncomms13806

Khodayari, A., Zomorrodi, A.R., Liao, J.C., Maranas, C.D., 2014. A kinetic model of Escherichia coli core metabolism satisfying multiple sets of mutant flux data. Metabolic engineering 25, 50–62. doi:10.1016/j.ymben.2014.05.014

Kim, W.J., Kim, H.U., Lee, S.Y., 2017. Current state and applications of microbial genome-scale metabolic models. Current Opinion in Systems Biology 2, 10–18. doi:10.1016/j.coisb.2017.03.001

Kirdar, A.O., Conner, J.S., Baclaski, J., Rathore, A.S., 2007. Application of Multivariate Analysis toward Biotech Processes: Case Study of a Cell-Culture Unit Operation. Biotechnology Progress 23, 61–67. doi:10.1021/bp060377u

Knepper, A., Heiser, M., Glauche, F., Neubauer, P., 2014. Robotic Platform for Parallelized Cultivation and Monitoring of Microbial Growth Parameters in Microwell Plates. Journal of Laboratory Automation 19, 593–601. doi:10.1177/2211068214547231

Knorr, B., Schlieker, H., Hohmann, H.-P., Weuster-Botz, D., 2007. Scale-down and parallel operation of the riboflavin production process with Bacillus subtilis. Bochem. Eng. J. 33, 263–274. doi:DOI: 10.1016/j.bej.2006.10.023

Koenig, L., Ramme, A.P., Faust, D., Lauster, R., Marx, U., 2018. Production of Human Induced Pluripotent Stem Cell-Derived Cortical Neurospheres in the DASbox ® Mini Bioreactor System. Application note 1–12.

Konstantinov, K.B., Cooney, C.L., 2015. White Paper on Continuous Bioprocessing May 20-21, 2014 Continuous Manufacturing Symposium. Journal of Pharmaceutical Sciences 104, 813–820. doi:10.1002/jps.24268

Kopp, J., Kolkmann, A.M., Veleenturf, P.G., Spadiut, O., Herwig, C., Slouka, C., 2019. Boosting recombinant inclusion body production—from classical fed-batch approach to continuous cultivation. Frontiers in Bioengineering and Biotechnology 7, 1–12. doi:10.3389/fbioe.2019.00297

Kourti, T., 2006. Process Analytical Technology Beyond Real-Time Analyzers: The Role of Multivariate Analysis. Critical Reviews in Analytical Chemistry 36, 257–278. doi:10.1080/10408340600969957

Krausch, N., Barz, T., Sawatzki, A., Gruber, M., Kamel, S., Neubauer, P., Cruz Bournazou, M.N., 2019. Monte Carlo Simulations for the Analysis of Non-linear Parameter Confidence Intervals in Optimal Experimental Design. Frontiers in Bioengineering and Biotechnology 7, 122. doi:10.3389/fbioe.2019.00122

Krause, M., Ukkonen, K., Haataja, T., Ruottinen, M., Glumoff, T., Neubauer, A., Neubauer, P., Vasala, A., 2010. A novel fed-batch based cultivation method provides high cell-density and improves yield of soluble recombinant proteins in shaken cultures. Microbial Cell Factories 9, 1–11. doi:10.1186/1475-2859-9-11

Kroll, P., Hofer, A., Ulonska, S., Kager, J., Herwig, C., 2017. Model-Based Methods in the Biopharmaceutical Process Lifecycle. Pharmaceutical Research 34, 2596–2613. doi:10.1007/s11095-017-2308-y

LaCroix, R.A., Palsson, B.O., Feist, A.M., 2017. A Model for Designing Adaptive Laboratory Evolution Experiments. Applied and Environmental Microbiology 83. doi:10.1128/AEM.03115-16

Lagaris, I.E., Likas, A., Fotiadis, D.I., 1998. Artificial neural networks for solving ordinary and partial differential equations. IEEE Transactions on Neural Networks 9, 987–1000. doi:10.1109/72.712178

Lee, J.H., 2011. Model predictive control: Review of the three decades of development. International Journal of Control, Automation and Systems 9, 415–424. doi:10.1007/s12555-011-0300-6

Lee, S.Y., 1996. High cell-density culture of Escherichia coli. Trends in Biotechnology 14, 98–105. doi:10.1016/0167-7799(96)80930-9

Lenski, R.E., Rose, M.R., Simpson, S.C., Tadler, S.C., 1991. Long-Term Experimental Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000 Generations. The American Naturalist 138, 1315–1341. doi:10.1086/285289

Lin, H., Hanschke, R., Nicklisch, S., Nietsche, T., Jarchow, R., Riemschneider, S., Meyer, S., Gupta, A., Hecker, M., Neubauer, P., 2001a. Cellular Responses To Strong Overexpression of Recombinant Genes in Escherichia Coli. Recombinant Protein Production with prokaryotic and eukaryotic cells. A comparative view on host physiology 1–19. doi:10.1007/978-94-015-9749-4_5

Lin, H.Y., Mathiszik, B., Xu, B., Enfors, S.O., Neubauer, P., 2001b. Determination of the maximum specific uptake capacities for glucose and oxygen in glucose-limited fed-batch cultivations of Escherichia coli. Biotechnology and Bioengineering 73, 347–357. doi:10.1002/bit.1068

López, D.C., Barz, T., Peñuela, M., Villegas, A., Ochoa, S., Wozny, G., 2013. Model-based identifiable parameter determination applied to a simultaneous saccharification and fermentation process model for bio-ethanol production. Biotechnology Progress 29, 1064–1082. doi:10.1002/btpr.1753

Luli, G.W., Strohl, W.R., 1990. Comparison of growth, acetate production, and acetate inhibition of Escherichia coli strains in batch and fed-batch fermentations. Applied and Environmental Microbiology 56, 1004–1011. doi:10.1128/aem.56.4.1004-1011.1990

Mahadevan, R., Edwards, J.S., Doyle, F.J., 2002. Dynamic Flux Balance Analysis of diauxic growth in Escherichia coli. Biophysical Journal 83, 1331–1340. doi:10.1016/S0006-3495(02)73903-9

Manahan, M., Nelson, M., Cacciatore, J.J., Weng, J., Xu, S., Pollard, J., 2019. Scale-down model qualification of ambr® 250 high-throughput mini-bioreactor system for two commercial-scale mAb processes. Biotechnology Progress 35, 1–13. doi:10.1002/btpr.2870

Markx, G.H., Davey, C.L., Kell, D.B., 1991. The permittistat: A novel type of turbidostat. Journal of General Microbiology 137, 735–743. doi:10.1099/00221287-137-4-735

Martin, G.A., Hempfling, W.P., 1976. A Method for the Regulation of Microbial Population Density during Continuous Culture at High Growth Rates. Archives of Microbiology 47, 41–47. doi:10.1007/BF00427865

McKinstry, W.J., Hijnen, M., Tanwar, H.S., Sparrow, L.G., Nagarajan, S., Pham, S.T., Mak, J., 2014. Expression and purification of soluble recombinant full length HIV-1 Pr55Gag protein in Escherichia coli. Protein Expression and Purification 100, 10–18. doi:10.1016/j.pep.2014.04.013

McMullen, J.P., Jensen, K.F., 2011. Rapid determination of reaction kinetics with an automated microfluidic system. Organic Process Research and Development 15, 398–407. doi:10.1021/op100300p

Mears, L., Stocks, S.M., Sin, G., Gernaey, K. V., 2017. A review of control strategies for manipulating the feed rate in fed-batch fermentation processes. Journal of Biotechnology 245, 34–46. doi:10.1016/j.jbiotec.2017.01.008

Meyer, R., 2017. Bioeconomy Strategies: Contexts, Visions, Guiding Implementation Principles and Resulting Debates. Sustainability 9, 1031. doi:10.3390/su9061031

Modak, J.M., Lim, H.C., Tayeb, Y.J., 1986. General characteristics of optimal feed rate profiles for various fed-batch fermentation processes. Biotechnology and Bioengineering 28,

1396–1407. doi:10.1002/bit.260280914

Monk, J.M., Lloyd, C.J., Brunk, E., Mih, N., Sastry, A., King, Z., Takeuchi, R., Nomura, W., Zhang, Z., Mori, H., Feist, A.M., Palsson, B.O., 2017. iML1515, a knowledgebase that computes Escherichia coli traits. Nature Biotechnology 35, 904–908. doi:10.1038/nbt.3956

Nakano, K., Rischke, M., Sato, S., Märkl, H., 1997. Influence of acetic acid on the growth of Escherichia coli K12 during high-cell-density cultivation in a dialysis reactor. Applied Microbiology and Biotechnology 48, 597–601. doi:10.1007/s002530051101

Narayanan, H., Sokolov, M., Morbidelli, M., Butté, A., 2019. A new generation of predictive models–the added value of hybrid models for manufacturing processes of therapeutic proteins. Biotechnology and Bioengineering 0. doi:10.1002/bit.27097

Narayanan, H., Luna, M.F., Stosch, M. von, Cruz-Bournazou, M.N., Polotti, G., Morbidelli, M., Butté, A., Sokolov, M., 2020. Bioprocessing in the Digital Age: The Role of Process Models. Biotechnology Journal 15, 1900172. doi:10.1002/biot.201900172

Neubauer, P., Lin, H.Y., Mathiszik, B., 2003. Metabolic load of recombinant protein production: Inhibition of cellular capacities for glucose uptake and respiration after induction of a heterologous gene in Escherichia coli. Biotechnology and Bioengineering 83, 53–64. doi:10.1002/bit.10645

Neubauer, P., Glauche, F., Cruz-Bournazou, M.N., 2017. Editorial: Bioprocess Development in the era of digitalization. Engineering in Life Sciences 17, 1140–1141. doi:10.1002/elsc.201770113

Nomikos, P., MacGregor, J.F., 1994. Monitoring batch processes using multiway principal component analysis. AIChE Journal 40, 1361–1375. doi:10.1002/aic.690400809

Orth, J.D., Thiele, I., Palsson, B.O., 2010. What is flux balance analysis? Nature Biotechnology 28, 245–248. doi:10.1038/nbt.1614

Orth, J.D., Conrad, T.M., Na, J., Lerman, J.A., Nam, H., Feist, A.M., Palsson, B., 2011. A comprehensive genome-scale reconstruction of Escherichia coli metabolism-2011. Molecular Systems Biology 7, 1–9. doi:10.1038/msb.2011.65

Øyås, O., Stelling, J., 2018. Genome-scale metabolic networks in time and space. Current Opinion in Systems Biology 8, 51–58. doi:10.1016/j.coisb.2017.12.003

Paalme, T., Kahru, A., Elken, R., Vanatalu, K., Tiisma, K., Raivo, V., 1995. The computer-controlled continuous culture of Escherichia coli with smooth change of dilution rate (A-stat). Journal of Microbiological Methods 24, 145–153. doi:10.1016/0167-7012(95)00064-X

Panula-Perälä, J., Šiurkus, J., Vasala, A., Wilmanowski, R., Casteleijn, M.G., Neubauer, P., 2008. Enzyme controlled glucose auto-delivery for high cell density cultivations in microplates and shake flasks. Microbial Cell Factories 7, 1–12. doi:10.1186/1475-2859-

7-31

Pfost, D.R., Bjornson, T.O., Coppock, R.M., Kowalski, C., Marquiss, S.A., Murray, D.S., Pfost, R.F., Sanford, B., Puckett, K.L., 1989. Automated multi-purpose analytical chemistry processing center and laboratory work station. US5104621A.

Pope, B., Kent, H.M., 1996. High Efficiency 5 Min Transformation of Escherichia Coli. Nucleic Acids Research 24, 536–537. doi:10.1093/nar/24.3.536

Puskeiler, R., Kaufmann, K., Weuster-Botz, D., 2005. Development, parallelization, and automation of a gas-inducing milliliter-scale bioreactor for high-throughput bioprocess design (HTBD). Biotechnology and Bioengineering 89, 512–523. doi:10.1002/bit.20352

Rajamanickam, V., Wurm, D., Slouka, C., Herwig, C., Spadiut, O., 2017. A novel toolbox for E. coli lysis monitoring. Analytical and Bioanalytical Chemistry 409, 667–671. doi:10.1007/s00216-016-9907-z

Randek, J., Mandenius, C.F., 2018. On-line soft sensing in upstream bioprocessing. Critical Reviews in Biotechnology 38, 106–121. doi:10.1080/07388551.2017.1312271

Reddy, R.K., Bhargava, R., 2010. Chemometric Methods for Biomedical Raman Spectroscopy and Imaging. In: Matousek, P., Morris, M.D. (Eds.), Emerging Raman Applications and Techniques in Biomedical and Pharmaceutical Fields. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 179–213. doi:10.1007/978-3-642-02649-2_8

Reed, J.L., Vo, T.D., Schilling, C.H., Palsson, B.O., 2003. An expanded genome-scale model of Escherichia coli K-12 (iJR904 GSM/GPR). Genome Biology 4, R54. doi:10.1186/gb-2003-4-9-r54

Ritz, D., Raley, J., Huff, K., Fernandez, J., Wu, J., Pu, Z., Marsh, D., Warr, S., Read, G.A., Finka, G., Patel, G.P., 2017. Use of Ambr250 As a Small Scale Model for Manufacturing-Scale Single-Use Bioreactors. p. 250.

Rosano, G.L., Ceccarelli, E.A., 2014. Recombinant protein expression in Escherichia coli: advances and challenges. Frontiers in Microbiology.

Ruano, P., Delgado, L.L., Picco, S., Villegas, L., Tonelli, F., Merlo, M., Rigau, J., Diaz, D., Masuelli, M., 2016. Increasing Recombinant Protein Production in E. coli by an Alternative Method to Reduce Acetate. Intech 13. doi:10.5772/57353

Rydenfelt, M., Garcia, H.G., Cox III, R.S., Phillips, R., 2015. The Influence of Promoter Architectures and Regulatory Motifs on Gene Expression in Escherichia coli. PLOS ONE 9, 1–31. doi:10.1371/journal.pone.0114347

Sawatzki, A., Hans, S., Narayanan, H., Haby, B., Krausch, N., Sokolov, M., Glauche, F., Riedel, S.L., Neubauer, P., Cruz Bournazou, M.N., 2018. Accelerated Bioprocess Development of Endopolygalacturonase-Production with Saccharomyces cerevisiae Using Multivariate Prediction in a 48 Mini-Bioreactor Automated Platform. Bioengineering 5. doi:10.3390/bioengineering5040101

Scheidle, M., Jeude, M., Dittrich, B., Denter, S., Kensy, F., Suckow, M., Klee, D., Büchs, J., 2010. High-throughput screening of Hansenula polymorpha clones in the batch compared with the controlled-release fed-batch mode on a small scale. FEMS Yeast Research 10, 83–92. doi:10.1111/j.1567-1364.2009.00586.x

Schmideder, A., Severin, T.S., Cremer, J.H., Weuster-Botz, D., 2015. A novel milliliter-scale chemostat system for parallel cultivation of microorganisms in stirred-tank bioreactors. Journal of Biotechnology 210, 19–24. doi:10.1016/j.jbiotec.2015.06.402

Schubert, J., Simutis, R., Dors, M., Havlik, I., Lübbert, A., 1994. Bioprocess optimization and control: Application of hybrid modelling. Journal of Biotechnology 35, 51–68. doi:10.1016/0168-1656(94)90189-9

Selvarasu, S., Ow, D.S.-W., Lee, S.Y., Lee, M.M., Oh, S.K.-W., Karimi, I.A., Lee, D.-Y., 2009. Characterizing Escherichia coli DH5α growth and metabolism in a complex medium using genome-scale flux analysis. Biotechnology and Bioengineering 102, 923–934. doi:https://doi.org/10.1002/bit.22119

Sezonov, G., Joseleau-Petit, D., D\textquoterightAri, R., 2007. Escherichia coli Physiology in Luria-Bertani Broth. Journal of Bacteriology 189, 8746–8749. doi:10.1128/JB.01368-07

Shiloach, J., Fass, R., 2005. Growing E. coli to high cell density - A historical perspective on method development. Biotechnology Advances 23, 345–357. doi:10.1016/j.biotechadv.2005.04.004

Shiloach, J., Kaufman, J., Guillard, A.S., Fass, R., 1996. Effect of glucose supply strategy on acetate accumulation, growth, and recombinant protein production by Escherichia coli BL21 (λDE3) and Escherichia coli JM109. Biotechnology and Bioengineering 49, 421–428. doi:10.1002/(SICI)1097-0290(19960220)

Šiurkus, J., Panula-Perälä, J., Horn, U., Kraft, M., Rimšeliene, R., Neubauer, P., 2010. Novel approach of high cell density recombinant bioprocess development: Optimisation and scale-up from microlitre to pilot scales while maintaining the fed-batch cultivation mode of E. coli cultures. Microbial Cell Factories 9, 1–17. doi:10.1186/1475-2859-9-35

Sokolov, M., Ritscher, J., MacKinnon, N., Bielser, J.M., Brühlmann, D., Rothenhäusler, D., Thanei, G., Soos, M., Stettler, M., Souquet, J., Broly, H., Morbidelli, M., Butté, A., 2017. Robust factor selection in early cell culture process development for the production of a biosimilar monoclonal antibody. Biotechnology Progress 33, 181–191. doi:10.1002/btpr.2374

Sousa, M., Santo, V.E., Becken, U., Serra, M., Lisboa, U.N. De, 2018. Culture of 3D Cell Aggregates in Perfusion in a DASbox ® Mini Bioreactor System 1–8.

Stachowiak, H., 1973. Allgemeine Modelltheorie. Springer.

Stosch, M. von, Oliveira, R., Peres, J., Feyo de Azevedo, S., 2014. Hybrid semi-parametric modeling in process systems engineering: Past, present and future. Computers and

Chemical Engineering 60, 86–101. doi:10.1016/j.compchemeng.2013.08.008

Stosch, M. Von, Oliveria, R., Peres, J., Azevedo, F. de, 2012. Hybrid Modeling Framework for Process Analytical Technology : Application to Bordetella Pertussis Cultures. Biotechnol. Prog 28, 284–291. doi:10.1002/btpr.706

Subramanian, G., Erm, S., Abner, K., Seiman, A., Adamberg, K., Vilu, R., 2017. Study of Cells in the Steady-State Growth Space. In: Continuous Biomanufacturing - Innovative Technologies and Methods. pp. 233–258. doi:10.1002/9783527699902.ch9

Takahashi, M.B., Leme, J., Caricati, C.P., Tonso, A., Fernández Núñez, E.G., Rocha, J.C., 2015. Artificial neural network associated to UV/Vis spectroscopy for monitoring bioreactions in biopharmaceutical processes. Bioprocess and Biosystems Engineering 38, 1045–1054. doi:10.1007/s00449-014-1346-7

Tarca, A.L., Carey, V.J., Chen, X. wen, Romero, R., Drăghici, S., 2007. Machine learning and its applications to biology. PLoS computational biology 3. doi:10.1371/journal.pcbi.0030116

Thomassen, Y.E., Sprang, E.N.M. van, Pol, L.A. van der, Bakker, W.A.M., 2010. Multivariate data analysis on historical IPV production data for better process understanding and future improvements. Biotechnology and Bioengineering 107, 96–104. doi:10.1002/bit.22788

Toeroek, C., Cserjan-Puschmann, M., Bayer, K., Striedner, G., 2015. Fed-batch like cultivation in a micro-bioreactor: screening conditions relevant for Escherichia coli based production processes. SpringerPlus 4. doi:10.1186/s40064-015-1313-z

Tohsato, Y., Ikuta, K., Shionoya, A., Mazaki, Y., Ito, M., 2013. Parameter optimization and sensitivity analysis for large kinetic models using a real-coded genetic algorithm. Gene 518, 84–90. doi:10.1016/j.gene.2012.11.080

Townshend, B., 2013. pyTecan [WWW Document]. URL https://github.com/btownshend/pyTecan (accessed 2.7.21).

Ündey, C., Ertunç, S., Mistretta, T., Pathak, M., 2009. Applied advanced process analytics in biopharmaceutical manufacturing: Challenges and prospects in real-time monitoring and control. IFAC Proceedings Volumes (IFAC-PapersOnline) 7, 177–182. doi:10.1016/j.jprocont.2010.05.008

Unthan, S., Radek, A., Wiechert, W., Oldiges, M., Noack, S., 2015. Bioprocess automation on a Mini Pilot Plant enables fast quantitative microbial phenotyping. Microbial Cell Factories 14, 1–11. doi:10.1186/s12934-015-0216-6

Vaitkus, V., Brazauskas, K., Repšytundefined, J., 2020. Soft-Sensors Based on Black-Box Models for Bioreactors Monitoring and State Estimation. In: Proceedings of the 2020 12th International Conference on Bioinformatics and Biomedical Technology, ICBBT 2020. Association for Computing Machinery, New York, NY, USA, pp. 43–47.

doi:10.1145/3405758.3405780

Villadsen, J., Nielsen, J., Lidén, G., 2011. Bioreaction Engineering Principles. Springer US. doi:10.1007/978-1-4419-9688-6

Viña Rodríguez, A., 2018. RNA virus detection and identification using techniques based on DNA hybridization.

Vital-Jacome, M., Buitrón, G., Moreno-Andrade, I., Garcia-Rea, V., Thalasso, F., 2016. Microrespirometric determination of the effectiveness factor and biodegradation kinetics of aerobic granules degrading 4-chlorophenol as the sole carbon source. Journal of Hazardous Materials 313, 112–121. doi:10.1016/j.jhazmat.2016.02.077

Wang, R.F., Kushner, S.R., 1991. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in Escherichia coli. Gene 100, 195–199.

Wang, X., Kruger, U., Irwin, G.W., 2005. Process monitoring approach using fast moving window PCA. Industrial and Engineering Chemistry Research 44, 5691–5702. doi:10.1021/ie048873f

Warr, S.R., Betts, J.P., Ahmad, S., Newell, K. V, Finka, G.B., 2013. Streamlined process development using the Micro24 Bioreactor system. BMC Proceedings 7, 6–8. doi:10.1186/1753-6561-7-s6-p36

Warr, S.R.C., 2014. Microbioreactors and Scale-Down Models: Growth of CHO Cells Using the Pall Micro24 MicroReactor System. In: Animal Cell Biotechnology. Humana Press, pp. 105–116. doi:10.1007/978-1-62703-733-4

Watson, T.G., 1969. Steady state operation of a continuous culture at maximum growth rate by control of carbon dioxide production. Journal of general microbiology 59, 83–89. doi:10.1099/00221287-59-1-83

Weuster-Botz, D., 2005. Parallel reactor systems for bioprocess development. Advances in Biochemical Engineering/Biotechnology 92, 125–143. doi:10.1007/b98916

Xu, B., Jahic, M., Enfors, S.-O., 1999. Modeling of overflow metabolism in batch and fed-batch cultures of Escherichia coli. Biotechnology progress 15, 81–90. doi:10.1021/bp9801087

Yazdani, A., Lu, L., Raissi, M., Karniadakis, G.E., 2020. Systems biology informed deep learning for inferring parameters and hidden dynamics. PLoS Computational Biology 16, 1–19. doi:10.1371/journal.pcbi.1007575

Yoshida, F., Yamane, T., Nakamoto, K.-I., 1973. Fed-batch hydrocarbon fermentation with colloidal emulsion feed. Biotechnology and Bioengineering 15, 257–270. doi:10.1002/bit.260150204

Youngblut, N., 2017. pyTecanFluent [WWW Document]. URL https://pypi.org/project/pyTecanFluent/ (accessed 2.7.21).

Zampieri, G., Vijayakumar, S., Yaneske, E., Angione, C., 2019. Machine and deep learning meet genome-scale metabolic modeling. PLoS Computational Biology 15, 1–24.

doi:10.1371/journal.pcbi.1007084

Zareef, M., Chen, Q., Hassan, M.M., Arslan, M., Hashim, M.M., Ahmad, W., Kutsanedzie, F.Y.H., Agyekum, A.A., 2020. An Overview on the Applications of Typical Non-linear Algorithms Coupled With NIR Spectroscopy in Food Analysis. Food Engineering Reviews 12, 173–190. doi:10.1007/s12393-020-09210-7

Zhang, D., Rio-Chanona, E.A. Del, Petsagkourakis, P., Wagner, J., 2019. Hybrid physics-based and data-driven modeling for bioprocess online simulation and optimization. Biotechnology and Bioengineering 116, 2919–2930. doi:10.1002/bit.27120

Zhu, X., Rehman, K.U., Wang, B., Shahzad, M., 2020. Modern Soft-Sensing Modeling Methods for Fermentation Processes. Sensors 20. doi:10.3390/s20061771

Zimmermann, H.F., Rieth, J., 2006. A Fully Automated Robotic System for High Throughput Fermentation. JALA - Journal of the Association for Laboratory Automation 11, 134–137. doi:10.1016/j.jala.2006.03.003

Zimmermann, H.F., John, G.T., Trauthwein, H., Dingerdissen, U., Huthmacher, K., 2003. Rapid evaluation of oxygen and water permeation through microplate sealing tapes. Biotechnology Progress 19, 1061–1063. doi:10.1021/bp025774t

Zweck, A., Holtmannspötter, D., Braun, M., Cuhls, K., Hirt, M., Kimpeler, S., 2015. Forschungs- und Technologieperspektiven 2030 Ergebnisband 2 zur Suchphase von BMBF-Foresight Zyklus II.

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