Platform Technologies for Automated Bioprocess Development

vorgelegt von
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von der Fakultät III – Prozesswissenschaften
der Technischen Universität Berlin
zur Erlangung des akademischen Grades

Doktor der Ingenieurwissenschaften
- Dr.-Ing. -

genehmigte Dissertation

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Tag der wissenschaftlichen Aussprache: 24. November 2017

Berlin 2018

Zusammenfassung

Die Entwicklung von Produktionsprozessen in der Biotechnologie ist zeit- und kostenintensiv, insbesondere bei der Produktion von therapeutischen Proteinen und gentechnisch verbesserten Enzymen. Neben allgemeinen Prozessgrößen wie Temperatur, pH und Medienzusammensetzung beeinflussen Produktionsstamm, Expressionssystem und Fusionspartner sowie Prozessführung und die Aufarbeitung des Produktes den Ertrag und damit die Wirtschaftlichkeit eines biotechnologischen Herstellungsprozesses. Die Anzahl an nötigen Laborexperimenten zur Optimierung ist meist zu groß, um manuell durchgeführt zu werden, sodass häufig nur bei einem Quasi-Optimum gearbeitet werden kann.

In den letzten zwei Jahrzehnten ist die Miniaturisierung, Parallelisierung und Automatisierung von Experimenten stark vorangeschritten, sodass heute tausende von Versuchen pro Woche durchgeführt werden können. Ob die Daten aus den kleinen Systemen prädiktiv für den Produktionsmaßstab eingesetzt werden können, ist jedoch nicht ohne Zweifel. Daher müssen für die Maßstabsvergrößerung in der Regel zusätzliche Versuchsreihen in Labor- und Pilotbioreaktoren durchgeführt werden.

Die Unterschiede zwischen Labor- und Produktionsbedingungen betreffen häufig die Kulturführung und die eingesetzten Medien. Meist wird ein Batch-Prozess mit Komplexmedium im Kleinmaßstab und ein Fed-Batch Prozess mit Mineralsalzmedium in der Produktion eingesetzt. Außerdem erlauben die Screening-Systeme nur Endpunktmessungen und es wird häufig erfahrungsbasiert oder nach Versuch und Irrtum gearbeitet. Die Datenaufzeichnung und -Auswertung geschieht meist manuell.

Um die Aussagekraft von Laborexperimenten im Kleinmaßstab zu verbessern, sollten Versuchsbedingungen gewählt werden, die dem Produktionsmaßstab entsprechen. Diese Prozessentwicklungsstrategie wird auch konsistente Bioprozessentwicklung genannt. Im Rahmen dieser Dissertation wurden Plattformtechnologien für konsistente Bioprozessentwicklung erarbeitet und in Form von drei Fallstudien auf unterschiedliche Fragestellungen angewendet.

Ein neuartiger Sensor zur Messung von Gelöstsauerstoff in Mikrowellplatten wurde in der ersten Publikation entwickelt. Der Sensor besitzt eine Ansprechzeit von 9,7 Minuten, was die verlässliche Bestimmung von Gelöstsauerstoffwerten in Anlagen ermöglicht, in denen Verzögerungen durch Transportzeiten vom Inkubator zum Photometer vorhanden sind. Mithilfe von *Escherichia coli* und *Saccharomyces cerevisiae* Kulturen konnte demonstriert werden, dass der Sensor Sauerstofflimitationen im Inkubator detektieren kann. In Kombination mit einem schnell ansprechenden Sensor ist eine umfassende Charakterisierung des automatisierten Kultivierungssystems möglich.

In der zweiten Publikation wurde ein Arbeitsablauf zur schnellen Optimierung des chemisch-enzymatischen Zellaufschlusses entwickelt. Mit Hilfe einer Software für experimentelles Design wurden Versuchspläne erstellt, die in einem Datenbanksystem für den Pipettierroboter abrufbar gespeichert wurden. In drei Experimenten wurde die optimale Mischung von EDTA, Lysozym, Triton X-100 und Polymyxin B bestimmt. Im Vergleich zu kommerziell erhältlichen Produkten wies der Puffer eine vergleichbare Performance auf.

Im Rahmen der dritten Publikation wurde das Produktbildungsprofil von Hefekulturen in parallelen miniaturisierten Fed-Batch Kulturen untersucht und eine Abhängigkeit der spezifischen Produktbildungsrate von der spezifischen Wachstumsrate festgestellt. Das Produktbildungsprofil wurde mit einer kontinuierlichen Kultivierung verglichen. Es konnte festgestellt werden, dass die aus den miniaturisierten Kulturen gewonnenen Daten mit dem 1,5 L Bioreaktor vergleichbar sind. Eine Charakterisierung von Hefestämmen in Mikrowellplatten bietet sich demnach an, um zeit- und kostenintensive Experimente in Bioreaktoren zu minimieren.

Die entwickelten Plattformtechnologien bieten eine Grundstruktur zur schnelleren Versuchsplanung, -durchführung und -auswertung. Durch die Beibehaltung der Rahmenbedingungen des Produktionsmaßstabes während der Produkt- und Prozessentwicklung können Entwicklungszeit und -kosten von biotechnologischen Produktionsprozessen verringert werden, was schlussendlich zu einer weiteren Verbreitung von nachhaltigen Produktionsmethoden führen wird.

Acknowledgements

I would like to thank the students I supervised in the past five years. Thanks to Michael, who has put endless efforts into the set-up and improvement of our robotic platform, Wojtek and Petra were able to help me out with the experimental work for the cell lysis paper. Gregor, Rick, Fabian and Robert helped carrying out the experiments for the yeast paper.

I am grateful for the support and scientific advice I received from Peter Neubauer throughout my PhD project. I also want to thank all my colleagues at BVT, who always had time for discussions and advice in the lab. Especially Julia, Nico, Sebastian, Mirja, Stefan, Christian, Andreas and of course our technicians Irmgard, Brigitte and Thomas.

For financial support, I would like to thank the Ministry of Research and Education. The results presented in this thesis were part of the joint research project “Autobio”. Also, I want to thank the whole project team: Gernot John and Sarina Arain from PreSens Precision Sensing GmbH, Andreas Raab, Detlef Goelling and Christine Lang from Organobalance GmbH, Daniel Meyer and Jörg Mampel from BRAIN AG, Ingrid Schmid and Joachim Aschoff from infoteam software AG and Norman Violet and Rudibert King from the department of measurement and control at TU Berlin. Thanks to Maciek Pilarek from TU Warsaw, with whom the cell lysis project made steady progress over the years. It was a fun time working with him.

I would like to thank my family and friends for their endless support. I also want to thank my uncle Matthias who has shown me the fascinating world of Biology a long time ago. I am grateful to be with Lena, who has helped me through all these years to find the time and maintain the motivation to finish my PhD.

List of original articles

The presented results have been published in the scientific journals Engineering in Life Sciences and Journal of Laboratory Automation. Authorized copies can be obtained from SAGE journals and Wiley VCH.

Paper I:

Glauche F, John GT, Arain S, Knepper A, Neubauer A, Goelling D, Lang C, Violet N, King R, Neubauer P. **Toward Microbioreactor Arrays: A Slow-Responding Oxygen Sensor for Monitoring of Microbial Cultures in Standard 96-Well Plates.** *J Lab Autom.* 2015 Aug;20(4):438-46
DOI: 10.1177/2211068215573924
Preprint version

Author's contributions: The requirements for the prototype sensor were defined in initial discussions with all authors. GTJ and SA designed and produced the prototypes. SA conducted initial tests with different sensors and wrote the corresponding parts of the manuscript. FG designed and conducted the cultivation experiments with the prototype sensor plate all other parts of the manuscript. NV and RK designed and conducted in silico tests of different prototype sensors. AK, AN, DG and CL were involved in the design and interpretation of the cultivation experiments. PN supported with study design and manuscript preparation as the principal investigator. All authors have read and approved the final version of the manuscript.

Paper II:

Glauche F, Pilarek M, Cruz Bournazou MN, 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 DOI: 10.1002/elsc.201600030

Preprint version

Author's contributions: FG developed the methods, designed the experiments and wrote the manuscript. MP and FG carried out the experiments and MP drafted parts of the manuscript and figures. MNCB developed the experimental design and carried out the statistical analysis of the raw data. PG established the protein quantification method on the liquid handler and assisted in carrying out the experiments. PN was the principle investigator and supported the experimental design, data interpretation and manuscript preparation. All authors have read and approved the final version of the manuscript.

Paper III:

Glauche F, Glazyrina J, Cruz Bournazou MN, Kiesewetter G, Cuda F, Goelling D, Raab A, Lang C, Neubauer P. **Detection of growth rate-dependent product formation in miniaturized parallel fed-batch cultivations.** *Eng. Life Sci.* 2017. 17: 1215-1220 DOI: 10.1002/elsc.201600029

Preprint version

Author's contributions: FG established the methods, designed and performed the experiments and prepared the manuscript. GK assisted in carrying out the 24-well plate experiments. FG, JG and FC planned and carried out the A-Stat cultures. DG, AR and CL supervised the strain generation and development of the activity assay. MNCB and PN supervised the study design, data evaluation and assisted the preparation of the manuscript. All authors have read and approved the final version of the manuscript.

Co-Authored publications:

Glazyrina, J, Krause, M, Junne, S, Glauche F, Strom D, Neubauer P, **Glucose-limited high cell density cultivations from small to pilot plant scale using an enzyme-controlled glucose delivery system.** *N. Biotechnol.* 2012, *29*, 235–42.

Neubauer P, Cruz Bournazou MN, 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.

Mayer S, Junne S, Ukkonen K, Glazyrina J, Glauche F, Vasala A, Neubauer P. **Lactose autoinduction with enzymatic glucose release: Characterization of the cultivation system in bioreactor.** *Protein Expr. Purif.* 2014, *94*, 67–72.

Knepper A, Heiser M, Glauche F, Neubauer P. Rob**otic Platform for Parallelized Cultivation and Monitoring of Microbial Growth Parameters in Microwell Plates.** *J. Lab. Autom.* 2014, *19*, 593–601.

Cruz Bournazou MN, Barz T, Nickel D, Lopez Cárdenas D, Glauche F, Knepper A, Neubauer P, **Online optimal experimental re-design in robotic parallel fed-batch cultivation facilities for validation of macro-kinetic growth models using *E. coli* as an example.** *Biotechnol. Bioeng.* 2016, *114*, 1–29.

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

Abstract

The development of production processes in biotechnology is a time and resource intensive task due to the vast design space to be screened. Therefore, processes are commonly performed at a local optimum. Within the past two decades, miniaturization, parallelization and automation of laboratory work have improved significantly and researchers are now able to carry out thousands of experiments per week in automated facilities.

In many cases there is a disagreement between the results of small-scale experiments and data from the production scale due to the different conditions, in which the cells are cultivated. Laboratory scale experiments are often performed as batch cultures in complex media without any instrumentation, while the production processes run under fed-batch conditions in instrumented bioreactors. This discrepancy may result in multiple rounds of experiments, until a feasible strain for scale-up is identified. Therefore, cultivation conditions should be kept consistent throughout the developmental line.

In this work, platform technologies for consistent bioprocess development are presented in the form of three publications. First, a novel dissolved oxygen sensor for screening facilities was developed, which can determine the aeration level of cultures grown in 96-well plates that are transported from the incubator to the plate reader. The long response time (t_{90}) of 9.7 min allows an estimation of the oxygenation status during incubation. The sensor detected oxygen limitation in fed-batch cultures of *E. coli* and *S. cerevisiae*.

In the second part, a workflow for rapid cell lysis buffer optimization using design of experiments (DoE) is presented. Experiments were planned with a DoE software and written as worklists for the liquid handling robot into a laboratory information management system (infoteam iLab-Bio). In three experimental runs, a lysis buffer composition for efficient release of beta-galactosidase from *E. coli* was determined.

The product formation profile of yeast strains was evaluated using parallel fed-batch cultures at the millilitre-scale in the third part. For comparison, A-stat cultivations in a 1.5 L bioreactor were performed, which showed comparable product formation rates.

Using these platform technologies, a framework for streamlined experimental planning, execution and data management can be established.

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List of Abbreviations

a.s.	Air saturation
ANN	Artificial neural networks
BCA	Bicinchoninic acid
DO	Dissolved oxygen
DoE	Design of experiments
EDTA	Ethylenediaminetetraacetic acid
EPG	Endo-polygalacturonase
FDA	U.S. Food and Drug Administration
GMP	Good manufacturing practice
GRAS	Generally regarded as safe
HT	High-throughput
LHS	Liquid handling system
LIMS	Laboratory information management system
MLR	Multiple linear regression
OD	Optical density
PLS	Partial least squares
QbD	Quality by design
SiLA	Standardization in laboratory automation
ßGal	ß-Galactosidase

1 Introduction

Within the past decades, a vast number of organisms, synthesis pathways and molecules were discovered and numerous methods to make use of this were established. The knowledge and technology obtained now serves as the basis for a sustainable, bio-based economy, which will ideally lead to the replacement of bulk chemicals derived from crude oil (Viaggi et al., 2012). Modern pharmacology and pharmaceutical production would not work without the help of cells and enzymes. In the near future, synthesis of novel polymers and therapeutic substances by engineered enzymes or synthetic cells will be possible (Weber and Fussenegger, 2011).

Although biotechnology is considered one of the key technologies of the 21st century, innovation and development of products is still consuming a comparatively high amount of time, personnel and capital. This is mainly due to the complexity of biological processes. Exploiting the synthesis capabilities of cells for commercial purposes requires a combination of deep scientific knowledge of the biological functions and a thorough understanding of process engineering. In contrast to other fields such as mechanical engineering or chemistry, this discrepancy has not yet been solved (Golembiewski et al., 2015).

While the initial stages of bioprocess development are mainly driven by natural sciences, engineering aspects usually come into play at a later stage. Consequently, the development does not follow a holistic approach with a strong focus on the final production scale, but is – especially at the very early stage of product development – driven by a high degree of experience-based decisions and trial-and-error. If applicable the industry heavily relies on existing process blueprints, the so-called platform technologies, to minimize risk during scale-up. However, in some cases this strategy may even increase the overall risk of economical failure (Neubauer et al., 2013).

To overcome these obstacles, the implementation of engineering aspects already at the early screening stage is of great importance. Keeping conditions comparable to the production scale while going through the developmental stages ensures robustness and decrease risk of failures during scale-up (Islam et al., 2008; Marques et al., 2010).

The aim of this thesis is to combine existing technologies for automation, cell cultivation, measurement of key process parameters, experimental planning and data management into platforms, which can be used to accelerate bioprocess development.

For rapid strain screening and process development, cultivation conditions should be defined in compliance to the production scale, which requires fed-batch operation of small scale cultures in most cases. Since large-scale bioreactors are monitored extensively, process monitoring needs to be established at the screening stage. Dissolved oxygen (DO), pH, cell density and product concentration were selected as the key parameters for this work. For DO, a reliable determination of the oxygenation state of micro-scale cultures in 96-well plates needs to be developed. Moreover, experiments in this scale should yield not only end-point data, but allow the determination of productivity profiles with respect to the strain's growth rate. Efficient experimental planning using design of experiments (DoE) methodology and rapid data evaluation is another goal, for which an integration of software packages and automation equipment needs to be established.

To give an overview on the current knowledge state and point out open questions, the subsequent literature review opens with a summary of the process development workflow. Then, the current state of technology in the field of miniaturized cultivation systems is presented, followed by a chapter on small-scale fed-batch technologies. In the final chapter, experimental design and data management in automated laboratories is reviewed.

2 Literature review

2.1 Bioprocess development – past, present and future

Fermentation processes were involved in food production and conservation for several thousand years without any knowledge on the scientific background. Since the early days of microbiology, microbes have been increasingly used for the industrial production of bulk chemicals, fine chemicals, as well as pharmaceuticals. The field of industrial biotechnology is nowadays considered to be one of the key technologies of the 21st century, paving the way into a sustainable bio-based economy (Bugge et al., 2016). Prominent examples of biotechnological products are amino acids (Hermann, 2003), 1-3-propanediol (Biebl et al., 1999), human insulin (Johnson, 1983) and citric acid (Shu and Johnson, 1948). The strains used during large-scale manufacturing of these products have gone through multiple rounds of engineering before they can produce economically feasible amounts (Ferrer-Miralles et al., 2009).

The development of bioproduction processes consists of three design steps: strain design, medium optimization and process design (Kumar et al., 2004). For strain development, several starting points are possible. A strain collection of natural isolates, random or directed mutagenesis of previously identified producer strains, or cells containing metagenome libraries from different habitats may serve as the initial pool of candidates for selection (Parekh et al., 2000). Metabolic engineering, multi -omics approaches and in silico strain design have increased the possibilities of strain improvement, but also raised the required experimental throughput (Sang et al., 2005).

Strain screenings are usually carried out in test tubes, shake flasks or microwell plates. To ensure rapid growth and cover a wide range of possible nutrient requirements, the strains are grown in rich media containing yeast extract or other complex additives. Due to the low oxygen transfer rate of surface-aerated cultivation vessels and the high oxygen demand of exponentially growing cultures, the cells are commonly facing oxygen limitation during initial screening, which is considered as a major risk for the validity of screening results (Zimmermann et al., 2006). Such a screening selects candidates, which are robust against oxygen limitation and are able to synthetize high amounts of product during the exponential growth phase. Based on these results, the top candidates are then transferred into benchtop bioreactors for strain evaluation and process development.

Since most production hosts show overflow metabolism at elevated substrate concentrations, the fed-batch mode is commonly applied in the bioreactor. This inconsistency in cultivation conditions may result in a failure of the candidate during scale-up, which leads to another round of initial small-scale experiments. After development of a fed-batch protocol with a high space-time yield, the process is scaled-up to pilot and then to production scale. The number of iterations before scale-up strongly depends on the experience of the personnel and the existence of platform technologies, which have been proven to work for similar processes (Sun and Chew, 2012). It is doubtful that production processes which originate from such a workflow will give the best possible space time yield and are robust against disturbances of the process.

To address the unsatisfactory process characterization, the Quality by Design (QbD) initiative was first introduced to the chemical and pharmaceutical production industries as part of the good manufacturing practice (cGMP). The expectations of the regulatory agencies were outlined in the process analytical technology (PAT) guidance by the US Food and Drug Administration (FDA) (FDA, 2004). QbD is defined in the International Conference on Harmonisation (ICH) guidelines as "a systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management (ICH, 2009)". In 2009, Rathore et al. have presented a roadmap for the implementation of QbD for biomanufacturing, which condensates the ICH guidelines into a multistep process (ICH, 2005; ICH, 2008; ICH, 2009). First, the product attributes, which are of special importance for the product safety and efficacy need to be identified. Then, the process needs to be designed in a manner to deliver these attributes and a process control strategy is developed. Finally, the production process needs to be monitored to ensure robust process performance (Rathore, 2009).

A reduction of time-to-market and overall risk can be achieved, if cultivation conditions are kept constant throughout the developmental line, resembling the production scale as closely as possible (Formenti et al., 2014). One possible solution is to grow cells in chemically defined medium under glucose-limited fed-batch conditions already at the screening stage. With improved aeration and dissolved oxygen monitoring, anaerobic conditions can be prevented (Hermann et al., 2003). When performing screening campaigns in automated facilities, experimental throughput can be drastically increased, which allows the screening of large libraries. Promising candidates are then transferred into parallel bioreactors with working volumes of 10 – 1000 mL for in-depth

characterization and process optimization (Figure 2.1). In case of known impact of gradients, which occur at production scale, on the strain's performance, scale-down simulators can be used to rule out possible pitfalls during scale-up (Neubauer and Junne, 2010). This process can be streamlined, if state of the art small-scale cultivation systems, sensors, experimental design and data management are used. The recent advances in these fields are described in the following chapters.

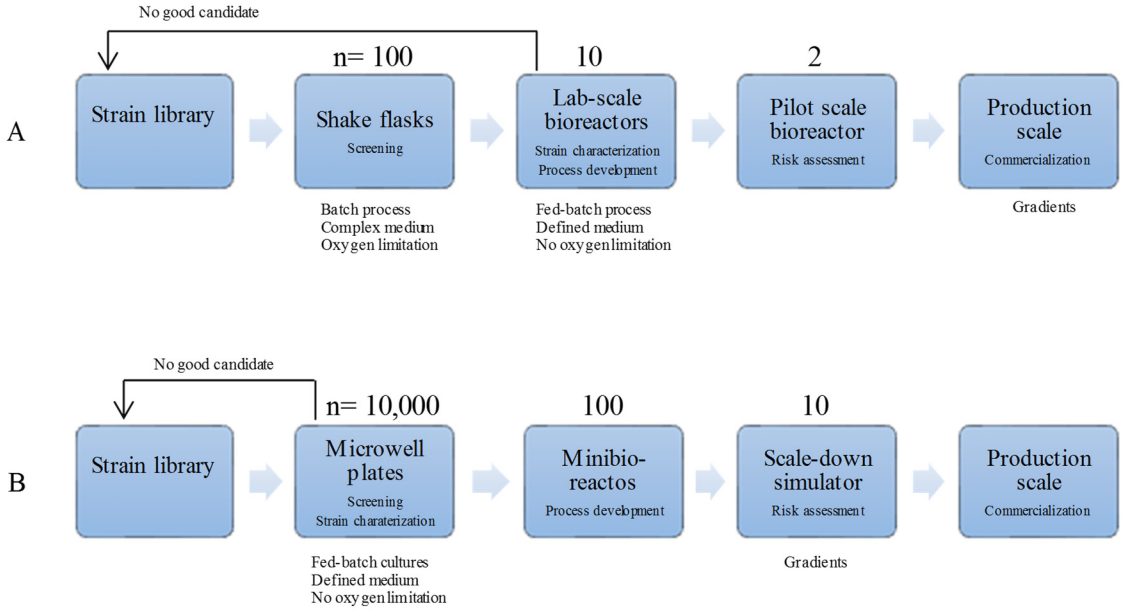

Figure 2.1.1 Overview of developmental stages in upstream process development: classical approach (A) versus consistent high-throughput approach (B). The estimated number of experiments n is stated above the developmental stages. If strain characterization did not reveal a promising candidate for scale-up, the process is repeated (adapted from (Sun and Chew, 2012)).

2.2 Parallel cultivation systems

Shake flasks

Shaken bioreactors are the most popular form of cultivation vessels (Bareither and Pollard, 2011). Historically, Erlenmeyer flasks have been the vessel of choice for liquid cultures as they were readily available in any chemical laboratory. They are made of borosilicate glass or plastic, feature a volume range from 25 mL to 5 L and can be equipped with baffles. The working volume is usually between 10 and 20% of the total volume depending on the shaking conditions.

Although being in use for more than a century, only within the last two decades shake flasks have been characterized with regards to their process engineering aspects (Büchs, 2001). The influence of operating conditions such as filling volume, shaking frequency and diameter on the gas transfer were included in a mathematical model by the group of Jochen Büchs (Maier and Büchs, 2001). Their precise characterization of liquid distribution in flasks of different sizes and geometries revealed that at certain conditions, oxygen transfer is negatively affected by out-of-phase behaviour of the liquid phase, when baffled flasks are used (Büchs et al., 2001). For unbaffled flasks, the mass transfer was described with a mathematical model validated with experiments using the sulphite oxidation method (Maier et al., 2004). An overview of gas-liquid mass transfer coefficients (k_La) of common small-scale cultivation systems is given in Table 2.2.1.

Shake flasks are the routinely used for cultivations of bacteria (Ibarra et al., 2002), yeast and fungi (Bushell et al., 1997; Veglio et al., 1998), insect and plant cells (Chattopadhyay et al., 2002; Marteijn et al., 2003), as well as animal cells (Jänicke et al., 2007). Several studies have demonstrated the impact of operating conditions such as shaking speed and orbit, flask closures such as cotton plugs or gas-permeable membranes (Ukkonen et al., 2011), and flask shape.

Despite the availability of these guidelines, the majority of cultivations is performed without awareness of this issue (Büchs, 2001). To shed light into the “black box”, the instrumentation level of shake flasks has been steadily improved over the past 20 years. Oxygen uptake rates can be measured on-line with a specific device (RAMOS – Respiratory Activity Monitoring System) (Anderlei and Büchs, 2001). Optical sensor systems (SFR Vario – Multiparameter Shake Flask Reader) allow the on-line measurement of dissolved oxygen (Schiefelbein et al., 2013; Tolosa et al., 2002;

Wittmann et al., 2003), pH (Schneider et al., 2010) and biomass (Schmidt-Hager et al., 2014). With customized lids and pumps, successful pH control in shake flasks was reported recently (Ude et al., 2015).

Microwell plates

For increased throughput at the screening stage, the most common format for cell propagation is the microwell plate (Weuster-Botz et al., 2006). The 96-well plate was invented in the 1950s for diagnostic tests (Takàtsy, 1955), but the format became increasingly popular for the miniaturization of enzymatic assays and polymerase chain reaction (Nakayama, 1998). High-throughput (HT) screening of compound libraries in drug discovery can be considered as one of the most prominent examples of microplate usage (Hüser et al., 2006).

Since the turn of the century, microplates were increasingly used for small-scale cultivations. Gas-liquid mass transfer was characterized for 96 well plates (Hermann et al., 2003), as well as 48 and 24 well plates (Duetz et al., 2000; Duetz and Witholt, 2001) resulting in recommendations on how to perform oxygen-demanding experiments with special lids (Figure 2.2.1). The sandwich covers, are commercially available as 'System Duetz' covers and work together with a clamp system to fixate the covers on the microplates. The covers allow headspace refreshment rates of one culture volume per minute while reducing evaporation to about 2 % of culture volume per day (Duetz, 2017).

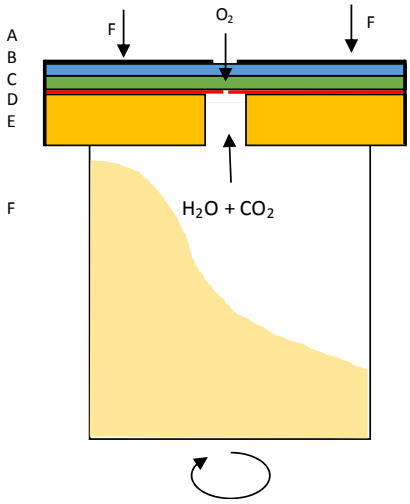

Figure 2.2.1 Cross-section of a single well of the 'System Duetz' sandwich cover. A: stainless steel lid (black), B: microfiber filter (blue), C: polytetrafluorethene filter (0.3 µm) lamitated between two polyester and polyamide fabrics (green), D: stainless steel foil with pinholes (red), E: silicone layers for hermetical sealing (yellow), F: culture broth. The sandwich cover is fixed onto the microwell plate with a defined force (F) of 400 N. Adapted from (Duetz, 2017)

Microwell plate cultures can be easily handled with multichannel pipets and liquid handling robots for simplified culture handling. Several examples of automated cultivation platforms were reported. Among them are screening campaigns for biotransformation reactions in deep well plates (Doig et al., 2002), yeast strain development in low well plates (Zimmermann and Rieth, 2006), monoclonal antibody process development in yeast (Barnard et al., 2010), expression and purification of proteins using *E. coli* (Vincentelli et al., 2011), at-line monitoring of microbial fed-batch cultures (Knepper et al., 2014) and mammalian cell line development (Daniels et al., 2016; Markert and Joeris, 2017).

Still, in most cases, microwell plate cultivations are carried out without any knowledge on the DO level (Duetz, 2007) or the growth dynamics of the culture. Even if instrumentation is desired, there are currently no commercially available solutions to measure DO in shaken microwell plates on robotic platforms. This issue was addressed in paper I (chapter 3.2).

To fill the gap between parallel benchtop bioreactors and microwell plates, the BioLector cultivation system was developed. It uses baffled microwell plates, which have a six-petal flower-shaped well geometry (Funke et al., 2009; Samorski et al., 2005). The system is able to generate growth and protein expression characteristics comparable to results from the L-scale (Kensy et al., 2009a) and can be integrated into liquid handling systems (Huber et al., 2009a; Rohe et al., 2012; Unthan et al., 2015).

In a scale-up study, Kensy et al. performed *E. coli* cultures expressing green fluorescent protein in the BioLector and a 2 L stirred tank bioreactor and found an excellent correlation, if the oxygen transfer in the microwell plate is matched to the bioreactor (Kensy et al., 2009a). The variation of filling volume, and consequently of mass transfer capacity is not only recommended for characterization of the screening system, but can also be used as a screening parameter. Käß et al. have made use of this set-up to determine the impact of oxygen availability on lysine production in *Corynebacterium glutamicum* (Käß et al., 2014). The same production host was studied by Unthan et al. on an automated platform with at-line lysine determination. Substrate uptake kinetics and product formation of 17 different strains were analysed in an automated manner (Unthan et al., 2015).

Despite the technological progress, researchers who work in microwell plates face several obstacles. The small culture volumes limit sampling mostly to end-point measurements. Although evaporation can be reduced with breathable sealing tape, a trade-off between oxygen transfer and moisture barrier is always needed. In addition, temperature inaccuracies caused by evaporation occur (Sieben et al., 2016). When plates with optical sensors are used, the read-out can be disturbed when expressing fluorescent proteins (Kunze et al., 2014).

Table 2.2.1 Gas-liquid mass transfer coefficient (k_La) of common small-scale cultivation systems.

Cultivation system	**Typical working volume [mL]**	**k_La [h^{-1}]**	**Reference**
Shake flasks	20 - 1000	50 - 400	(Glazyrina et al., 2011)
Deepwell plates	1 - 3	100 – 190	(Duetz et al., 2000)
Lowwell plates	0.15 - 0.2	50-150	(Hermann et al., 2003)
Minibioreactor	8 - 12	700 - 1500	(Puskeiler et al., 2005a)

Stirred miniature bioreactors

Stirred miniature bioreactors are superior to shaken systems with regards to mixing and mass transfer. They mirror conventional benchtop bioreactors, but scaled down by a factor of 10 to 100. In the group of Dirk Weuster-Botz, a miniaturized stirred tank system was developed and commercialized (Weuster-Botz et al., 2002). The temperature-controlled reaction block contains 48 disposable vessels with baffles and sensors for dissolved oxygen and pH. A k_La value of at least 700 h^{-1} was measured at working volumes of 8 – 12 mL (Puskeiler et al., 2005a). Among many other publications, the group reported successful discrimination of four different riboflavin producing *B. subtilis* strains (Vester et al., 2009), comparability to stirred tank cultivations of *Streptomyces tendae* (Hortsch et al., 2011) and the methylotrophic yeast *Komagataella pastoris* (Schmideder et al., 2016).

Another example is the µ-24 MicroReactor (Pall GmbH), which is capable of measuring and controlling dissolved oxygen, pH and temperature. It consists of a disposable cassette containing 24 bioreactors with a working volume of 4- 6 mL (Isett et al., 2007). Since maximal k_La values of 53 h^{-1} were reported, the device is better suitable for animal cells than microbial cultures (Betts et al., 2014). Still, the device was used in a study to optimize Cr(VI) reduction catalysed by *Shewanella oneidensis* (Tang et al., 2006).

Another type of minibioreactor is the Ambr system. It is a stirred and sparged disposable reactor system with pH and dissolved oxygen sensors and is mainly designed for animal cell culture. The system was found to be a superior scale-down model for early-stage process development than shake flasks (Hsu et al., 2012). DO and pH can be controlled individually (Kim et al., 2012) and the system is available in two sizes: 15 and 250 mL working volume. The 15 mL system was applied in comparative studies with stirred tank reactors and comparable profiles for CHO cell growth and antibody production were found (Moses and Manahan, 2012). A comprehensive characterization of the cultivation system revealed that the specific power input is much higher than in large vessels, which is necessary for achieving sufficient k_La values for aerobic growth. Moreover, instead of a turbulent flow, the flow regime is transitional. Despite the differences to large scale vessels, but due to the similar control possibilities, the comparability of scales is given (Nienow et al., 2013).

Recently, the 250 mL Ambr system was reported to be a reliable scale-down model based on power input and k_La. Comparable product titers and cell counts were reached in the Ambr system and at the 5 and 1000 L scale (Xu et al., 2017). An overview of commercially available miniaturized cultivation systems is given in Table 2.2.2.

Table 2.2.2 Overview of commercially available parallel small-scale bioreactor systems.

Adapted from (Baumann and Hubbuch, 2017) and (Fernandes et al., 2011).

Name	**Manufacturer**	**Reactors**	**V_R [mL]**	**k_La [h^{-1}] / OTR [mmol (L h^{-1})]**	**Sensors**	**Feeding/ Automation**	**Applications (Organism / Product)**
Ambr	Sartorius AG	48	10-15	k_La ~ 380	pH DOT	X / X	CHO cells / monoclonal antibodies (Hsu et al., 2012)
							E. coli / biomass (Betts et al., 2016)
BioLector	m2p-labs GmbH	48	0.8-2.4	k_La >600	pH DOT	X / X	*C. glutamicum/* Cutinase (Rohe et al., 2012)*;*
					Biomass		*H. polymorpha* / GFP (Kensy et al., 2009b)
bioREACTOR 48	2mag AG	48 or 8	8 – 15	k_La 720-1440	pH DOT	X / X	*Bacillus subtilis* / Riboflavin (Knorr et al., 2007)
Bioscreen C Pro	Oy Growth Curves Ab Ltd	200	0.4	N. a.	None	- / -	*Listeria monocytogenes* (Begot et al., 1996)
							Saccharomyces cerevisiae (Weiss et al., 2004)
Cellstation	Fluorometrix corp.	12	up to 35	N. a.	pH DOT	X / -	mouse hybridoma cells / antibody (Ge et al., 2006)
DASbox	Eppendorf AG	24	60-250	N.a.	Sampler	X / -	*E. coli* / GFP (Brognaux et al., 2013)
							Cardiomyocytes (Kempf et al., 2015)
HexaScreen	Telstar Life Sci. Sol.	6	10-15	N. a.	Sampler	- / -	Adherend and suspended animal cells (Fontova et al., 2006)
Mico-24	Pall corp.	24	3-7	OTR >300	pH DOT	X / -	CHO cells / IgG1 (Chen et al., 2009)
							S. cerevisiae, E. coli P. pastoris / biomass (Isett et al., 2007)
Micro-Flask	Applikon Biotechnology	96	1	OTR 12-51	None	- / -	*P. putida* / biomass (Duetz and Witholt, 2001)
Micro-Matrix	Applikon Biotechnology	24	1-5	OTR >300	pH DOT	X /X	(Choi and van Breemen, 2008)
Multifors	Infors HT	6	10-1000	N.a.	Sampler	X / -	*Y. lipolytica* / α-ketoglurate dehydrogenase (Holz et al., 2011)
							H_2O_2-adapted bifidobacteria (Mozzetti et al., 2010)
SimCell	Seahorse Bioscience Inc.	6 per array	0.7	N.a.	pH DOT	X / X	CHO cells (Amanullah et al., 2010)
Xplorer	HEL	8	100-4000	kLa < 400	Sampler		*E. coli* / biomass (Gill et al., 2008)

2.3 Small-scale fed-batch technologies

For consistent bioprocess development, strain screening and media optimization should be performed under conditions relevant to the final production scale (Noorman, 2011). If the industrial process is performed under fed-batch conditions, this cultivation mode should be kept throughout all developmental stages. The control of the cellular metabolism by glucose limitation has several advantages, which are important for small-scale cultivations. Due to the direct correlation of nutrient availability and cellular respiration, oxygen limitation can be avoided by adjusting the feed rate to the oxygen transfer capacity of the cultivation system. Ideally, the k_La of the screening system should match the production scale, which is known to simplify scale-up (Micheletti et al., 2006). Several solutions for performing small-scale fed-batch experiments exist and were reviewed recently (Krause et al., 2016).

These systems can be divided into mechanical and non-mechanical methods. With the ongoing progress in microelectronics and -fluidics, liquid dosing in the nanolitre-range became technically feasible. Funke et al, for example have developed a 48-well plate containing micro channels, which allow precise dosing of liquids from reservoir wells into culture wells. The plate can be used on the BioLector platform (Funke et al., 2010). The same group has also developed a diffusion-based feeding on a microplate, that contains a reservoir and the culture wells are connected with polyacrylamide gel channels (Wilming et al., 2014). Furthermore, a prototype microfluidic dosage unit for the 48-minibioreactor system developed in Dirk Weuster-Botz' group was reported. The unit can perform fed-batch cultures with double-sided pH adjustment. However, investigations of long-term stability and reproducibility are still necessary (Gebhardt et al., 2011) and until now, no finished product is available.

Non-mechanical methods for small-scale fed-batch cultures were already reported in the 1950s, when Tyrell et al. began experimenting with biphasic systems containing nutrient agar and liquid medium and discovered its beneficial attributes for bacterial cultures (Tyrrell et al., 1958). The system was later adapted for ammonia feeding of *Streptomyces* cultures (Lübbe et al., 1986) and served then as the basis of the FeedBead® technology (Jeude et al., 2006). In this growth system, silicone elastomer discs containing crystalline glucose are added to the liquid growth medium. The diffusion-based glucose release can

be adjusted by the number of beads added to the shake flask. For microwell-plates, the same amount of elastomer depots is immobilized in each well, which restricts the use to an identical feed rate throughout the plate. The plates were used in a study to synchronize parallel precultures of *E. coli* and *H. polymorpha* (Huber et al., 2009b).

Contrary to the diffusion-based system, the EnBase® cultivation technology allows the control of the feed rate without any external pumps or other equipment. In the growth medium, a polysaccharide is gradually degraded by a biocatalyst releasing a glucose into the culture medium. The glucose release is dependent on the amount of enzyme added to the culture and the medium pH, until the polysaccharide pool reaches a certain lower threshold.

The first version of the EnBase growth system consisted of two gel-layers and a liquid mineral salt medium (Panula-Perälä et al., 2008). Starch is constantly diffusing from the gel into the medium, in which the glucoamylase enzyme is releasing glucose monomers from the polysaccharide. By changing the polymer from starch to a soluble derivative, the gel-layers became obsolete, which simplified the preparation and handling while remaining growth control (Krause et al., 2010). The growth system has then later been changed from liquid medium to pre-sterilized tablets to simplify handling and is currently commercially available as EnPresso® medium for bacteria and yeast. Scale-up studies showed comparable protein production profiles in enzyme-based fed-batch cultures in microwell plates and shake flasks when compared to bioreactor fed-batch processes up to 100 L scale (Glazyrina et al., 2012). The medium was found to work well with miniature bioreactors resulting in improved cell density and product yield compared to traditional batch media (Hortsch and Weuster-Botz, 2011).

The successful production of more than 50 different target proteins with the EnBase growth system is described in the literature (Krause et al., 2016); most of the studies are using *E. coli* as a host organism. Among these target molecules are enzymes (Nowicki et al., 2015; Panula-Perälä et al., 2014; Veselá et al., 2016), proteins containing disulfide bonds (Matos et al., 2014; Nguyen et al., 2011), antibody fragments (Gaciarz et al., 2016; Ukkonen et al., 2013; Zarschler et al., 2013) and bioactive peptides (Gatte-Picchi et al., 2014; Jaitzig et al., 2014). Recently, a first mathematical model of the glucose release kinetics in the EnBase system was proposed (Herold et al., 2017).

The growth system was also successfully adapted to the requirements of yeast cultures. For Pichia pastoris, the slow glucose feed was found to be beneficial as it did not repress the AOX1 promotor after induction with methanol (Panula-Perälä et al., 2014). Promising results from parallel cultivation of a variety of yeast strains in deepwell plates for whole-cell biotransformation were also published (Grimm et al., 2012).

Especially for cases, in which conventional cultivation approaches failed, the so-called difficult-to-express proteins, harnessing the host cells protein production machinery via the cultivation conditions has led to the successful production of active soluble protein. A prominent example is the production of an RNAse inhibitor, which strongly aggregated when expressed in *E. coli* under standard conditions, but was produced correctly folded in the cytosol after screening of 45 different vectors under fed-batch conditions (Siurkus et al., 2010). The yield was further increased by the addition of a reducing agent, a low expression temperature and co-expression of the chaperonin GroELS (Šiurkus and Neubauer, 2011a) and dithiothreitol was determined as a key factor for both cytoplasmic and periplasmic expression (Šiurkus and Neubauer, 2011b). The outcome of these investigations strongly depended on tight metabolic control, which is only possible under glucose-limited conditions.

Another example is the production of the nonribosomal peptide valinomycin in *E. coli*, which is synthetized by the nonribosomal peptide synthetase subunitis Vlm1 (370 kDa) and Vlm2 (284 kDa). Coexpression of this large protein complex in complex batch medium resulted in a short valinomycin production phase of 4h, which corresponds to the exponential growth phase. Under fed-batch conditions, however, the production phase was prolonged since the culture grows continuously. After an applying design of experiments (DoE) methodology to optimize cultivation conditions, a 5.2-fold improvement compared to the initial batch cultivation was achieved (Li et al., 2014).

Immunoglobulins are an important class of proteins for a wide range of therapeutic and diagnostic applications. Usually, the yield of functional antibody fragments is quite low. With the help of a controlled growth strategy and optimized production strains, Zarschnler et al. reported a yield of up to 200 mg L^{-1} of soluble single domain antibodies in the cytoplasm of *E. coli* (Zarschler et al., 2013). With a combination of an *E. coli* strain optimized for cytoplasmic production of disulfide bonds and the EnBase technology, Gaciarz et al. could produce single-chain antibody fragments with a yield of up to 240 mg L^{-1} (Gaciarz et al., 2016).

Most of the studies described in this chapter report product yield at the end-point of the cultivation. However, valuable information can be gained from the dynamics of product formation, especially during the first hours of glucose-limited growth, which was investigated in this thesis. The feasibility of using enzyme-based glucose delivery for the determination of product formation rates in parallel fed-batch cultures is described in paper III (chapter 3.4).

Especially when combining HT cultivation systems with fed-batch media, thorough experimental planning, process monitoring, and data handling is required. Methods for the effective utilization of the experimental capabilities are described in the following chapter.

2.4 Experimental design and data management

The advances in laboratory automation and small-scale up- and downstream unit operations have increased the experimental throughput immensely. The acceptance and adaptation of these technologies was encouraged by the QbD initiative of the FDA (Bhambure et al., 2011). To make use of the increased experimental throughput efficiently, a combination of expert knowledge, DoE and mathematical modelling is necessary (Baumann and Hubbuch, 2017).

As an example, if a parallel cultivation system is performing an experiment with eight 96 well plates, which can be read out and sampled at-line at a rate of bi-hourly measurements for 24 hours, the analysis of four parameters (e.g. DO, optical density, extracellular glucose and acetate levels) would result in the generation of 36,864 data points. For processing and safe storage of the vast amount of data generated by HT experiments, a laboratory information system (LIMS) should be used. This chapter will give a brief overview of the methods and tools available to plan experiments and handle data in the automated laboratory.

Statistical experimental planning, or DoE, is a concept for investigating the relationship between the input and the output variables of a system, which are commonly named factors and responses. Its strength lies in the reduction of experiments needed to find an optimum and the identification of interactions between factors and their influence on the responses. The classical experimental design is the factorial design, which can contain qualitative and quantitative factors. The initial step is usually a screening for important factors using the corner experiments of the design space. Then, a subset of these factors can be repeated at different levels in order to predict the optimum (Mandenius and Brundin, 2008).

Alternatively, both steps can be combined when using a computer-generated D-optimal design. In this case, an algorithm selects the levels of each factor based on the number of experiments selected by the user (Lutz et al., 1996). Another scenario is mixture design, which is applied for the optimization of growth media and buffers. The concentration of components is transformed into intervals between 0 and 1, which results in a symmetric triangle for a three-variable design (Eriksson et al., 1998).

Regardless of the chosen design, the response surface is evaluated with the same methods. Commonly, multiple linear regression (MLR), which uses linear or quadratic terms for each factor, as well as interaction terms, is used. When the design is not orthogonal, partial least squares (PLS) regression can be applied (Mandenius and Brundin, 2008).

Since the complexity of cellular growth and target protein production cannot be described by linear or quadratic equations sufficiently, alternatively artificial neural networks (ANN) can be trained to describe complex nonlinear relationships (Glassey et al., 1994). More information on the mechanisms behind the interaction of the factors require the integration of nonlinear differential equations. For parameter estimation of these equations, DoE strategies can assist in reducing the experimental work (Takors et al., 1997). Recently, hybrid model approaches, which combine ANNs with mechanistic models were introduced, which allow to adapt the complexity of the microkinetic model to the available experimental data (von Stosch et al., 2016). Further reduction of the amount of experimental effort can be achieved, if the experiments are evaluated and re-designed during cultivation using optimal experimental design. Initial studies, which prove the effectiveness of this approach were recently published by our group. An average 50-fold reduction of the variation coefficient for parameter estimates compared to the sequential method was found for the fitting of an *E. coli* model (Cruz Bournazou et al., 2016).

Another important issue is the integration of up- and downstream process development. Recently, Baumann et al. have published a study in which they have combined protein expression optimization using micro-scale cultivations in the BioLector system with *in silico* chromatography optimization. Although the highest recovery was obtained from the best-performing cultivation, the optimum purity was among the low-yield fermentations. This indicates that an integrative approach can be a powerful method to tackle problematic impurities, which could lower the overall production costs significantly (Baumann et al., 2015).

Apart from the efficient planning of experiments, data handling, storage and laboratory instrument control are equally important for efficient usage of the equipment in an automated environment. LIMS were developed since the early days of automation. In 1973, the first symposium on *"Guidelines for Defining and Implementing the Computerized Laboratory System"* (Gibbon, 1996) was held.

Since then, LIMS steadily improved alongside the hardware and software packages, especially for biochemical and DNA sequence related data, which are generated on large amounts since the early 2000s. However, the integration of LIMS with a variety of equipment such as liquid handlers, incubators and analytical devices still requires extensive programming skills (Chapman, 2003). Many LIMS are proprietary software suites, which hinders adaptation to the end-users requirements (Liotta and Petricoin, 2003).

To overcome these obstacles when setting up an automated laboratory, graphical programming languages such as LabVIEW were proven to be helpful (Elliott et al., 2007) and standardization efforts were made, which lead to the SiLA-standard (Standardization in Lab Automation) (Bär et al., 2012). Some groups have even developed open-source software for the scheduling and control of hardware, such as Clarity from Harvard University (Delaney et al., 2013). Another example is the iLab software framework from infoteam Software AG, which was used in this thesis. It consists of a modular structure for various tasks such as DoE, data logging, visualization and a graphical user interface. All information are stored in a SQL database, which can be accessed by SiLA compatible devices (Schmid and Aschoff, 2016).

Besides data handling and experimental planning, method development requires a significant amount of time before any automation procedure can be performed routinely. At first, the decision is needed if a custom-made solution should be done by the equipment manufacturer or if the methods should be developed in-house. Contrary to diagnostic tests or screening routines of thousands of strains, in bioprocess development the laboratory needs to be very flexible. Organisms with different metabolite profiles, products with different detection methods, experiments with changing plate layouts and cultivation routines. Therefore, a modular framework is necessary to avoid excessive adjustment and testing of methods. To utilize the equipment optimally, scheduling of the equipment usage is necessary. The integration of hardware and software into process development platforms was done in this work, and will be described in more detail in paper II.

3 Results

3.1 Structure of this work

In the recent years, significant technological advancements in all fields related to bioprocess development were made. Miniaturized bioreactor systems evolved from prototypes and concept studies into commercial products, which are routinely used in industry and academia. Coupled with automation systems and sensor technology, the cultivation throughput has increased drastically. Fed-batch technologies for the mL and µL scale were developed and the scientific community is increasingly using these advanced growth systems. Although experimental planning, execution and data management can be automated, the full potential of this is often not used.

So far, only few attempts to integrate these technologies into a novel process development workflow were made, none of which aimed at keeping cultivation conditions comparable throughout the developmental line. Therefore, the aim of this thesis is to develop methods for consistent bioprocess development and combine them into platform technologies for accelerated transition of product ideas into processes at the industrial scale.

In the following chapters, case studies in the form of three publications are presented. In paper I, a new type of DO sensor is developed. The system was designed for automated fed-batch cultivations in the µL-scale and validated with *E. coli* and *S. cerevisiae* cultures.

In paper II, a workflow for convenient experimental planning, performance and evaluation is developed for cell lysis buffer screening and optimization. The native beta-galactosidase of *E. coli* served as a model protein for the determination of cell lysis efficiency.

The combination of parallel fed-batch cultivations and at-line product formation measurement is presented in paper III. The product formation rates in 1 mL cultures of *S. cerevisiae* cultures secreting a pectinolytic enzyme were found to be comparable to 1.5 L A-stat bioreactor cultivations.

3.2 Paper I

Toward microbioreactor arrays – a slow responding oxygen sensor for monitoring of microbial cultures in standard 96-well plates

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Keywords: Oxygen sensor, response time, high-throughput, microbial cultures

Abbreviations: a.s., air saturation; **DO**, Dissolved oxygen; **OD_{600},** Optical density at 600 nm]

3.2.1 Abstract

In this study, a slow responding chemooptical sensor for dissolved oxygen (DO) integrated into a 96-well plate was developed. The slow response time ensures that the measured oxygen value does not change much during plate transport to the microplate reader. The sensor therefore permits *at-line* DO measurement of microbial cultures. Moreover, it eliminates the necessity of individual optical measurement systems for each culture plate, as many plates can be measured successively. Combined with the 96-well format, this increases the experimental throughput enormously.

The novel sensor plate (Slow OxoPlate) consists of fluorophores suspended in a polymer matrix that were placed into u-bottom 96-well plates. Response time was measured using sodium sulfite and a t_{90} value of 9.7 min was recorded. For application, DO values were then measured in *E. coli* and *S. cerevisiae* cultures grown under fed-batch like conditions. Depending on the DO sensor's response time, different information on the oxygenation state of the culture plate was obtained: a fast sensor variant detects disturbance through sampling while the slow sensor indicates oxygen limitation during incubation.

A combination of the commercially available OxoPlate and the Slow OxoPlate enables operators of screening facilities to validate their cultivation procedures with regards to oxygen availability.

3.2.2 Introduction

Dissolved oxygen (DO) is a crucial parameter for aerobic bioprocesses since the solubility of oxygen is low in aqueous solutions containing nutrients and salts while exponentially growing cells have a high oxygen demand. The volumetric oxygen consumption may exceed the oxygen transfer rate, especially at high cell densities (Suresh et al., 2009). Oxygen limitation has severe effects on growth and product formation (Glazyrina et al., 2012; Zimmermann et al., 2006) and is therefore monitored during production processes in bioreactors (Suresh et al., 2009). In contrast to that, especially the screening for microbial production strains is mostly carried out under uncontrolled conditions without any process monitoring (Neubauer et al., 2013).

To overcome this lack of information, several commercial systems for online DO monitoring in shake flasks (Schneider et al., 2010), minibioreactors (Puskeiler et al., 2005a) and microplates (Funke et al., 2009) were developed. They all share the disadvantage of a limited number of parallel cultivations and are therefore not suitable if hundreds or thousands of putative production strains need to be screened.

96-well plates are a common format for HT screening programs. They have been characterized with regards to oxygen transfer (Duetz et al., 2000) and mixing time and have been equipped with sensors for DO (Arain et al., 2006; Mercier et al., 2014), and pH (John et al., 2003). In a common laboratory automation set-up, numerous 96-well plates are incubated in a so-called plate hotel. To read out signals or take samples, the plates have to be removed from the hotel and processed. During that time, steady decrease of DO due to the cells' respiration occurs. In order to estimate the state of a culture during the cultivation in such a set-up, the sensor needs to have a "memory" function, i.e. it should show a slow response.

Therefore, it was the aim of this study to develop an oxygen sensor for at-line measurements in 96-well plates. Due to the slow response, the oxygen value measured in the plate reader does not change much compared to the one on the shaker. With the sensor, discrimination between aerobic and anaerobic conditions should be possible. The functionality of the prototype sensor then needs to be proven by response time measurements using sodium sulfite and microbial culture broth. Then the sensor is applied to monitor growth conditions of microliter-scale fed-batch cultures.

3.2.3 Material and Methods

Oxygen sensors

The dissolved oxygen in bacterial and yeast cultures was measured with optical oxygen sensors embedded in 96-well microplates (Microlon 600, Greiner, Frickenhausen, Germany). Additionally, to the sensors with slow response, commercially available microplates with an oxygen sensor with fast response (OxoPlate®, PreSens, Regensburg, Germany; t_{90} < 30 s) were used. The response time t_{90} is the time when oxygen has decreased to 90 % of its total decrease. The sensors consist of a fluorescent, oxygen-sensitive dye and an inert reference dye. Both dyes are integrated in a polymer. Furthermore, a microsensor (NTH-PSt1-L5-TS-NS40/0.8-NOP, PreSens; t_{90} < 2 s) was tested to obtain the actual oxygen content within the sample for comparison with the ones detected with the sensor plates with slower response.

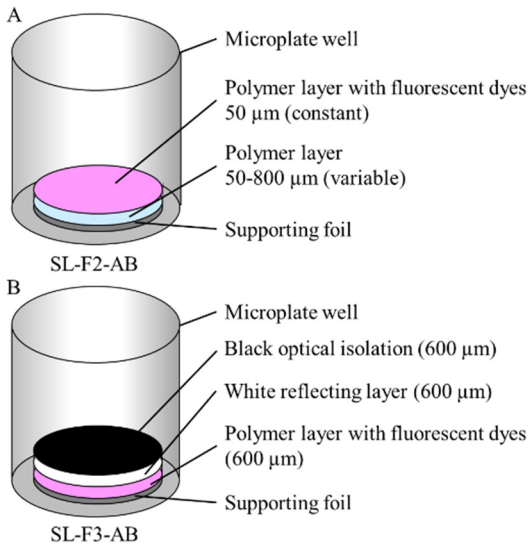

Figure 3.2.1. Schematic drawing of prototype sensor setups. (**A**) Two-layer sensor foil SL-F2-AB with variable polymer layer (50–800 µm) and constant fluorescent polymer layer (50 µm). (**B**) Three-layer sensor foil SL-F3-AB with fluorescent polymer layer, white reflecting layer, and black isolation (600 µm each). Five-millimeter spots were bonded into flat-bottom 96-well microplates.

Manufacturing of the sensor plates with slow response

Different approaches were tested to obtain a sensor with a reproducible response time of approx. 10 min. As the original oxygen sensor of the commercial OxoPlate responds within 30 s, it was tried to enlarge the response time by using different polymer layers and by increasing the layer thickness. Sensor foils with different layers and thicknesses were produced using a knife coating device (ZAA 2300, Zehntner GmbH, Sissach, Switzerland) to spread the liquid sensor material with a constant thickness onto a supporting foil. The dyes were dissolved in a hydrophobic polymer. The 2-layer sensor foils (SL-F2-AB, PreSens; Figure 3.2.1 A) consisted of a polymer layer with thicknesses between 50-800 µm and a polymer layer containing the fluorescent dyes with a constant thickness of 50 µm. The 3-layer sensor foils (SL-F3-AB, PreSens; Figure 3.2.1 B) consisted of a layer with fluorescent dyes, a white, reflecting layer and a black layer as optical isolation. Each of the 3 layers had a thickness of 600 µm. 5mm spots were cut out of these sensor foils and bonded with silicone glue (No. 692-542, RS Components, Mörfelden-Walldorf, Germany) into the wells of a 96-well, flat-bottom microplate (Greiner). The supporting foil faced the bottom of the microplate.

In a third approach, liquid sensor materials with volumes between 2 and 130 µL were pipetted into round-bottom 96-well microplates (Greiner). The sensors were produced using a hydrophilic (HG-T-AB, PreSens) and a hydrophobic polymer (SL-T-AB, PreSens), respectively. The sensors were dried under atmospheric conditions for at least 24 h. After drying, the polymer layer adheres to the bottom of the microplate. For volumes of more than 50 µL, successive addition of smaller volumes was also tested, with a minimum drying time of 4 h before adding the next volume. Four wells per microplate and volume were used. For the best sensor (Slow OxoPlate), plates with 96 sensors were produced, sealed in an opaque bag and beta irradiated. Figure 3.2.2 shows the set-up of the sensors as well as pictures of the Slow OxoPlate.

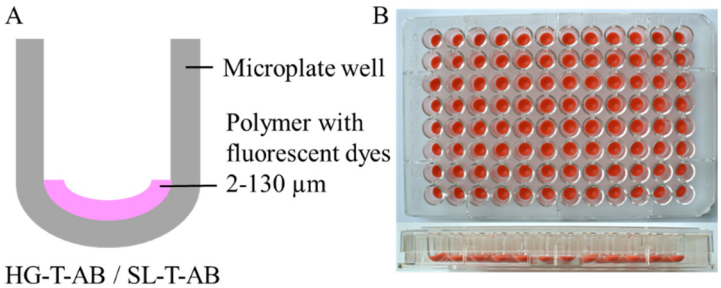

Figure 3.2.2 Third sensor set-up: liquid sensor materials pipetted into round-bottom microplates. **(A)** Schematic drawing of a u-bottom well containing air-dried polymer with fluorescent dyes. **(B)** Picture of the final product (top and side view)

Sensor characterization

For determination of the precision and resolution of the Slow OxoPlate, the sensors were calibrated using air-saturated water (cal100) and oxygen-free water (cal0). For cal100, about 20 mL of deionized water was put into a 50 mL glass vessel, closed with a screw cap and shaken vigorously for approx. 2 min. Then the glass was opened and moved gently to avoid oversaturation. For cal0, 0.2 g of sodium sulfite was dissolved in 20 mL deionized water in a closed glass vessel and moved slightly to dissolve the sulfite. 24 wells of the Slow OxoPlate were filled with 200 µL of cal100 and 280 µL of cal0 for the round-bottom plates and 380 µL of cal0 for the flat-bottom plates, respectively. The wells with cal0 were closed with strips of adhesive foil (EasySeal, Greiner) to minimize oxygen ingress. The plate was incubated in a microplate reader Fluoroskan Ascent (Thermo Fisher Scientific, Dreieich, Germany) for 1 h at 37 °C. The fluorescence signals were measured at 37 °C for 20 min with a measurement interval of 2 min, using the filter combinations 544 / 650 nm for the indicator dye and 544 / 590 nm for the reference dye. At these wavelenghts, most fluorescent media ingredients do not display fluorescence. The calibration constants k_{100} and k_0 were calculated as the average values of the respective intensity ratios of the indicator and reference dye.

Differing from this procedure, for DO calculation of the response time measurements, k_{100} was taken from the start value and k_0 from the end value of the respective measurement.

Calculation of DO from fluorescence data

DO (in % air saturation = % a.s.) was calculated from the ratios I_R of the fluorescence intensities of the indicator and reference dye and the calibration values k_{100} and k_0 using the Stern-Volmer equation:

$$DO = 100 \cdot \left(\frac{k_0}{I_R} - 1\right) / \left(\frac{k_0}{k_{100}} - 1\right) \quad \text{(Eq.1)}$$

Response time measurement

80 µL of cal100 were put into the 4 wells containing the sensors, covering all of the sensor material. The fluorescence intensities (544/650 nm and 544/590 nm) were measured with the Fluoroskan Ascent microplate reader at room temperature with an interval of 1 min for 2 h until the signals were constant. Then a new measurement was started, measuring

cal100 for 5 min with an interval of 1 min. After that, the plate was ejected and 200 µL (U bottom plates) or 300 µL (flat bottom plates) of cal0 containing $5 \cdot 10^{-4}$ M of cobalt nitrate as a catalyst were added rapidly with an 8-channel pipette and carefully mixed once. The wells were covered with strips of adhesive foil (EasySeal, Greiner) and the measurement continued with an interval of 0.25 min for at least 2.5 h until the signals were constant. After converting the signals into oxygen values, the response time t_{90} was determined as the time in which the oxygen decreased to 10 % of its original value of about 100 % air saturation.

Precision and resolution

For the Slow OxoPlate, precision and resolution at 37 °C were determined. Precision was calculated as the maximum standard deviation of 4 sensors measured at the same time. Resolution was calculated as the maximum standard deviation of 5 succeeding measurement points of a kinetic of one sensor. As target values for these specifications, the ones of the standard OxoPlate (PreSens) were applied. The precision of this plate is given as +/- 5% a.s. for cal100 and +/- 2 % a.s. for cal0, the resolution as +/- 2% a.s. for cal100 and 0.5 % a.s. for cal0.

Strains

For bacterial cultivation experiments, *Escherichia coli* W3110 and BL21 were used. The BL21 strain was transformed with the plasmid pDgPNP, which has been constructed for heterologous expression of a purine nucleoside phosphorylase from *Deinococcus geothermalis* (Szeker et al., 2011). For yeast cultivations, Saccharomyces *cerevisiae* AH22 harboring the plasmid pPG6 for secretion of an endo-polygalacturonase from *Aspergillus niger* (Lang and Looman, 1995) was used. Both strains were stored at -80°C in media containing 20 % glycerol.

Media and cultivation conditions

E. coli cultures were grown in EnPresso® B or a prototype growth system based on EnBase® technology (EnPresso® B Defined), while baker's yeast cells were grown in EnPresso® Y Defined medium (all from BioSilta Ltd. Cambridge, UK). Unless otherwise

stated, cultures with EnPresso growth systems were prepared according to the manufacturer's instructions. All cultivation experiments were performed at 30°C.

Microbial cultures were carried out in different systems and scales. Shake flask cultivations were performed with 25 mL EnPresso B using a 125 mL Ultra Yield Flask™ covered with AirOtop Enhanced Seal™ (Thomson Instrument Company, Oceanside, USA). For controlled glucose release, 1.5 U L^{-1} of biocatalyst (Reagent A) was added and the flasks were shaken in a Kuhner LT-X incubator (Adolf Kühner AG, Basel, Switzerland) at 200 rpm, 25 mm amplitude. Microplate cultures were carried out in two different ways. For first experiments with optimal aeration, the sensor plates were covered with "System Duetz" sandwich covers (Enzyscreen B.V., Haarlem, The Netherlands) and shaken at 300 rpm, 50 mm amplitude.

For automated reading and sampling, experiments were performed on a robotic platform, which was previously described (Knepper et al., 2014). Briefly, it consists of several instruments attached to a liquid handling robot (Hamilton Microlab Star, Hamilton Bonaduz AG, Bonaduz, Switzerland). The system can incubate up to eight 96-well plates and analyze samples with a microplate reader and a flow cytometer. The culture plates were sealed with XPierce™ foil (Excel Scientific Inc., Victorville, USA) and incubated on an orbital shaker (Hamilton FAME incubator). For measurements with the microsensor, the on-deck shaker Teleshake 95 (Inheco GmbH, Munich, Germany) was used.

Prototype sensor tests with bacterial cultures

Sensor tests with microbial cultures were performed using *E. coli* W3110 cells grown in shake flasks overnight to an optical density at 600 nm (OD_{600}) of 6 - 8. From the culture, different volumes (100, 150, 200 µL) were then transferred in triplicates to the sensor plates. The plates were covered with sandwich covers and shaken for one hour, in order to reach equilibrium conditions. Then, the plates were manually placed into a Biotek Synergy MX plate reader (Biotek Instruments Inc., Winooski). The delay until measurement was less than one minute. For both sensor plate types (Slow OxoPlate, OxoPlate), the same reader settings were used. The excitation wavelength for both fluorophores was 540 nm. The emitted fluorescence light of the indicator was measured at 650 nm, while the reference fluorophore emission was measured at 590 nm. A time-

resolved measurement from 0 - 500 µs was performed with a sensitivity setting of 50. Measurements were carried out every minute for 8 min. DO values were calculated from the raw data using Gen5 v1.09 (BioTek Instruments).

For comparison of the oxygen values generated with the microplates with an external reference instrument, a DO microsensor (PreSens) was applied. The Slow OxoPlate was shaken at different speeds on the Teleshake 95, which is located on the robotic platform, for 30 min. The microsensor was calibrated according to the manufacturer's instructions. A gripper transported the plate from the shaker to a position where the microsensor DO measurement was carried out. The transport time was 12 s. Due to the rapid signal decrease, the microsensor was only submerged in one well for 5 s. Afterwards, the sensor plate was transported to the microplate reader.

Cultivation on the robotic platform

E. coli BL21 pDgPNP glycerol stock (maintained at -80°C) was plated on LB agar containing 100 µg mL^{-1} ampicillin and incubated overnight at 30°C. The grown cell material was then washed from the agar plate using 3 mL of EnPresso B Defined [Prototype]. The yeast preculture was grown as a batch culture in EnPresso Y Defined containing 20 g L^{-1} of glucose for 24 hours.

The main cultures were prepared in Erlenmeyer flasks with cells from the precultures corresponding to an initial OD_{600} of 0.15. Then, 170 µl per well were distributed into the Slow OxoPlate and the OxoPlate, sealed and placed onto the robotic platform. The liquid handler added Reagent A in the predefined amounts (*E. coli*: 1 U L^{-1}, *S. cerevisiae*: 3 U L^{-1}). The plates were transported into the FAME incubator and shaken at 1000 rpm (2 mm amplitude). For DO measurement and liquid handling steps (OD_{600} measurement, addition of Reagent A), a sampling method was programmed using the Hamilton VenusOne Method Editor v4.2.1.6670. With the sampling method, the user is able to define sampling frequency and individual events using an MS Excel spreadsheet. Every two hours, the plates were transported from the shaker to the microplate reader for the DO measurements. For OD_{600} measurements, 5 µL samples were taken after 6, 14, 23, 33 and 43 hours. This automated sampling procedure included 30-fold dilution of the samples to a total volume of 150 µL in standard flat bottom 96-well plates (Greiner) and measurement at 600 nm in the microplate reader. After overnight incubation, glucose-release was further increased by addition of reagent A to the cultures.

3.2.4 Results

Development of a Slow OxoPlate sensor

The robotic platform used in this study is equipped with an incubator and a spectrophotometer, which are attached to a liquid handler. In order to read out fluorescence signals from a sensor plate, a total transport time from the shaker to the spectrophotometer of 26 s was measured. Since larger platforms may have longer transport times, a minimum response time (t_{90}) of ten minutes was defined.

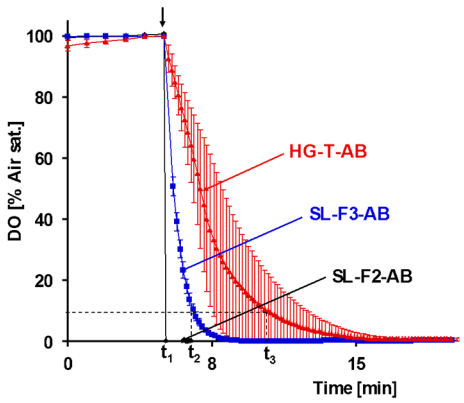

Figure 3.2.3 Response time measurements of the foil-based sensors SL-F2-AB, SL3-AB and the pipetted sensor HG-T-AB. After measuring oxygen at air saturation for 5 min with a measurement interval of 1 min, sulfite was added and the solution mixed. The arrow shows the time point of sulfite addition (t_1). The response times of SL-F2-AB (t_2) and SL-F3-AB (t_3) were determined.

The 2-layer SL-F2-AB foil sensors showed inhomogeneity of the sensor foil at layer thicknesses of 200 µm or more. For the response time measurements, the most homogeneous parts of the foil were used. The response time was < 10 s for all layer thicknesses and thus much too low for the target response time of 10 min (Figure 3.2.3). The 3-layer SL-F3-AB foil sensor revealed excellent reproducible results, but again the response time was only 1.0 – 1.2 min. The production of even thicker layers to enhance the response time was not possible due to the inhomogeneity of such thick sensor foils. Thus, foil sensors were not further investigated.

The pipetted sensor using a hydrophilic polymer (HG-T-AB) showed response times of 2.4 – 7.9 min for the maximum volume of 130 µL/well, which was still too low for this application. Furthermore, the reproducibility of the response times of the 4 different sensors was rather bad for volumes above 50 µL. Thus, higher volumes than 130 µL to increase the response time were not tried.

The pipetted sensor using a hydrophobic polymer (SL-T-AB) showed also a bad reproducibility for high volumes of liquid sensor material of above 75 µL. For these

volumes, surface defects in the sensor layer were visible due to inhomogeneous drying. Thus, the sensor material was applied to the microplate well in smaller volumes step by step. After addition of the first volume of sensor material, it was dried for at least 4 h at ambient air before applying the next volume. This improved the reproducibility dramatically. A volume of 2 x 15 µL gave a response time of 9.7 min. This sensor was further characterized with regards to precision, resolution, reproducibility of the response time and the oxygen values after 1 min.

The response times of 96 sensors pipetted from a different batch of liquid sensor material was determined and compared to the response times of the 4 sensor spots of the previous test. The results were very similar with 9.6 +/-0.8 min (Figure 3.2.4) compared to 9.7 +/- 0.6 min. The average oxygen values after 1 min were 91.4 +/- 4.1 % a.s. compared to 90.2 +/- 1.8 % a.s. from the previous test. This shows that the sensor can be produced in a very reproducible way. The slow response enables detection of the approximate oxygen value in the shaken culture after moving the microplate from the robotic system to the reader, as the measured oxygen decrease is quite small. The precision at 37°C for this sensor was determined as +/- 0.6% a.s. (cal0) and +/- 4.8 % a.s. (cal100), the resolution with +/- 0.05% a.s. (cal0) and +/- 0.2 % a.s. (cal100), respectively. These values lie within the target values of the OxoPlate. The Slow OxoPlate fulfills the requirements for this application and was used for the oxygen measurements during bacterial and yeast cultivation.

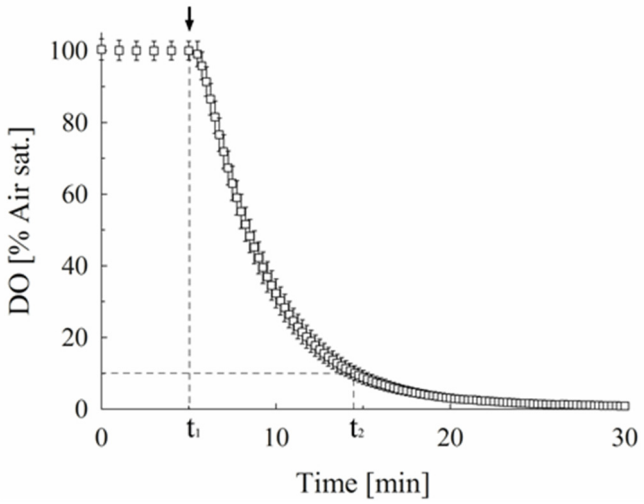

Figure 3.2.4 Response time measurements in the Slow OxoPlate with the sodium sulfite method. The arrow indicates the time point of sulfite addition (t_1). The decreasing DO was then measured with an interval of 15 s. 9.6 min after sulfite addition, oxygen dropped below 10 % a.s (t_2).

Proof-of-concept for microbial cultivation monitoring

In order to investigate the signal change during plate transport, the oxygen consumption of bacterial cultures was measured with the fast responding OxoPlate and the slow responding Slow OxoPlate. Cultures which were grown under glucose-limited fed-batch conditions were taken for these experiments, as they are characterized by a lower oxygen consumption rate than cells growing at their maximum growth rate. Preliminary experiments in shake flasks equipped with DO sensors were carried out, in order to determine the time, at which glucose limitation occurred. This was characterized by an increase of DO until 90% a.s. or above, which was reached after overnight incubation.

Then, the shake flask culture was transferred into the sensor plates, which were closed with sandwich covers for well-defined aeration and shaken for one hour. The fluorescence signals were then recorded for 8 min (Figure 3.2.5) without additional shaking. The Slow OxoPlate shows an initial DO value of 95 %, followed by a linear decrease. At the end-point, a DO value of 82 % was detected. In contrast to that, the signal of the OxoPlate declines from 70 % to 30 % within four minutes. The decline is then slowing down, reaching 21 % at the end-point.

Aerobic conditions were detectable with both sensor types, which means that both sensor types could be applied on the robotic platform for bacterial cultures under glucose limitation. However, in this experiment cells were slow growing, and the initial DO was close to 100% air saturation, which does not resemble the actual situation during a strain screening.

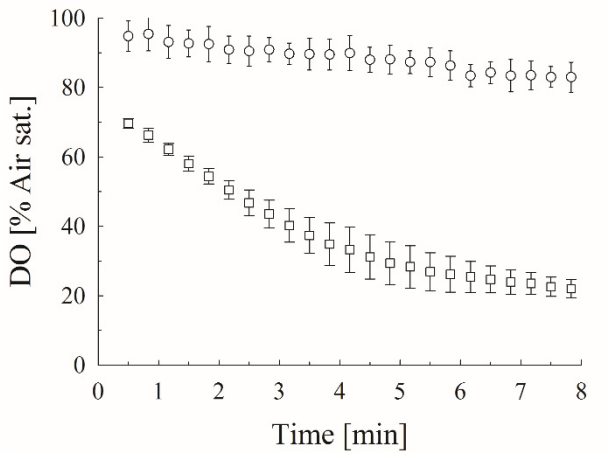

Figure 3.2.5 Triplicate DO measurements of *E. coli* W3110 cultures grown in EnPresso B at 30°C. Open circles: Slow OxoPlate; open squares: OxoPlate. The cultures were grown in shake-flasks overnight, and then 200 µL per well were transferred into Slow OxoPlates and OxoPlates. After 1 hour of incubation at 300 rpm (50 mm amplitude), the plates were measured in a microplate reader without additional shaking.

Cultures with a high growth rate, which are commonly used in screening experiments under batch conditions, exhibit lower DO levels than the slow growing cells in the first experiment. Therefore, lower initial DO levels were tested in a subsequent investigation. A DO micro sensor served as a reference system for rapid at-line measurement and an on-deck shaker was used to shorten the transport times.

At lower shaking frequencies DO values declined (Figure 3.2.6). However, even at 400 rpm, DO levels above 20% were detected with the Slow OxoPlate. The microsensor measurements draw a different picture. Even at delay times of 20 seconds from stopping the shaker until measurement, it was not possible to measure DO signals that closely resemble the sensor plate results. In addition, the microsensor was mounted above the shaken plate for on-line DO measurements. After stopping the shaker, a decline of DO signal from 89 % to 59 % in 75 seconds was recorded (data not shown). Since glucose-limited cultures were taken for these experiments, it can be expected that these effects are much more severe with exponentially growing microbial cultures.

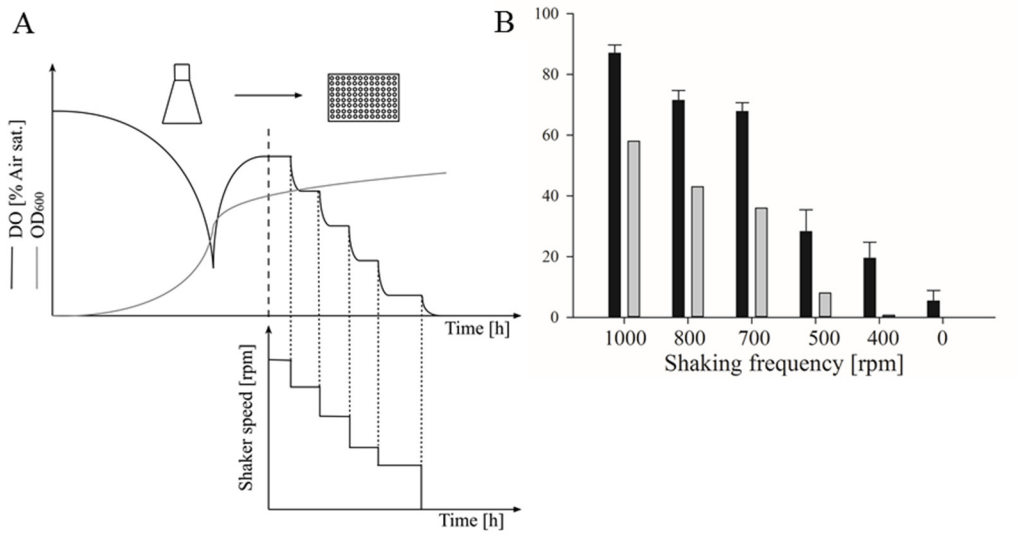

Figure 3.2.6 Comparison of at-line microsensor and Slow OxoPlate measurements of *E. coli* W3110 cells incubated at different shaking velocities. (A) Schematic outline of the experiment. Cells were grown overnight in a shake-flask, until glucose-limited conditions were reached. Then, the culture was transferred into a Slow OxoPlate, which was incubated at different shaking velocities. After changing the shaking speed, the plate was incubated for 30 min until the next measurement. (B) Dissolved oxygen levels at different shaking speeds measured at-line with the microsensor (grey) and the Slow OxoPlate (black).

Comparison of slow and fast responding sensor plates in an automated cultivation

In order to demonstrate the ability to detect oxygen limitation during HT cultivation experiments, a reference experiment for cell line screening on a robotic platform was performed. Two culture plates were prepared for comparison of both sensor plates – the Slow OxoPlate and the OxoPlate. Both were prepared with the same layout: Cultures with EnPresso B and EnPresoo Y Defined were inoculated with *E. coli* BL21 pDgPNP and *S. cerevisiae* AH22 pPG6, respectively. After an initial growth phase of 10 to 20 hours, glucose-release was further increased by adding reagent A. The plates were taken from the incubator every two hours for DO measurement, combined with OD_{600} measurements at certain time points.

For both yeast and bacterial cultures, the typical DO curves of fed-batch fermentations were recorded. However, different DO profiles were obtained from the sensor types tested. For *E. coli*, the OxoPlate showed a decline followed by an increase in DO the first few hours (Figure 3.2.7 A). This trend is not detectable with the Slow OxoPlate. After 20 hours 12 U L^{-1} reagent A were added, which provided a faster glucose release and thus a higher growth rate. Both sensor plates detect a drop in DO at that time. However, the OxoPlate indicates an oxygen limitation phase of 8 hours, while this phase was appeared to be less than 4 hours when looking at the data from the Slow OxoPlate. For *S. cerevisiae*, the obtained results were similar (Figure 3.2.7 B). Addition of reagent A after 10 hours resulted in a decrease of DO, indicated by both sensors. A short phase of oxygen limitation was detected with the Slow OxoPlate, while the OxoPlate data indicated an anaerobic phase of 10 hours.

The OxoPlate was able to detect the initial exponential phase of the culture after inoculation. Due to the delay between stopping of the aeration and the measurement, the oxygen decreased further during plate transport. After the first few hours, both cultures grow under substrate limitation, which is indicated by an increased DO level. This phase was not detectable with the Slow OxoPlate, which proves its capability of serving as an indicator for the DO concentration during shaking. After increasing the glucose-release rate, the OxoPlate indicated severe oxygen limitation in both cultures. In comparison to the data from the slow sensor, we conclude that this is mainly due to the delayed measurement. Only a short oxygen limitation phase of four hours is observed with the Slow OxoPlate.

With the reference cultivation presented in this study, the functionality of the Slow OxoPlate was demonstrated. Oxygen limitations in microbial cultures in 96-well plates can be detected through at-line DO monitoring.

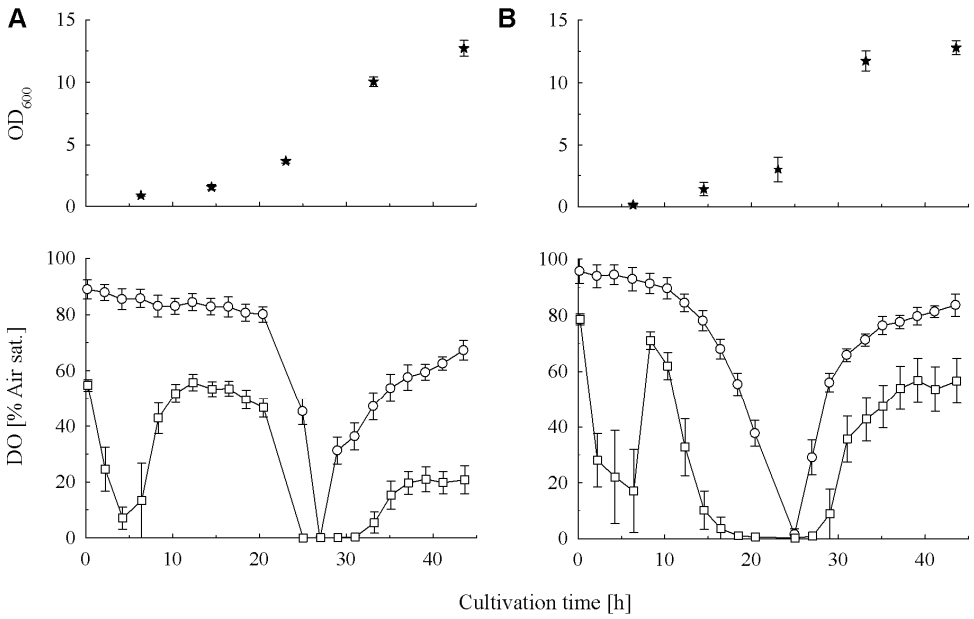

Figure 3.2.7 Application of slow and fast responding sensor plates for fed-batch cultures of *E. coli* BL21 pDgPNP (A) and *S. cerevisiae* AH22 pPG6 (B). Open circles: Slow OxoPlate, open squares: OxoPlate, filled stars: OD600. Cells were grown in EnPresso growth system for 44 h with DO measurement intervals of 2 h. The arrows indicate the time-point of supplementation with additional reagent A for increased glucose-release.

3.2.5 Discussion

In this study, we present the development of a new sensor type for standard 96-well plates. The targeted response time and reproducibility was achieved by sequential pipetting of 2 x 15 µL of liquid sensor material into the plates. Sensor characterization using sodium sulfite showed a response time of 9.7 minutes and further tests using microbial cultures indicated that the system can be applied for culture monitoring. Therefore, a reference experiment on a robotic platform was performed, in which DO level monitoring helped to identify oxygen limitation phases in *E. coli* and *S. cerevisiae* cultures.

Each of the two sensor types was able to deliver information on different oxygenation states of the culture. The OxoPlate was able to detect disturbances caused by the measurements. Data on the DO level at the time of sampling may serve as guideline for sampling frequency and time points. Signals obtained from the Slow OxoPlate closely reflected the DO curves obtained from on-line sensor systems such as the PreSens

SensorDish Reader or Shake Flask Reader, which underlines that the right approach for sensor design was chosen. The importance of the adjustment of aeration conditions in small-scale cultures was pointed out by different research groups. For example, Stockmann and colleagues have reported a severe impact of oxygenation on the screening of phytase producing *H. polymorpha* strains (Stöckmann et al., 2003).

Within the study, the application of glucose-limited cultures has been a good choice since their stable oxygen consumption rate over time made the adjustment of DO level by adjustment of the shaking frequency possible. Moreover, growing *E. coli* using the EnBase technology has been found to be an important factor for the scale up of recombinant protein production processes (Glazyrina et al., 2012; Siurkus et al., 2010). In future work, we want to prove that the combination of small-scale sensor technology and HT fed-batch cultivation can be seen as an enabler for process design based on QbD principles (Long et al., 2014).

In summary, the detection of oxygen limitation in aerobic microbial cultures using standard 96-well plates equipped with fluorescent sensor spots was successfully performed. By increasing the sensor's response time, at-line determination of a rapidly changing parameter such as DO was possible. With the new sensor, optimization of growth conditions on a reference plate is possible. HT screening programs can afterwards be carried out using standard 96-well plates. The conditions of such screening experiments are comparable to bioreactor cultivations in terms of oxygen availability, which helps in reducing the risk of difficulties during scale-up.

Acknowledgements

The authors are thankful for assistance carrying out the experimental work and programming. Michael Heiser, Susan Bigesse and Christopher Skopnik were involved in parts of the study. Moreover, technical and material support by BioSilta Ltd. and PreSens Precision Sensing GmbH was appreciated. The authors acknowledge financial support by the German Federal Ministry of Education and Research (BMBF) within the Framework Concept "Research for Tomorrow's Production" (project no. 02PJ1150, AUTOBIO project), as well as project management support by the Project Agency Karlsruhe (PTKA-PFT).

3.3 Paper II

Design of Experiments based high-throughput strategy for development and optimization of efficient cell disruption protocols

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Keywords: Bioprocess development, Cell disruption, Design of Experiments (DoE), High-throughput lysis buffer optimization, Laboratory automation /

Abbreviations: ßGal, ß-galactosidase; **BCA**, bicinchoninic acid; **DoE,** Design of Experiments; **EDTA**, ethylenediaminetetraacetic acid; **HT**, High-throughput; **LHS**, Liquid handling system; **SiLA,** Standardization in Lab Automation

3.3.1 Abstract

Efficient and reproducible cell lysis is a crucial step during downstream processing of intracellular products. The composition of an optimal lysis buffer should be chosen depending on the organism, its growth status, the applied detection methods, and even on the target molecule. Especially for high-throughput applications where sample volumes are limited, the adaptation of a lysis buffer to the specific campaign is an urgent need.

Here, we present a general Design of Experiments (DoE) based strategy suitable for eight constituents and demonstrate the strength of this approach by the development of an efficient lysis buffer for Gram-negative bacteria which is applicable in a HT format in a short time. The concentrations of four lysis-inducing chemical agents: EDTA, lysozyme, Triton X-100 and polymyxin B were optimized for maximal soluble protein concentration and ß-galactosidase activity in a 96-well format on a Microlab Star liquid handling platform under DoE methodology. The resulting lysis buffer showed the same performance as a commercially available lysis buffer.

The developed protocol resulted in an optimized buffer within only three runs. The established procedure can be easily applied to adapt the lysis buffer to other strains and target molecules.

3.3.2 Introduction

Cell lysis is an important step in industrial production of biomolecules, as well as in miniaturized high-throughput (HT) screening setups. The composition of an optimal lysis buffer is dependent on the target organism but also depends on the cultivation conditions such as medium and cultivation temperature, as well as on the applied cell density. Furthermore, it must be composed in view of the target molecule of the purification process and may differ from small molecules to proteins, DNA and RNAs. For a target protein, all further factors need to be considered, such as the state of oligomerization, redox state, compartmental localization and cofactors.

A combination of chemical, enzymatic and physical cell disruption methods is the standard in industrial and laboratory scale and this is normally optimized for a specific situation (Balasundaram et al., 2009; Gehmlich et al., 1997; Zhao and Yu, 2001). However, effective methods like high pressure homogenization (known as French Press) cannot be easily applied in HT screening systems. HT-compatible methods are ultrasound, bead milling and the combination of chemical and enzymatic methods.

Although a variety of newly developed micro-scale cell disruption methodologies exist (Lin and Cai, 2009), e.g. the application of microfluidic compact discs, nano-scale barbs or electric pulses (Vaara, 1992), which have the advantage of working without any additional, potentially interfering chemical compound. But the application of such mechanical or physical lysis methods adapted to the micro-scale applies specialized prototyped apparatuses which are not easily applied on existing liquid handling systems (LHS) or are limited to specific applications. Another option is the application of head-inducible autolytic vectors (Xu et al., 2006). In contrast, chemo-enzymatic cell disruption methods can be easily applied in microwell-plate based experiments without any significant increase of costs and any need to use additional equipment, and they can be adapted to new screening tasks in short time.

A tremendous variety of chemicals and enzymes have been applied for the disruption of microorganisms so far, i.e. chaotropic agents (e.g. ethanol, guanidine-HCl, guanidine-SCN) (Gehmlich et al., 1997), anionic (e.g. sodium dodecyl sulphate (SDS)), non-ionic (e.g. Triton, Tween or Brij family) and zwitterionic (CHAPS) detergents (Zhao and Yu, 2001), chelating agents (e.g. EDTA) (Vaara, 1992), organic solvents (e.g. toluene,

butanol) (de Smet et al., 1978), cationic polypeptide antibiotics (e.g. polymyxins) (Daugelavicius et al., 2000) and foreign peptidoglycan digesting enzymes (e.g. lysozyme) (Pierce et al., 1997) are the main classes of lysis buffer components.

Cell lysis buffers are commercially available from several manufacturers, e.g. BugBuster® of Merck Millipore, or SoluLyse® of Genlantis, which were applied in a comparative study using an expression library by Listwan et al. (Listwan et al., 2010). However, since the formulation is proprietary, not all lysis-inducing components are known. In the case of protein assays, detergents and EDTA mainly can interfere with chromophore formation (Olson and Markwell, 2007). For purification of expressed polyhistidine-tagged recombinant proteins, lysis buffers should not contain EDTA, which as chelate disturbs the immobilized metal ion affinity chromatography (Bornhorst and Falke, 2000). Therefore, the ideal solution would be to optimize the lysis buffer composition depending on the specific demands of the expression host's cell wall, the target protein and the subsequent downstream steps. Hence a fast and cost efficient framework for the development or adaptation of a specific cell disruption mixture will allow a higher degree of specification for each application. In order to maximize the efficiency of the development process, Design of Experiments (DoE) (Balasundaram et al., 2009; Mayr and Bojanic, 2009) is a standard way to plan the experimental setup in a liquid handling station. The applications of optimal experimental planning is beneficial for a wide variety of operations in upstream and downstream bioprocess development (Gehmlich et al., 1997; Zhao and Yu, 2001). In formulation development, the design space can get very large, especially in HT screening facilities were the large number of costly and time-consuming experiments makes intuitive design very complex and inefficient. Consequently, liquid handling robotics and laboratory automation have to be coupled to experimental design programs which select the best combination of experiments for a given task including correct evaluation of datasets.

The aim of this study was to use state of the art methods to design and carry out experiments on a LHS in order to create a framework for fast, cost effective and efficient optimization of cell lysis buffers. The applicability of the method is demonstrated with the development of a lysis buffer for purification of soluble expressed recombinant proteins released from *Escherichia coli* cells.

The DoE-based strategy can then be easily applied for re-optimization of the buffer composition suitable for other strains and target proteins within a few days of work.

3.3.3 Materials and Methods

Strains

Bacterial cultivations were carried out using *Escherichia coli* BL21. The strain was transformed with the plasmid pDgPNP for heterologous expression of purine nucleoside phosphorylase from *Deinococcus geothermalis* (Szeker et al., 2011). Media were supplemented with 100 mg L^{-1} ampicillin to maintain plasmid stability. All strains were cryopreserved at -80 °C, in media containing 20% glycerol.

Cultivation conditions

All cell lysis experiments were performed using *E. coli* cells obtained from glucose-limited fed-batch cultivations. For this purpose, the EnBase® technology (BioSilta Ltd., Cambridge, UK) was applied. By enzymatic degradation of a polymer, quasi-constant feeding of cells can be carried out using EnBase media. In this study a medium for bacterial cultures in ready-made tablets was used (EnPresso B). The kit contains medium tablets, glucose-releasing "Reagent A" and complex "Booster" tablets.

Pre-cultures were carried out at 37 °C in 5 mL of Luria-Bertani (LB) broth (10 g L^{-1} peptone, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride, pH 7.0) using a 125 mL UltraYield Flask™ covered with AirOtop Enhance Seal™ (Thomson Instrument Company, Oceanside, USA). The pre-culture was shaken at 250 rpm with 25 mm amplitude in a Kühner LT-X incubator (Adolf Kühner AG, Basel, Switzerland) for 6 hours. For main cultures, 50 mL of EnPresso B (BioSilta Ltd.) medium was prepared in a 250 mL UltraYield Flask™, according to the manufacturer's instructions. The optical density at 600 nm (OD_{600}) of the preculture was measured in an Ultrospec 2100 pro spectrophotometer (GE Healthcare Europe GmbH, Freiburg Germany) in order to inoculate the main culture with an initial OD_{600} of 0.2 AU. Immediately after inoculation, 1.5 U L^{-1} of Reagent A (BioSilta Ltd.) was added for controlled glucose release. The culture was incubated at 250 rpm and 30 °C overnight. Intracellular recombinant protein production was induced using 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG). At the time of induction, 3 U L^{-1} of Reagent A and a tablet of a complex "Booster" mix (BioSilta Ltd.) were added. The culture was continuously incubated for another 24 hours, until the shake flask was cooled down on ice for cell harvest. At the end-point of the

cultivation, OD_{600} was measured and the harvest volume (V_H) was calculated in order to normalize the harvested cells to 1 mL of culture taken at an OD_{600} of 5 AU:

$$V_H = \frac{5\ mL}{OD_{600}} \quad (1)$$

The culture broth was distributed into 96-microtube racks (HJ Bioanalytik GmbH, Erkelenz, Germany) and centrifuged at 3 000 g and 4 °C for 15 min. The supernatant was removed thoroughly and the cell pellets were stored at -20 °C.

Experimental design and automated mixture preparation

All experiments were planned and evaluated using MKS Umetrics MODDE® 10 (MKS Umetrics AB, Umeå, Sweden). Experimental plans were imported into a laboratory automation database (iLab-Bio, infoteam software AG, Bubenreuth, Germany) via the MODDE-Q interface (Umetrics AB). With the help of a graphical user interface, worklists for the liquid handling robot were generated. The software calculated the individual volumes for each component, based on the concentration of stock solutions and the working volume. A method for mixture design on the LHS (Hamilton Microlab Star, Hamilton Bonaduz AG, Bonaduz, Switzerland) was programmed using the Hamilton VenusOne software. In brief, it enables the operator to import worklists containing volumes from the iLab-database and distribute the respective compounds into a 96-deep well plate (Ritter GmbH, Schwabmünchen, Germany) for convenient fully-automated preparation of mixtures with up to 8 components.

Six compounds were selected for evaluation: ethylenediaminetetraacetic acid (EDTA), guanidine hydrochloride (guanidine-HCl), lysozyme, polymyxin B, Triton X-100 and Tween 20. For mixture design, all solutions were prepared as tenfold concentrates to allow easy mixing of stock solutions with the liquid handler to a final volume of 500 µL. A basic binding buffer (80 mM sodium phosphate buffer, 40 mM imidazole, 0.5 M sodium chloride, pH 7.6) which allows further purification steps was used. Dilutions for cell lysis screening were prepared in binding buffer containing an EDTA-free blend of protease inhibitors (cOmplete EDTA-free, Roche Diagnostics GmbH, Mannheim, Germany) on the liquid handling robot. BugBuster® Protein Extraction Reagent (Merck Millipore, Billerica MA, USA) supplemented with 1800 U mL^{-1} of lysozyme served as a reference system. All lysis buffers were supplemented with 25 U mL^{-1} of Benzonase® Nuclease (Merck Millipore) for viscosity reduction of cell lysates.

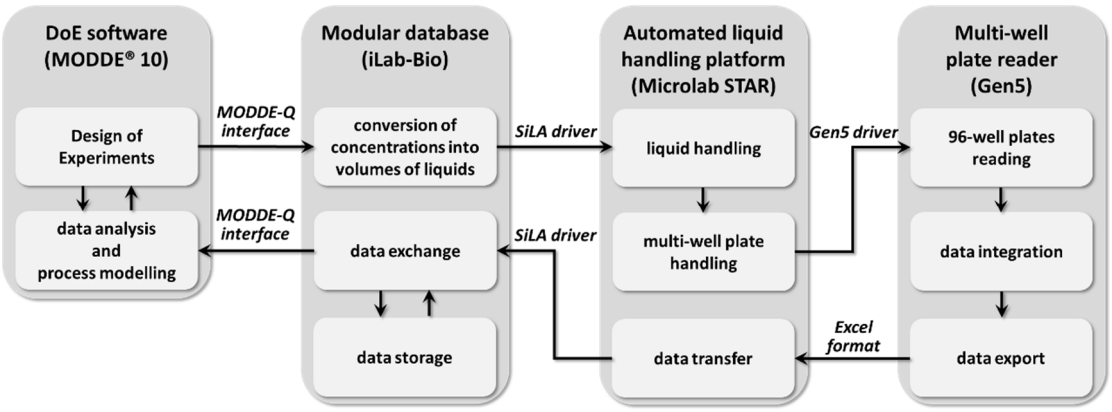

Figure 3.3.1 Integrated system of data transmission and processing. The Experiments are planned in the DoE software (MODDE® 10), and translated into worklists for the LHS with the mod-ular database (iLab-Bio). The LHS manages fully automated multi-channel pipetting of chemicals, and performs read-outs in the multi-well plate reader (Gen5) after the cell lysis procedure. The results are manually integrated and saved as spreadsheets, which are then automatically imported back into the iLab-Bio database via the LHS control software. Finally, the experimenter can use the DoE software to analyze the data statistically and determine the optimal region.

Cell disruption

The frozen cell pellets were incubated at room temperature for app. 5 min. Then, 300 µL of lysis buffer was added simultaneously to all 96 tubes of the rack, using the 96 pipettor head of the LHS. The pellets were resuspended immediately afterwards, by 30 aspiration-and-dispense cycles of 300 µL. Incubation was carried out for 20 min at room temperature, followed by centrifugation at 4 000 g for 20 min at 4°C. Then, the supernatants were transferred into fresh 96-microtubes for further analysis.

BCA assay

Protein quantification of the soluble fraction was carried out with a bicinchoninic acid (BCA) (Smith et al., 1985) protein assay kit (BioVision Inc., Milpitas, USA) according to the manufacturer's instructions. In brief, 200 µL of BCA working reagent was distributed into a 96-well flat bottom plate (Greiner Bio-One, Frickenhausen, Germany). Then, 25 µL of sample was added and the plate was incubated for 30 min. at 37 °C. After cooling down for 5 min, absorption was measured at 562 nm in a Biotek Synergy MX plate reader (BioTek Instruments Inc., Winooski, USA), connected to the liquid handling station using the Gen5 software (BioTek Instruments Inc.). Standard curves were prepared using bovine serum albumin (Sigma-Aldrich, Munich, Germany) in concentrations ranging from 0.025 to 2 mg mL^{-1}.

β-galactosidase assay

β -galactosidase (β-gal) activity was measured in flat-bottom 96-well plates using o-nitrophenyl-β-D-galactopyranoside as the substrate. 10 µL of sample were added to 160 µL of Z-buffer (60 mM NaH_2PO_4, 40 mM Na_2HPO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, pH 7.0) and incubated at 30 °C for 3 min. Then, 32 µL of o-nitrophenyl-β-D-galactopyraonside solution (4 mg mL^{-1} in 50 mM Tris-HCl, pH 8.0) were added simultaneously to all wells using the LHS. The increase of absorbance at 420 nm was measured every 30 s for 10 min. One enzyme unit (U) is defined as the amount of enzyme releasing 1 µmol o-nitrophenol per min under the defined reaction conditions.

3.3.4 Results

To simplify the planning, execution and data evaluation for DoE-based experiments, a workflow of proceeding was established in this study. Independent software solutions for (i) DoE (MODDE® 10), (ii) data handling (iLab-Bio), (iii) liquid handling (VenusOne) and (iv) microplate reading (Gen5) were connected using customized interfaces (Figure 3.3.1). This combination of commercially available software packages is user-friendly and allows planning and data handling of large experimental setups.

During validation of the integrated automated workflow, preliminary experiments showed that Triton X-100 outperformed Tween 20, as well as tests using guanidine-HCl exhibited a significantly negative influence on ß-gal activity (data not shown). Therefore, for the main experiments, four components were selected for the screening and the optimization steps (Table 3.3.1).

The experiments were planned as D-optimal designs, which have the advantage of flexible boundaries and restrictions. For the screening phase, a design with duplicates of 32 experiments and 4 center points was used. In addition, triplicates of mixtures with three out of four parameters at the highest concentration, triplicates of basic buffer as a negative control and BugBuster as a reference buffer were included. In total, the first round consisted of 91 experiments (72 from MODDE + 19 controls). The experiments were randomized over the plate. The concentrations of lysis-inducing agents were automatically converted into volumes of stock solutions to be pipetted by the LHS. After incubation and centrifugation, the supernatant was analyzed for both, protein concentration using the BCA assay and β-gal activity.

Since each buffer composition gave an individual background signal in the protein assay, blank measurements of all buffers were performed. As a reference cell lysis system, the BugBuster® Protein Extraction Reagent was used.

Table 3.3.1 Factors and concentration ranges of ingredients added to cell lysis buffers applied for screening and optimization.

Component	1st run (screening)	2nd run (optimization)
Benzonase [$\times 10^2$ U mL^{-1}]	2.5	2.5
EDTA [mM]	0.5 – 10	-
Lysozyme [$\times 10^3$ U mL^{-1}]	0.3 – 9	4.5 – 13.5
Polymyxin B [µM]	0.1 – 50	20 – 60
Triton X-100 [%]	0.1 – 2	0.94 – 2.82

The data was fitted using the partial least squares (PLS) regression, which gave a good fit for both responses (Table 2). Apart from EDTA, all other factors, showed positive and synergistic effects. The response surface was investigated for extreme points, which resulted in a recommendation for high concentrations of lysozyme, polymyxin B and Triton X-100. The predicted activity of released β-gal for the optimium is 58 % of the activity obtained with the commercial cell lysis kit considered as reference.

In order to get a better picture of the optimal region and improve the lysis efficiency, a second D-optimal experiment was performed in the optimal region. The design space was shifted to higher concentrations of the three buffer's ingredients with positive influence, i.e. lysozyme, polymyxin B and Triton-X100. The concentration ranges of lysis-inducing agents were defined around the optimal region of the first experiment with 50 % surpluses and insufficiencies. Again, duplicates of 32 runs with 4 center points were selected. The results were combined with the data from the previous experiment, resulting in a model for the complete design space, which is depicted in Figure 3.3.2. The final model showed high reproducibility but with a reduced goodness of fit, and prediction precision. The maximum predicted ß-gal activity of the model is 0.35 U mL^{-1}, which is 92 % of the activity obtained using BugBuster.

Based on the model obtained, it can be concluded that high lysozyme (> 9 000 U mL^{-1}) and high Triton X-100 (> 2 %) levels at moderate Polymyxin B concentrations (35 µM) are necessary to accomplish efficient *E. coli* cell disruption. Interestingly, at a detergent concentration of around 2 %, the concentration of soluble protein reaches a saturation point. At detergent levels greater than 2 %, only the enzyme activity increased, but not the soluble protein concentration. At high lysozyme concentrations, less soluble protein is detected with increasing detergent concentration compared to low lysozyme levels. Polymyxin B appears to have a negative effect on protein content and enzyme activity at the given concentration range of used cationic polypeptide antibiotic. Finally, β-gal activity is negatively influenced by EDTA, especially at moderate polymyxin B concentrations.

Table 3.3.2 Comparison of the statistics parameters summarizing the fit of the models used for screening (1st run) and optimization (2nd run)

Statistical parameters	Responses			
	Screening		Optimization	
	Soluble protein concentration	β-gal activity	Soluble protein concentration	β-gal activity
R^{2} [a]	0.76	0.81	0.55	0.74
Q^{2} [b]	0.62	0.71	0.50	0.69
Reproducibility[c]	0.77	0.84	0.75	0.92

[a] Coefficient of determination.

[b] Future prediction precision.

[c] Variation of the replicates compared to overall variability.

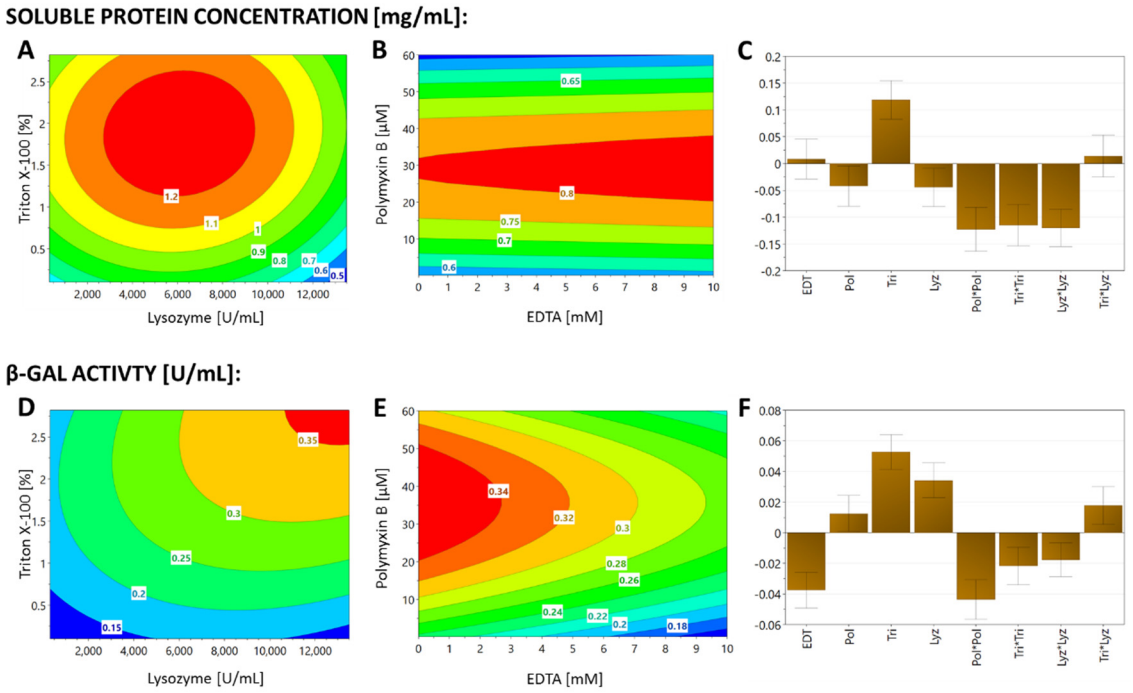

Figure 3.3.2 Response surface and coefficient plots for soluble protein concentration (upper row; A-C), and for β-gal activity (lower row; D-F). Influence of Triton X-100 and lysozyme (for 30 µM polymyxin B and 0 mM EDTA) on soluble protein concentration (A) and on β-gal activity (D), as well as influence of polymyxin B and EDTA (for 2.82 % Triton X-100 and 13.5 × 103 U mL-1 lysozyme) on soluble protein concentration (D) and on β-gal activity (E) are presented. Coefficient plots for soluble protein concentration (C) and β-gal activity (F) indicating positive or negative influence of the factors on the responses.

In summary, we see that the integration of independent software units for DoE, data handling, liquid handling and plate reading enhances the efficiency of method development in HT miniaturized-systems. The presented workflow has been applied for screening cell-lysis agents and choosing those with a positive effect on chemo-enzymatic disruption of *E. coli* cells. Performing the optimization step and combining both datasets gave more detailed information on the influence of the additives. The detergent (i.e. Triton X-100) and muramidase (lysozyme) B) were found to be crucial for efficient cell lysis, while the cationic polypeptide antibiotic (i.e. polymyxin) assisted the break-down of the cells.

3.3.5 Discussion

The appearance of commercially available laboratory automation systems for liquid and plate handling gave rise to the introduction of HT technologies in the field of bioprocess engineering and applied biotechnology. However, increased experimental throughput requires more sophisticated methods for experimental planning and data evaluation. In our view, a fully-integrated platform of interacting units for DoE, data management, experimentation, and analysis will be a milestone in increasing productivity of laboratories. Up to date, some attempts in the matter of integrated automation of bioprocess development have been achieved and recently reviewed (Balasundaram et al., 2009; Long et al., 2014; Mayr and Bojanic, 2009; Sparkes et al., 2010).

In this study, we demonstrate the effective interaction of different modules which minimizes the human interaction and especially enables complex experimentation schemes in an automated way. However, human involvement was still needed in most steps, e.g. in designing the experimental plan, in programming and initiating the liquid handling unit, in data treatment obtained from the plate reader, as well as in modification the plan of experiments for the next round. Such a strategy is in accordance with previously published automated HT platforms for bioprocess development (Welsh et al., 2015; Wu and Zhou, 2014). Further extension of such an automated flow can be envisioned by a full closed-loop system as recently published by Wu and Zhou (Wu and Zhou, 2014). Such an “intelligent” system could perform the modification of experimental plans for the next round automatically (e.g. optimization step) based on the results obtained from a previous round (e.g. screening step).

In this report, an automated workflow functionally combined the above-mentioned units into one HT screening and optimization platform which has been used for development of a chemo-enzymatical cell lysis buffer for *E. coli* cells. The developed lysis buffer emerged from six components in only three experimental runs, and finally resulted in a similar disruption efficiency compared to a commercial system.

EDTA, lysozyme, polymyxin B and Triton X-100 have been taken into consideration for the optimization step, which were ordinarily used in previously reported studies focused on chemo-enzymatic disruption of bacterial cells [2, 3, 7 ,8]. Optimization of the considered lysis buffer gave us more detailed answers on the influence of lysing factors mentioned above on *E. coli* disruption yield and on stability of the released intracellular

enzyme. As an outcome, the lysis buffer containing high amount of nonionic detergent (i.e. Triton X-100) and lytic enzyme (i.e. lysozyme), with low-mid amount of cationic polypeptide antibiotic (i.e. polymyxin B) at low concentrations of chelating agents (i.e. EDTA) has been found to yield highest activity of the intracellular target enzyme.

We considered that the developed lysis buffer, as an EDTA-free variant, extends applicability of the lysing system also in the case of methodologies which use cell-lysates free of chelating agent for further enzymatic assays, i.e. if binding of divalent metal ions (e.g. Ca^{2+}, Co^{2+}, Mg^{2+} or Mn^{2+}) influences on results of the enzymatic assays as essential cofactors, or by displacing the intrinsic factors (Bisswanger, 2014; Goddard and Reymond, 2004). Alternatively, sonication could be used as applied as the reference method in a study by Listwan *et al*. (Listwan et al., 2010), which also points out the importance of testing a larger variety of proteins for validation of a lysis method.

The cells pellets, which have been used as biomass for lysis, were taken from glucose-limited fed-batch cultures using EnBase® technology (Krause et al., 2010; Panula-Perälä et al., 2008). Such culture conditions prevent over-feeding of the cells, which is relevant for scale-up to production scale (Glazyrina et al., 2012; Siurkus et al., 2010), however fed-batch grown cells are also more difficult to lyse. Many of previously published data on methods for lysis of bacterial cells miss details on the cultivation conditions, or the cells used in experiments came from batch cultures with complex media, which makes it hard to compare the outcome of different studies. In the context of bioprocess development, the cell wall composition of *E. coli* grown under glucose limitation should be recognized as a reference point for further comparative studies on the topic of the efficient disruption of Gram-negative bacteria.

In general, the overall DoE-aided methodology, which was applied for the development of a lysis buffer designed for *E. coli* cells, shows a great potential for application flexibility. This gives a robust possibility for very fast, cost effective, no time- and no labor-consuming re-optimization of the end-user-specified solution for chemo/enzymatically induced lysis of whichever bacterial strain, or other type of cells. Such a strategy makes also possible to easily improve the efficiency of further downstream processes of the target intracellular molecules purification, and it is not limited to only proteins but also for wide range of other intracellular products, as small molecules, DNA, plasmids, RNAs, and others (Pilarek et al., 2013).

Conclusions

A functional integration of software units for DoE, data handling, liquid handling and multi-well plate reader supplied by various producers has been developed. Based on this, a HT miniaturized-format platform for cell lysis buffer screening and its further optimization has been developed.

The chemo-enzymatic lysis buffer, containing nonionic surfactant, muramidase, cationic polypeptide antibiotic and low concentrated chelating agent, has been recognized as supporting the most effective conditions for releasing of soluble proteins from *E. coli*, as well as for retaining the active structure of the released intracellular enzyme. The developed lysis buffer showed the same performance than a commercially available product.

Summarizing, the idea of the DoE-aided rapid optimization of a lysis buffer suitable for maximization of recombinant protein production, and to provide maximal activity of intracellular enzyme released from Gram-negative bacteria has been fulfilled. Moreover, the presented methodology of the HT automated lysis of cells can be easily adapted to changed lysis-inducing constituents, as well as various strains of *E. coli* or other species of Gram-negative bacteria.

Acknowledgements

The authors are thankful for assistance carrying out the experimental work and programming. Michael Heiser (TU Berlin) and Wojciech Stępień (Warsaw University of Technology) were involved in initial parts of the study. The close collaboration with infoteam software AG, namely Ingrid Schmid and Joachim Aschoff was very helpful. The authors would also like to thank Mirja Krause (University of Oulu) and Andreas Knepper (TU Berlin) for methodology suggestions and fruitful discussions. Moreover, technical and material support by BioSilta Ltd. was appreciated. The MODDE-Q license was kindly provided by Umetrics AB. The authors acknowledge financial support by the German Federal Ministry of Education and Research (BMBF) within the Framework Concept “Research for Tomorrow’s Production” (project no. 02PJ1150, AUTOBIO).

Conflict of interest statement

The authors have declared no conflict of interest.

3.3.6 Practical application

The efficiency of a cell lysis procedure is influenced by the organism and growth status and affects the consecutive downstream processing. For HT applications, the adaptation to the specific campaign is an urgent need since the low sample volume requires efficient chemical or enzymatic lysis.

Here we present a computer-aided design of experiments (DoE) procedure for the optimization of a multi-agent buffer for the lysis of Gram-negative bacteria with up to eight components. The optimization is exemplarily shown at the release of cytoplasmic β -Galactosidase from *Escherichia coli* cells.

The power of the approach lies in the functional integration into an automated HT robot-based screening platform of independent software packages for (i) DoE, (ii) data processing, (iii) liquid handling and (iv) spectrophotometric read-out.

The presented protocol may be applied for any lysis buffer optimization for bacterial, plant or animal cell cultures or for quantitative assay development.

3.4 Paper III

Research article

Detection of growth rate-dependent product formation in miniaturized parallel fed-batch cultivations

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Keywords: Polygalacturonase, high-throughput, recombinant protein, fed-batch, change-stat

Abbreviations: [**EPG,** Endo-polygalacturonase; **OD_{600},** Optical density at 600 nm]

3.4.1 Abstract

Saccharomyces cerevisiae is a popular expression system for recombinant proteins. In most cases, production processes are performed as carbon-limited fed-batch cultures to avoid aerobic ethanol formation. Especially for constitutive expression systems, the specific product formation rate depends on the specific growth rate. The development of optimal feeding strategies strongly depends on laboratory scale cultivations, which are time and resource consuming, especially when continuous experiments are carried out. It is therefore beneficial for accelerated process development to look at alternatives.

In this study, *S. cerevisiae* AH22 secreting a heterologous endo-polygalacturonase (EPG) was characterized in microwell plates with an enzyme-based fed-batch medium. Through variation of the glucose release rate, different growth profiles were established and the impact on EPG secretion was analyzed. Product formation rates of 200- 400 U $(g_X\ h)^{-1}$ were determined. As a reference, bioreactor experiments using the change-stat cultivation technique were performed. The growth-dependent product formation was analyzed over dilution rates of D= 0.01 – 0.35 with smooth change of D at a rate of 0.003 h^{-2}. EPG production was found to be comparable with a q_p of 400 U $(g_X\ h)^{-1}$ at D= 0.27 h^{-1}.

The presented results indicate that parallel miniaturized fed-batch cultures can be applied to determine product formation profiles of putative production strains. With further automation and parallelization of the concept, strain characterization can be performed in shorter time.

3.4.2 Introduction

Yeasts are utilized for large-scale production of proteins and small molecules. One popular host organism is *Saccharomyces cerevisiae* due to its well-characterized genome, robust growth characteristics and the GRAS (generally regarded as safe) status. Since it is a Crabtree-positive yeast, industrial production processes exclusively apply the fed-batch technique (Porro et al., 2005). Selection and evaluation of production strains, however, is mostly done in batch cultures. This may cause problems during industrialization of production processes (Neubauer et al., 2013). Ideally, the cultivation conditions should remain as similar as possible during scale-up (Siurkus et al., 2010).

Strain variants obtained from mutant libraries or strain collections are commonly screened in parallel batch cultures and evaluated from end-point measured data. Under these conditions, oxygen limitation, aerobic ethanol formation and medium pH instability often occur, which can severely affect the outcome of strain screening experiments (Scheidle et al., 2010).

In the last decade, a number of key enabling technologies for consistent bioprocess development have been commercialized, such as microwell plates equipped with sensors (Arain et al., 2006; Glauche et al., 2015) and lids for improved aeration (Duetz et al., 2000), minibioreactor systems (Puskeiler et al., 2005b) and fed-batch media for small-scale cultures (Jeude et al., 2006; Panula-Perälä et al., 2008). These technologies are expected to significantly improve bioprocess development and scale-up (Long et al., 2014).

One major factor for yeast fed-batch development is the determination of feeding profiles, which avoid excessive ethanol formation, while maintaining high product formation rates within the mass-transfer boundaries of the bioreactor. The classical approach for investigations of the productivity at different dilution rates is to perform a series of chemostat experiments, which is very tedious and has been recently scaled down to mini-bioreactors (Schmideder et al., 2015). Another option for accelerated data collection from continuous cultures is the change-stat technique. With a smooth change of the dilution rate the culture is kept in a quasi-steady-state and a wide range of growth rates can be investigated. The technique has been successfully applied to bacteria (Adamberg et al., 2009; Nahku et al., 2010) and yeasts (Paalme et al., 1997; Van Sluis et al., 2001), but so

far there has been no attempts to investigate recombinant protein production in yeast (Valgepea et al., 2015). The change-stat technique has been successfully applied for the investigation of protein secretion at different growth rates. The technology could be combined with miniaturized continuous cultivation systems (Schmideder et al., 2015) in order to characterize production strains, e.g. for improved productivity with processes at higher growth rates (Klein et al., 1999).

The aim of this study was to determine the specific product formation rate (q_P) in relation to the corresponding specific growth rate (μ) of a putative production strain significantly faster than using chemostat cultures. Microwell plate cultures with parallel enzyme-based fed-batch experiments performed at different glucose release rates were applied to characterize the production strain. The results were compared to change-stat continuous cultivations.

3.4.3 Materials and Methods

Yeast strain and culture storage

Cultivations were carried out using Saccharomyces cerevisiae AH22 (leu2-1, leu2-112, his4-519, can1, cir^+, mating type a) harboring the plasmid pPG6, which was constructed for heterologous expression of polygalacturonase from Aspergillus niger (Lang et al., 1997; Lang and Looman, 1995).

Ethyl methanesulfonate mutagenesis was applied with subsequent mutant selection on pectin agar plates. One colony, which showed improved pectin hydrolysis was isolated from the mutagenesis experiment (strain pPG6 M27). The cell bank was stored at -80°C in minimal medium containing 20 % glycerol.

Media

The cultivations were performed in a minimal medium based on WMVIII (Lang and Looman, 1995) containing glucose or 20 g L^{-1} EnPump glucose polymer (BioSilta Ltd., Cambridge, UK). Glucose is released from the EnPump polymer, when Reagent A is added. The composition of the modified WMVIII (mWM8) was as follows: $NH_4H_2PO_4$ 0.25 g L^{-1}, NH_4Cl 5.48 g L^{-1}, $MgCl_2 \cdot 6\ H_2O$ 0.25 g L^{-1}, $CaCl_2 \cdot 2\ H_2O$ 0.1 g L^{-1}, KH_2PO_4 2.0 g L^{-1}, $MgSO_4 \cdot 7\ H_2O$ 0.55 g L^{-1}, myo-inositol 75 mg L^{-1}, sodium glutamate 1.5g L^{-1} $ZnSO_4 \cdot 7\ H_2O$ 1.75 mg L^{-1}, $FeSO_4 \cdot 7\ H_2O$ 0.5 mg L^{-1}, $CuSO_4 \cdot 5\ H_2O$ 0.1 mg L^{-1}, $MnCl_2 \cdot 4\ H_2O$ 0.1 mg L^{-1}, Na2MoO4 · 2 H_2O 0.1 mg L^{-1}, nicotinic acid 10 mg L^{-1}, pyridoxin-HCl 25 mg L^{-1}, thiamine HCl 10 mg L^{-1}, biotin 2.5 mg L^{-1}, calcium panthotenate 50 mg L^{-1}, histidine 100 mg L^{-1}.

A-Stat cultivation

The preculture was inoculated from a cryo vial into 100 mL mWM8 containing 50 g L^{-1} glucose in a 500 mL UltraYield flask™ covered with AirOtop Enhance Seal™ (Thompson Instrument Company, Oceanside, USA), which was incubated at 30°C and 180 rpm overnight in a shaken incubator (Kühner LT-X, Kühner AG, Basel, Switzerland). The continuous culture was carried out in a 3.7 L bioreactor (KLF 2000, Bioengineering AG, Wald, Switzerland) with a working volume of 1.5 L at 30°C. The preculture was transferred into the bioreactor containing 1.5 L mWM8 with 15 g L^{-1} of glucose. The pH was controlled at 6.0 by addition of 10 % H_3PO_4 and 25 % ammonia. After an initial batch phase, the continuous culture was initialized by feeding medium into the reactor via a

voltage-controlled pump and harvesting culture broth through an overflow. The culture was stabilized at a dilution rate of 0.07 h^{-1} for at least five retention times to ensure steady-state conditions. Samples were taken for the analysis of optical density (OD_{600}), biomass, medium composition and EPG activity. The dilution rate was then increased, and in a subsequent experiment decreased, by an acceleration factor of 0.003 h^{-2} according to the following formula:

$$D(t) = D_0 + a \cdot t \qquad (1)$$

This acceleration factor has been previously used in a study by Adamberg et al. (Adamberg et al., 2009), which served as the main reference for this study.

Strain characterization in 24 well plates

Precultures were grown in 125 mL UltraYield flasks with 20 mL of mWM8 containing 40 g L^{-1} glucose. Cultures were incubated for 48 hours at 30 °C and 250 rpm (25 mm amplitude) on an orbital shaker. For the main culture, mWM8 with 20 g L^{-1} of EnPump polysaccharide (BioSilta Ltd.) was inoculated with the volume of the preculture corresponding to an initial OD_{600} of 0.1 and glucose release was initiated by the addition of 1 U L^{-1} of reagent A (BioSilta Ltd.). The main cultivation was performed in 24 well sensor plates (OxoDish, HydroDish, PreSens) with a filing volume of 1.1 mL covered with 'System Duetz' lids (Enzyscreen B.V., Heemstede, The Netherlands) and shaken at 300 rpm (50 mm amplitude). After an initial overnight phase, variations of the glucose-release were introduced by supplementing duplicate wells in each sensor plate with 1 – 30 U L^{-1} of reagent A. After an adaptation phase of 3 h, samples for OD_{600} and EPG measurement were taken using a liquid handling robot (Hamilton Microlab Star, Hamilton Bonaduz AG, Bonaduz, Switzerland). The OD_{600} values were converted to biomass with a predetermined factor of 0.38 and the growth rate and product formation rates were calculated using (2) and (3):

$$\mu = \frac{\ln(X_2)-\ln(X_1)}{t_2-t_1} \quad (2) \qquad ; \qquad q_p = \frac{EA_2-EA_1}{t_2-t_1} \times \frac{2}{X_2-X_1} \quad (3)$$

X= Biomass, EA= volumetric enzyme activity, t = sampling time

Biomass determination

Samples taken from microwell plate cultures were measured in 0.9 % NaCl or EPG assay buffer using flat-bottom 96-well plates (Greiner Bio-One, Frickenhausen, Germany) for OD_{600} determination in the microplate reader. One OD unit of the plate reader corresponds to a cell dry weight of 380 mg L^{-1}. Shake flask and bioreactor samples were diluted in 0.9 % NaCl solution and measured in a cuvette spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, Glattburg, Switzerland). Dry biomass was determined from bioreactor samples as follows: 2 mL of culture broth were centrifuged in pre-dried Eppendorf tubes at 21,500 g, the supernatant was discarded, and the cell pellet was dried at 75°C until a constant weight was recorded.

Analysis of medium composition

The culture supernatant was analyzed for glucose, ethanol, ammonia, and glutamate levels with enzymatic test kits. Calibration curves for the respective concentration ranges were prepared for all assays.

Glucose was determined using the Hexokinase FS test (DiaSys, Holzheim, Germany) in 96-well plates or cuvettes. In microwell plates, 10 µL of sample were added to 190 µL of test solution and incubated for 15 minutes until read-out at 340 nm. In cuvettes, 1 mL of test solution was applied to 10 µL of sample. Ethanol, ammonia and glutamate were measured using test kits for 1 mL cuvettes according to the manufacturer's instructions (R-Biopharm, Darmstadt, Germany).

EPG activity assay

The determination of EPG activity was performed in 96-well plate format using the liquid handling robot. A colorimetric assay using 3-Methyl-2benzothiazolinonehydrazone (MBTH), which was developed for test tubes (Anthon and Barrett, 2002; Honda and Kazuaki Kakehi Yoshiko Nishimura, 1981) was adapted to the 96-well format. Polygalacturonic acid (20 g L^{-1}) in 100 mM sodium acetate buffer (pH 4.5) served as a substrate. After an incubation time of 15 min, the reducing ends of the released galacturonic acid were quantified using 7 mM MBTH in a two-step reaction. The first step was carried out at 65 °C for 15 min in a thermal cycler, while the second step required the addition of acidic Fe^{3+} solution (10 mM $NH_4Fe(SO_4)_2 \cdot 12\ H_2O$, 51.5 mM sulfamic acid in 250 mM HCl) and took place for 15 min at room temperature. The color complex was detected at 620 nm and galacturonic acid served as a standard. One unit of enzyme releases 1 µmole of reducing sugar from the substrate per minute.

3.4.4 Results

State-of-the-art characterization of a production strain with regards to its product formation profile is typically carried out in continuous fermentation systems (Figure 3.4.1), which results in the acquisition of data from a limited number of steady states. A significant reduction of experimental efforts can be achieved using the change-stat method. Here, the whole growth space can be analysed, which leads to a more detailed description of the strain in shorter time. Finally, individual fed-batch experiments can be carried out at different feed-rates. The most time-efficient option is to un the cultures in parallel and sample during the transition phase of exponential to glucose-limited growth.

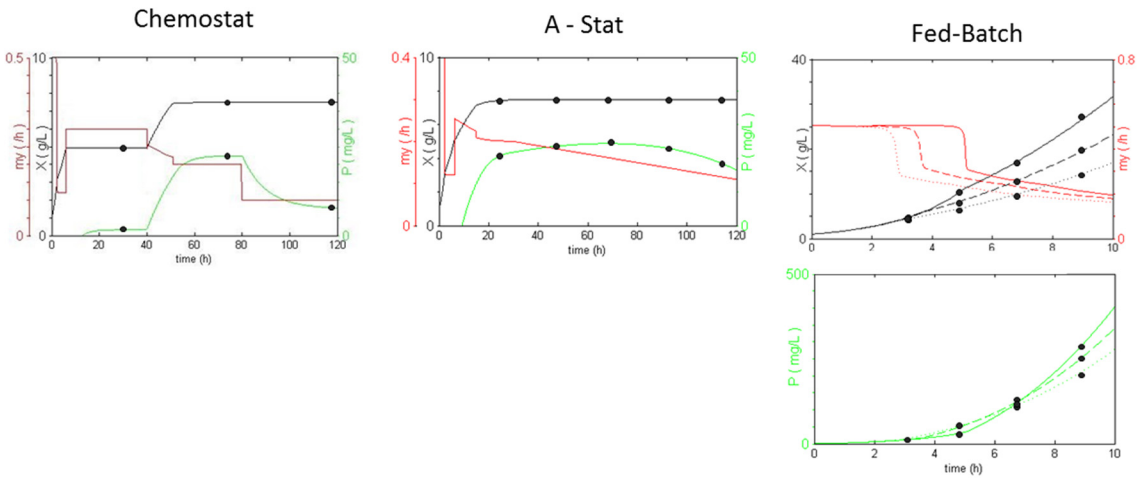

Figure 3.4.1 Schematic overview of methods to determine growth-rate dependent product formation. In a chemostat experiment, product formation at a limited number of steady states can be measured. The adapta-stat is a chemostat with a smooth change of the dilution rate, which allows measurements in a quasi-steady-state. In fed-batch cultures with constant feeding, the growth rate decreases abruptly once substrate limitation is reached. When monitoring cell density and product accumulation over time, a relation between μ and q_P can be drawn.

A-Stat Fermentation

For an efficient quantification of EPG-secretion at a wide range of dilution rates, the change-stat technique is known to be very beneficial. We performed cultivations with a working volume of 1.5 L that started as a batch culture with a subsequent chemostat for five residence times. Then, the dilution rate was smoothly increased (accellerostat, A-stat) or decreased (deceleration-stat, De-stat) with a linear profile until D = 0.01 h^{-1} for De-stat and wash-out for A-stat.

The De-stat experiment was performed under glucose-limitation at an initial D of 0.12 h^{-1}, which was below the D, at which aerobic ethanol formation was expected. From the EPG activity data obtained, it could be concluded that from the initial dilution rate until D ≈ 0.07 h^{-1}, the product formation rate remained constant in the interval between a dilution rate D of 0.12 h^{-1} and 0.07 h^{-1}. A further decrease of D caused a drastic decrease of q_P and the experiment was ended at q_P < 70 U $(g_x\ h)^{-1}$. To compare the different cultivation protocols, a second change-stat experiment was carried out starting from a chemostat at D = 0.07 h^{-1}. The dilution rate was increased with the same acceleration factor. From at-line enzymatic ethanol and glucose measurements, an onset of ethanol formation at D ≈ 0.2 h^{-1} was detected, while glucose accumulation was visible at D ≈ 0.28 h^{-1} (Figure 3.4.2). The biomass concentration remained constant at 6.5 g L^{-1} until D = 0.2 h^{-1} and then gradually decreased until D = 0.35 h^{-1}, where at a biomass concentration of 1.2 g L^{-1} the cultivation was stopped. EPG was detected at all dilution rates, while at D< 0.05, productivity decreased significantly. Dilution rates of 0.08 – 0.11 resulted in an EPG production rate of 250 – 400 U $(g_x\ h)^{-1}$ in the De-stat experiment, In the A-stat experiment, a maximum product formation rate of 400 U $(g_x\ h)^{-1}$ at D = 0.27 was found.

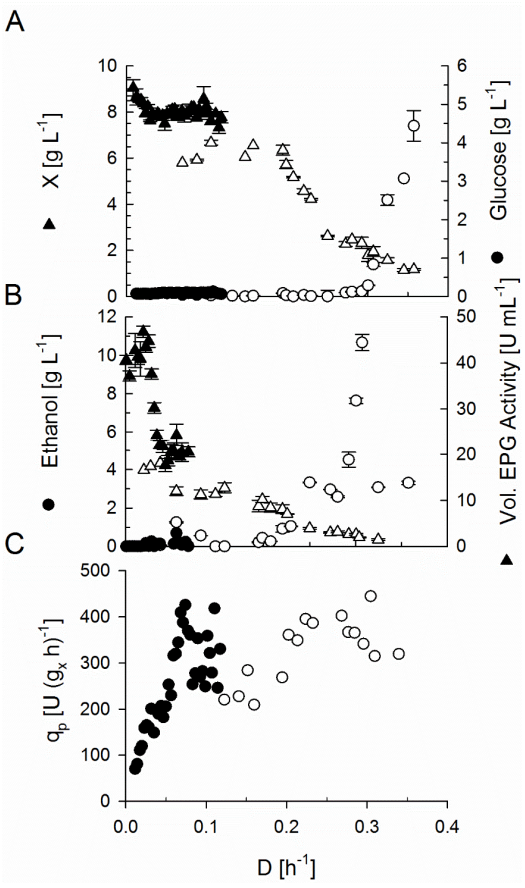

Figure 3.4.2. Growth space characterization of *S. cerevisiae* AH22 pPG6 M27 in change-stat cultivations. A-Stat (open symbols) and De-Stat (closed symbols) cultivations were performed (A) Biomass and Glucose concentrations. (B) Ethanol concentration and volumetric EPG activity. (C) Specific EPG formation rate (q_P)

The change-stat experiments confirmed that growth-rates below the onset of the Crabtree effect are necessary for the efficient production of EPG, as there is no considerable benefit from cultivations at growth rates above the threshold level of ethanol production. Depending on the selected cultivation mode, the product formation profile differs, which indicates an influence of the cell's history on the obtained results. In-depth analysis of the cell's metabolic state would be necessary to investigate this matter more closely.

Strain characterization in parallel fed-batch cultures at the mL scale

We propose to determine the specific product formation rate (q_P) at a wide range of specific growth rates (μ), parallel miniaturized fed-batch cultures to obtain the same results in shorter time. This is now possible due to the advances in automation and miniaturization. A 24-well-plate system with online DO and pH measurement and improved aeration was applied. Glucose feeding was performed with the enzyme-based glucose delivery system (EnBase) combined with the mWM8 medium. After an overnight phase with 1 U L^{-1} of reagent A for constant glucose release, the cultures were supplemented with 1 – 30 U L^{-1} to introduce variations in the growth pattern (Figure 3.4.3). Samples were analyzed for biomass and EPG activity at-line, i.e. during the experiment, with the robotic platform.

The parallel fed-batch cultures showed different optima for biomass and EPG production. At glucose release rates from 1.5 to 4 U L^{-1}, volumetric yield of active EPG was highest, while peak biomass formation was detected with 6 U L^{-1}. From the first three data points, μ and q_P were calculated and it was found that the range was comparable to the A-stat results (Figure 3D)

As an example, a maximal production rate of 218.8 U $(g_x\ h)^{-1}$ at a growth rate of 0.095 h^{-1} was identified for cultures grown with 1.5 U L^{-1}. However, due to the rapid decrease of the growth rate at constant glucose release rates, the range of growth rates was narrower than in the continuous cultures. Moreover, the optimum for EPG secretion was found to be at μ= 0.05 - 0.1, which is considerably lower than in the A-stat experiments.

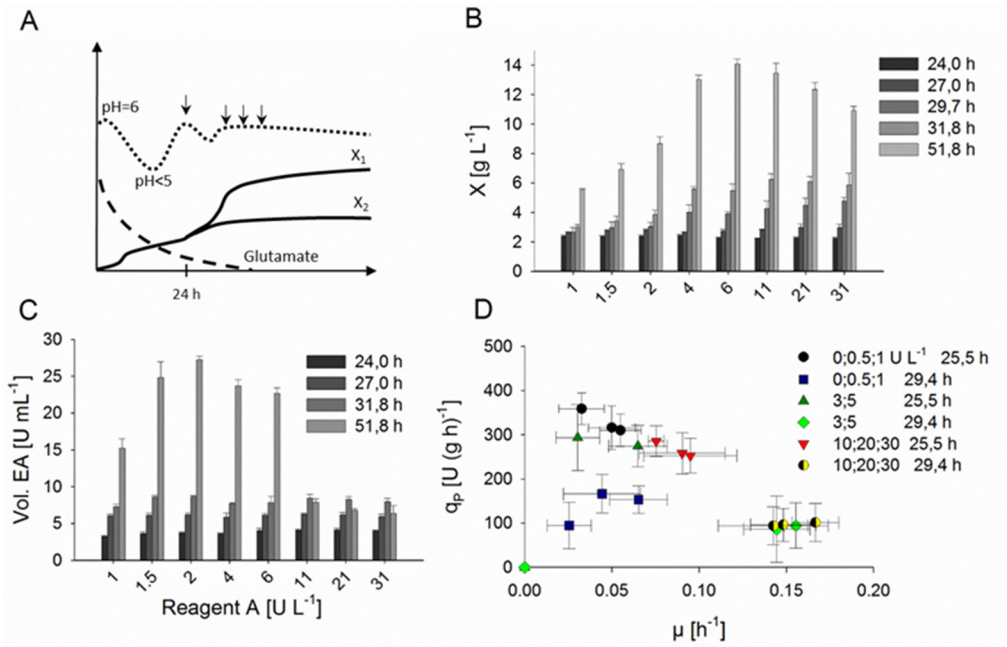

Figure 3.4.3 Strain characterization in parallel fed-batch cultures. (A) Schematic overview of the experimental set-up: After an initial batch phase, indicated by a pH drop (dotted line), glutamate consumption stabilized pH (dashed line). Additional reagent A supplementation (arrow) induces a short batch phase, after which samples are taken for OD_{600} and EPG measurements (dashed arrows). (B) Biomass data from cultures grown with 1 – 31 U L^{-1} reagent A. (C) Volumetric EPG activity of the culture supernatant. (D) Correlation of product formation rate (q_P) and specific growth rate.

3.4.5 Discussion

Choosing the right feeding strategy for the best space time yield is key for the development of a biotechnological production process. Traditionally, the determination of the relation of μ and q_P is done in chemostat cultures exclusively (Kocharin and Nielsen, 2013; Liu et al., 2013; Rebnegger et al., 2014; Vos et al., 2015). Due to the long experimental time to reach steady state, even the characterization of a single strain is very labour intensive, which usually rules out the chance to evaluate several putative production strains. In an effort to reduce experimental times, change-stat methods have been proposed.

In this study, the use of parallel fed-batch cultures is compared to the change-stat method to further reduce experimental time and costs. First, the change-stat technique was applied to characterize the entire growth space. The concept of determining product formation at different growth profiles was then applied to parallel fed-batch cultures in 24-well plates

with at-line OD_{600} and EPG determination allowed an estimation of the µ-dependent specific EPG-production rate.

Growth-dependent product formation was detected with a stable secretion level of 200-400 U $(g_x\ h)^{-1}$ over a wide range of dilution rates. However, the product formation profiles indicate that the operation mode of the change-stat culture has an influence on the strain behavior. This observation was confirmed by the subsequent microwell plate experiments, in which the product formation profile indicated a lower optimum than in the continuous cultures.

The fed-batch approach offers significant experimental time reduction and a simplified experimental set-up. While it is very demanding to perform chemostat cultures in micro- and milliliter reactors, reproducible fed-batch fermentations are easy to perform and only require minimal expenses for material and chemicals, compared to bioreactor experiments. Moreover, the determination of µ-dependency of q_P in fed-batch cultures provides good information about the dynamic changes, which are not obtained from chemostat experiments. Further development of this approach through a combination with mechanistic models will provide a real breakthrough for process development (Cruz Bournazou et al., 2016).

For future studies, automation can be further advanced using on-line biomass sensing or automated cell separation, as they are used by others (Rohe et al., 2012; Unthan et al., 2015). The µ-dependent protein secretion could be performed in pH-controlled minibioreactor systems, which could improve the predictive power of the obtained results (Vester et al., 2009). In conclusion, fed-batch process development can be accelerated with small-scale fed-batch cultures which may replace the need for chemostat and change-stat experiments.

Acknowledgements

The authors would like to thank BioSilta Ltd. and PreSens GmbH for technical support and Rick Nolte, Franziska Jehle and Michael Heiser for carrying out parts of the experimental work. The financial support by the German Federal Ministry of Education and Research (BMBF) within the Framework Concept “Research for Tomorrow’s Production” (project no. 02PJ1150, AUTOBIO project) was greatly acknowledged.

3.4.6 Practical application

When designing fed-batch processes, the optimal specific growth rate for efficient production is a key target parameter, which needs to be experimentally determined. Instead of the traditional method, using serial chemostat experiments, we have applied change-stat cultures to determine the optimal growth rate for efficient product secretion of a S. cerevisiae AH22 strain expressing fungal polygalacturonase. For even faster evaluation of the strain, parallel fed-batch cultivations with enzyme-based glucose delivery were performed. From these small-scale experiments, we could investigate influence of the specific growth rate (μ) on the specific production rate (q_P), which lead to results comparable to the bioreactor scale obtained in two weeks instead of several months.

4 Discussion

Automation, computer-aided design and mathematical modelling are among the main reasons for the technological advances in the manufacturing industries within the past decades. In many fields such as aerospace, automotive and mechanical engineering, mathematical models and artificial intelligence play nowadays a major role in the initial design and constant improvement of products and processes. Worldwide, initiatives to advance manufacturing facilities into intelligent factories are emerging (Li et al., 2017). Due to the complexity of cellular response on the environmental conditions, innovation in biotechnology heavily relies on experimental data. Therefore, the developmental cost of new products and processes strongly depends on prior knowledge gained from similar projects and the individual skills of the researchers.

Although laboratory automation has led to tremendous scientific breakthroughs in the past, such as the sequencing of the human genome, the sole increase of experimental throughput will not enable scientists to determine the global optimum of a production process in short time. In the process development laboratory of the future, intelligent experimentation platforms must perform cultivations under conditions that are relevant to the industrial scale. In addition, a rational reduction of the amount of experiments is necessary. The decision, which experiments are carried out should be made based on statistical methods or mathematical models.

Within the framework of this thesis, three elements necessary for accelerated bioprocess development are presented. Firstly, dissolved oxygen monitoring at the screening stage is necessary to avoid selection of strains under anaerobic conditions. Secondly, the productivity of putative production strains should be determined under fed-batch conditions already in parallel small-scale experiments. Thirdly, experimental planning of up- and downstream procedures should be performed using DoE to ensure systematic selection of the appropriate process conditions.

When screening for production strains or product variants from large libraries, aerobic conditions can be maintained using fed-batch growth media. However, the glucose release needs to be adjusted to the respective growth behaviour of the cells, which requires DO monitoring in the microwell format. Although there are already solutions for DO sensing at the µL-scale (Demuth et al., 2016), these methods only work accurately with

microbial cultures, when the shaking of the sensor plate is not interrupted. One option to detect anaerobic conditions with at-line optical read-out is to increase the response time of the sensor. The DO sensor developed in paper I allows researchers to measure DO in automated screening facilities, which transport multiple plates from shaken incubators to microplate readers. Due to the long response time, possible oxygen limitation of the cultures during shaking can be determined. In combination with a fast DO sensor (Arain et al., 2005; Arain et al., 2006), critical phases, in which the cultures should not be disturbed, can be identified. When validating a screening facility for aerobic cultivation procedures, a combination of both sensor plates allows the operator to determine the aeration status of the culture during shaking and the DO level during measurement. Especially for larger facilities with plate transport times of several minutes, the identification of critical time points is advantageous.

Mostly, microwell plate cultures are only sampled at the endpoint for biomass and product titre determination. This practise omits any growth dynamic and product formation profiling. To demonstrate that valuable and relevant data can be obtained when sampling microwell plate cultures frequently, product formation profiles of parallel fed-batch cultures were presented in paper III. The variation of glucose release resulted in a set of growth profiles for each strain. With this method, the cultures' product formation rate at several different growth rates can be determined simultaneously.

As a reference, change-stat cultures were chosen since this method of performing continuous cultures has several advantages. The smooth change of dilution rate keeps the cells in a quasi-steady-state, which allows sampling at a wide range of physiological states in shorter time compared to standard chemostat cultures (Valgepea et al., 2015). Still, continuous cultures cannot be performed for a large strain collection, even when using parallel benchtop bioreactor systems (Liu et al., 2013; Rebnegger et al., 2014). Parallel fed-batch experiments in the mL-scale could serve as an improved form of secondary screening combined with an initial productivity profiling. Since the medium pH cannot be controlled in shaken plates and the glucose-release is pH-dependent, the experiments should be performed in a pH-controlled environment such as a parallel minibioreactor system. This has been done with a *K. lactis* strain producing ß-galactosidase as a step between strain screening and process development. Growth-rate dependent productivity was characterized, which simplified the scale-up to pilot scale (Wellenbeck et al., 2017).

Even though a substantial amount of data in today's laboratories is stored digitally, experimental planning and record keeping is still done in paper-based form in most laboratories (Dirnagl and Przesdzing, 2016). If software packages are used for DoE-based experiments, the typical workflow is to calculate pipetting volumes based on the experimental plan, print out the instructions and then carry out the experiment manually.

In paper II, a workflow for cell lysis buffer optimization using DoE and robotics is presented. Experimental designs and the corresponding pipetting volumes are stored in a database, which can be accessed by the automation platform for method execution, while results are written back into the system for statistical analysis and optimum prediction. The methodology has several advantages such as the avoidance of errors when transferring data manually and the complete documentation of experimental and computational steps. When working with experimental designs, similar samples are usually grouped to simplify pipetting steps. This may cause unwanted corner effects and ideally, samples should be randomly placed (Malo et al., 2006). With the presented workflow, layouts can be generated in a randomized manner without the risk of errors during liquid handling or back-transfer of data.

In summary, laboratory automation will become an integral part in the laboratory of the future, if the full potential of this technology is being used. Therefore, it is necessary to combine robotics with powerful computational methods for experimental planning, scheduling and evaluation. Although there are many examples of bioprocess development in literature applying high throughput technologies, it is often not stated how the data for planning and evaluation is processed and stored. If the information flow is automated, multiple rounds of experimentation in a closed-loop system can be established.

An example for such a closed-loop system is the platform described by Wu et al. It consists of a liquid handling robot, a vacuum station and a plate reader, which are run by an agent-based software package. Different entities in the software package provide features such as data storage, experimental design, execution and data treatment. These building blocks can communicate with each other. The platform was developed for a lysozyme precipitation optimization, After several iterations of buffer optimization, the optimal pH and ion strength was determined (Wu and Zhou, 2014).

For upstream process development, several rounds of iterations might not be suitable, since experiments can last for days or weeks. Another option is to apply optimal experimental re-design. During the experiment, the available data is analysed and the experiment is re-designed to maximize information content. Since the data from enzymatic tests is available after a certain time window, which is then sliding to the next step, the method was introduced as "Sliding Window Optimal Re-Design (SWORD)" (Cruz Bournazou et al., 2016; Nickel et al., 2017). The online redesign of cultivation has also been performed in benchtop bioreactors (De-Luca et al., 2016).

At present, model-based approaches are usually limited to single unit operations. To use the full potential of a thorough mathematical description of the production process and ultimately enable researchers to minimize experimental effort, holistic models of up- and downstream processing are needed. Currently, the trend moves towards integrated HT process development of cultivation and purification, which yields promising results (Ladd Effio et al., 2016). The implementation of machine learning and online experimental redesign would certainly push the boundaries even further. An overview of the necessary tasks, which need to be translated into automated protocols leading from the initial product idea to the process blueprint, which can be scaled-up is given in Figure 4.1.

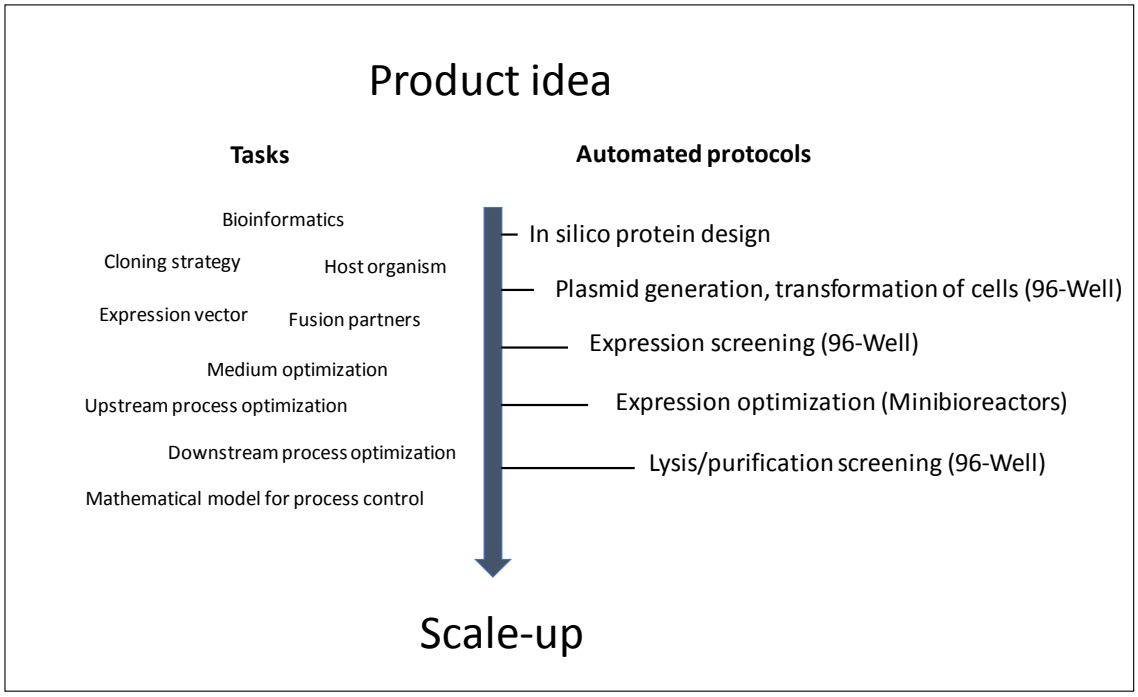

Figure 4.1 Combination of automated protocols for effective development of bioprocesses

In the bioprocess development laboratory of the future, all steps from protein design to the final production process are interconnected. The decisions made by scientists and engineers are supported by artificial intelligence, models and smart robotic facilities. All unit operations are available as experimental modules, which can be combined like building blocks of a process flowsheet. A universal knowledge base on process development strategies is combined with computerized experimental planning and evaluation. The combination of knowledge-based process design, HT experimentation and computer simulations allows the reduction of experimental work while maximizing knowledge output.

In this future scenario, process development would be carried out in the following way: Once a target molecule is chosen, a plasmid library based on the recommendations of the corresponding software is created and cloned into a producer strain collection. An automated expression screening in microwell plates is carried out and a subset of promising candidates is selected for growth model adaptation in miniature bioreactors using the SWORD method. Samples from the bioreactors are further processed in cell disruption and purification steps, to determine the final product yield and purity. This data collection is then used to calibrate a model of the production process, which can be used for scale-up and process control.

Although still limited to a few applications, the automation of scientific discovery using artificial intelligence has already begun revolutionizing the way mankind is gaining knowledge. In 2004, King et al. have reported the successful generation and usage of a robot scientist. The term summarizes an automated laboratory facility that applies methods from artificial intelligence. The system, which was called Adam, was able to generate hypotheses, run experiments, interpret the results and then repeat the cycle (King et al., 2004).

The platform technologies described in this thesis are building blocks for automated bioprocess development. In combination with additional modules for product purification, analytics and model-based experimental design, an integrated process development platform can be established. If this route is followed consequently, a significant reduction of process development time and costs can be achieved.

5 References

Adamberg K, Lahtvee P-J, Valgepea K, Abner K, Vilu R. 2009. Quasi steady state growth of *Lactococcus lactis* in glucose-limited acceleration stat (A-stat) cultures. *Antonie Van Leeuwenhoek* **95**:219–226.

Amanullah A, Otero JM, Mikola M, Hsu A, Zhang J, Aunins J, Schreyer HB, Hope JA, Russo AP. 2010. Novel micro-bioreactor high throughput technology for cell culture process development: Reproducibility and scalability assessment of fed-batch CHO cultures. *Biotechnol. Bioeng.* **106**:57–67.

Anderlei T, Büchs J. 2001. Device for sterile online measurement of the oxygen transfer rate in shaking flasks. *Biochem. Eng. J.* **7**:157–162.

Anthon GE, Barrett DM. 2002. Determination of reducing sugars with 3-methyl-2-benzothiazolinonehydrazone. *Anal. Biochem.* **305**:287–289.

Arain S, John GT, Krause C, Gerlach J, Wolfbeis OS, 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 Actuators, B Chem.* **113**:639–648.

Arain S, Weiss S, Heinzle E, John GT, Krause C, Klimant I. 2005. Gas sensing in microplates with optodes: Influence of oxygen exchange between sample, air, and plate material. *Biotechnol. Bioeng.* **90**:271–280.

Balasundaram B, Harrison S, Bracewell DG. 2009. Advances in product release strategies and impact on bioprocess design. *Trends Biotechnol.* **27**:477–485.

Bär H, Hochstrasser R, Papenfuß B. 2012. SiLA. *J. Lab. Autom.* **17**:86–95.

Bareither R, Pollard D. 2011. A review of advanced small-scale parallel bioreactor technology for accelerated process development: Current state and future need. *Biotechnol. Prog.* **27**:2–14.

Barnard GC, Kull AR, Sharkey NS, Shaikh SS, Rittenhour AM, Burnina I, Jiang Y, Li F, Lynaugh H, Mitchell T, Nett JH, Nylen A, Potgieter TI, Prinz B, Rios SE, Zha D, Sethuraman N, Stadheim T a, Bobrowicz P. 2010. High-throughput screening

and selection of yeast cell lines expressing monoclonal antibodies. *J. Ind. Microbiol. Biotechnol.* **37**:961–971.

Baumann P, Hahn T, Hubbuch J. 2015. High-throughput micro-scale cultivations and chromatography modeling: Powerful tools for integrated process development. *Biotechnol. Bioeng.* **112**:2123–2133.

Baumann P, Hubbuch J. 2017. Downstream process development strategies for effective bioprocesses: Trends, progress, and combinatorial approaches. *Eng. Life Sci.* **17**:1142–1158.

Begot C, Desnier I, Daudin JD, Labadie JC, Lebert A. 1996. Recommendations for calculating growth parameters by optical density measurements. *J. Microbiol. Methods* **25**:225–232.

Betts JPJ, Warr SRC, Finka GB, Uden M, Town M, Janda JM, Baganz F, Lye GJ. 2014. Impact of aeration strategies on fed-batch cell culture kinetics in a single-use 24-well miniature bioreactor. *Biochem. Eng. J.* **82**:105–116.

Betts J, Stacey A, Zoro B, Biotech SS. 2016. A Novel Automated Micro Bioreactor for High Density Fed Batch Microbial Screening Applications. Internal Poster from Sartorius, Royston UK.

Bhambure R, Kumar K, Rathore AS. 2011. High-throughput process development for biopharmaceutical drug substances. *Trends Biotechnol.* **29**:127–135.

Biebl H, Menzel K, Zeng AP, Deckwer WD. 1999. Microbial production of 1,3-propanediol. *Appl. Microbiol. Biotechnol.* **52**:289–297.

Bisswanger H. 2014. Enzyme assays. *Perspect. Sci.* **1**:41–55.

Bornhorst JA, Falke JJ. 2000. Purification of Proteins Using Polyhistidine Affinity Tags. *Methods Enzymol.* **326**:245–254.

Brognaux A, Han S, Sørensen SJ, Lebeau F, Thonart P, Delvigne F. 2013. A low-cost, multiplexable, automated flow cytometry procedure for the characterization of microbial stress dynamics in bioreactors. *Microb. Cell Fact.* **12**:100.

Büchs J. 2001. Introduction to advantages and problems of shaken cultures. *Biochem. Eng. J.* **7**:91–98.

Büchs J, Lotter S, Milbradt C. 2001. Out-of-phase operating conditions, a hitherto unknown phenomenon in shaking bioreactors. *Biochem. Eng. J.* **7**:135–141.

Bugge MM, Hansen T, Klitkou A. 2016. What is the bioeconomy? A review of the literature. *Sustain.* **8**.

Bushell ME, Dunstan GL, Wilson GC. 1997. Effect of small scale culture vessel type on hyphal fragment size and erythromycin production in *Saccharopolyspora erythraea*. *Biotechnol. Lett.* **19**:849–852.

Chapman T. 2003. Automation on the move. *Nature* **421**:661–666.

Chattopadhyay S, Srivastava a K, Bisaria VS. 2002. Optimization of culture parameters for production of podophyllotoxin in suspension culture of *Podophyllum hexandrum*. *Appl. Biochem. Biotechnol.* **102**–**103**:381–93.

Chen A, Chitta R, Chang D, Amanullah A. 2009. Twenty-four well plate miniature bioreactor system as a scale-down model for cell culture process development. *Biotechnol. Bioeng.* **102**:148–160.

Choi Y, van Breemen RB. 2008. Development of a screening assay for ligands to the estrogen receptor based on magnetic microparticles and LC-MS. *Comb. Chem. High Throughput Screen.* **11**:1–6.

Cruz Bournazou MN, Barz T, Nickel D, Lopez Cárdenas D, Glauche F, Knepper A, Neubauer P. 2016. Online optimal experimental re-design in robotic parallel fed-batch cultivation facilities for validation of macro-kinetic growth models using *E. coli* as an example. *Biotechnol. Bioeng.* **114**:1–29.

Daniels C, Rodriguez J, Lim E, Wenger M. 2016. An integrated robotic system for high-throughput process development of cell and virus culture conditions: Application to biosafety level 2 live virus vaccines. *Eng. Life Sci.* **16**:202–209.

Daugelavicius R, Bakiene E, Bamford DH. 2000. Stages of polymyxin B interaction with the *Escherichia coli* cell envelope. *Antimicrob. Agents Chemother.* **44**:2969–2978.

De-Luca R, Galvanin F, Bezzo F. 2016. A methodology for direct exploitation of available information in the online model-based redesign of experiments. *Comput. Chem. Eng.* **91**:195–205.

Delaney NF, Rojas Echenique JI, Marx CJ. 2013. Clarity. *J. Lab. Autom.* **18**:171–177.

Demuth C, Varonier J, Jossen V, Eibl R, Eibl D. 2016. Novel probes for pH and dissolved oxygen measurements in cultivations from millilitre to benchtop scale. *Appl. Microbiol. Biotechnol.* **100**:3853–3863.

Dirnagl U, Przesdzing I. 2016. A pocket guide to electronic laboratory notebooks in the academic life sciences. *F1000Research*:1–11.

Doig SD, Pickering SCR, Lye GJ, Woodley JM. 2002. The use of microscale processing technologies for quantification of biocatalytic Baeyer-Villiger oxidation kinetics. *Biotechnol. Bioeng.* **80**:42–49.

Duetz W. 2017. general_principle. http://enzyscreen.com/sandwich_covers_general_principle.htm.

Duetz W a. 2007. Microtiter plates as mini-bioreactors: miniaturization of fermentation methods. *Trends Microbiol.* **15**:469–475.

Duetz W a., Rüedi L, Hermann R, O'Connor K, Büchs J, Witholt B. 2000. Methods for intense aeration, growth, storage, and replication of bacterial strains in microtiter plates. *Appl. Environ. Microbiol.* **66**:2641–2646.

Duetz W a., Witholt B. 2001. Effectiveness of orbital shaking for the aeration of suspended bacterial cultures in square-deepwell microtiter plates. *Biochem. Eng. J.* **7**:113–115.

Elliott C, Vijayakumar V, Zink W, Hansen R. 2007. National Instruments LabVIEW: A Programming Environment for Laboratory Automation and Measurement. *J. Lab. Autom.* **12**:17–24.

Eriksson L, Johansson E, Wikström C. 1998. Mixture design - Design generation, PLS analysis, and model usage. *Chemom. Intell. Lab. Syst.* **43**:1–24.

FDA. 2004. Guidance for Industry PAT: A Framework for Innovative Pharmaceutical Development, Manufacuring, and Quality Assurance. *FDA Off. Doc.*:16.

Fernandes P, Carvalho F, P.C. Marques M. 2011. Miniaturization in Biotechnology: Speeding up the Development of Bioprocesses. *Recent Pat. Biotechnol.* **5**:160–173.

Ferrer-Miralles N, Domingo-Espín J, Corchero JL, Vázquez E, Villaverde A. 2009.

Microbial factories for recombinant pharmaceuticals. *Microb. Cell Fact.* **8**:17.

Fontova A, Soley A, Gálvez J, Sarró E, Lecina M, Rosell J, Riu P, Cairó J, Gòdia F, Bragós R. 2006. Multiple automated minibioreactor system for multifunctional screening in biotechnology. *Conf Proc IEEE Eng Med Biol Soc.*:632–635.

Formenti LR, Nørregaard A, Bolic A, Hernandez DQ, Hagemann T, Heins A-L, Larsson H, Mears L, Mauricio-Iglesias M, Krühne U, Gernaey K V. 2014. Challenges in industrial fermentation technology research. *Biotechnol. J.* **9**:727–738.

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

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. *Biotechnol. Bioeng.* **103**:1118–1128.

Gaciarz A, Veijola J, Uchida Y, Saaranen MJ, Wang C, Hörkkö S, Ruddock LW. 2016. Systematic screening of soluble expression of antibody fragments in the cytoplasm of E. coli. *Microb. Cell Fact.* **15**:22.

Gatte-Picchi D, Weiz A, Ishida K, Hertweck C, Dittmann E. 2014. Functional analysis of environmental DNA-derived microviridins provides new insights into the diversity of the tricyclic peptide family. *Appl. Environ. Microbiol.* **80**:1380–1387.

Ge X, Hanson M, Shen H, Kostov Y, Brorson KA, Frey DD, Moreira AR, Rao G. 2006. Validation of an optical sensor-based high-throughput bioreactor system for mammalian cell culture. *J. Biotechnol.* **122**:293–306.

Gebhardt G, Hortsch R, Kaufmann K, Arnold M, Weuster-Botz D. 2011. A new microfluidic concept for parallel operated milliliter-scale stirred tank bioreactors. *Biotechnol. Prog.* **27**:684–690.

Gehmlich I, Pohl HD, Knorre W a. 1997. Laboratory-scale permeabilization of *Escherichia coli* cells for recovery of a small recombinant protein - Staphylokinase. *Bioprocess Eng.* **17**:35–38.

Gibbon GA. 1996. A brief history of LIMS. *Lab. Autom. Inf. Manag.* **32**:1–5.

Gill NK, Appleton M, Baganz F, Lye GJ. 2008. Design and characterisation of a miniature stirred bioreactor system for parallel microbial fermentations. *Biochem. Eng. J.* **39**:164–176.

Glassey J, Montague GA, Ward AC, Kara B V. 1994. Artificial neural network based experimental design procedure for enhancing fermentation development. *Biotechnol. Bioeng.* **44**:397–405.

Glauche F, John GT, 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. *J. Lab. Autom.* **20**:438–446.

Glazyrina J, Krause M, Junne S, Glauche F, Storm D, Strom D, Neubauer P. 2012. Glucose-limited high cell density cultivations from small to pilot plant scale using an enzyme-controlled glucose delivery system. *N. Biotechnol.* **29**:235–42.

Glazyrina J, Materne E, Hillig F, Neubauer P, Junne S. 2011. Two-compartment method for determination of the oxygen transfer rate with electrochemical sensors based on sulfite oxidation. *Biotechnol. J.* **6**:1003–1008.

Goddard J, Reymond J. 2004. Enzyme assays for high-throughput screening. *Curr. Opin. Biotechnol.* **15**:314–322.

Golembiewski B, Sick N, Bröring S. 2015. The emerging research landscape on bioeconomy: What has been done so far and what is essential from a technology and innovation management perspective? *Innov. Food Sci. Emerg. Technol.* **29**:308–317.

Grimm T, Grimm M, Klat R, Neubauer A, Palela M, Neubauer P. 2012. Enzyme-based glucose delivery as a high content screening tool in yeast-based whole-cell biocatalysis. *Appl. Microbiol. Biotechnol.* **94**:931–937.

Hermann R, Lehmann M, Büchs J. 2003. Characterization of gas-liquid mass transfer phenomena in microtiter plates. *Biotechnol. Bioeng.* **81**:178–186.

Hermann T. 2003. Industrial production of amino acids by coryneform bacteria. *J. Biotechnol.* **104**:155–172.

Herold S, Krämer D, Violet N, King R. 2017. Rapid process synthesis supported by a

unified modular software framework. *Eng. Life Sci.* **17**:1202–1214.

Holz M, Otto C, Kretzschmar A, Yovkova V, Aurich A, Pötter M, Marx A, Barth G. 2011. Overexpression of alpha-ketoglutarate dehydrogenase in *Yarrowia lipolytica* and its effect on production of organic acids. *Appl. Microbiol. Biotechnol.* **89**:1519–1526.

Honda S, Kazuaki Kakehi Yoshiko Nishimura IC. 1981. Determination of Carbohydrates By Condensation With 3-Methyl-2-Benzothiazolinonehydrazone. *Anal. Chim. Acta.* **131**:293–296.

Hortsch R, Krispin H, Weuster-Botz D. 2011. Process performance of parallel bioreactors for batch cultivation of *Streptomyces tendae*. *Bioprocess Biosyst. Eng.* **34**:297–304.

Hortsch R, Weuster-Botz D. 2011. Growth and recombinant protein expression with *Escherichia coli* in different batch cultivation media. *Appl. Microbiol. Biotechnol.* **90**:69–76.

Hsu WT, Aulakh RPS, Traul DL, Yuk IH. 2012. Advanced microscale bioreactor system: A representative scale-down model for bench-top bioreactors. *Cytotechnology* **64**:667–678.

Huber R, Ritter D, Hering T, Hillmer A-K, Kensy F, Müller C, Wang L, Büchs J. 2009a. Robo-Lector - a novel platform for automated high-throughput cultivations in microtiter plates with high information content. *Microb. Cell Fact.* **8**:42.

Huber R, Scheidle M, Dittrich B, Klee D, Büchs J. 2009b. Equalizing growth in high-throughput small scale cultivations via precultures operated in fed-batch mode. *Biotechnol. Bioeng.* **103**:1095–1102.

Hüser J, Lohrmann E, Kalthof B, Burkhardt N, Brüggemeier U, Bechem M. 2006. High-throughput Screening for Targeted Lead Discovery. *High-Throughput Screen. Drug Discov. Vol. 35*:15–36.

Ibarra RU, Edwards JS, Palsson BO. 2002. Escherichia coli K-12 undergoes adaptive evolution to achieve in silico predicted optimal growth. *Nature* **420**:186–189.

ICH. 2005. Quality Risk Management Q9. *ICH Harmon. Tripart. Guidel.*:1–23.

ICH. 2008. ICH pharmaceutical quality system Q10. *WHO Drug Inf.* **22**:177–181.

ICH. 2009. Pharmaceutical Development Q8. *ICH Harmon. Tripart. Guidel.* **8**:1–28.

Isett K, George H, Herber W, Amanullah A. 2007. Twenty-four-well plate miniature bioreactor high-throughput system: Assessment for microbial cultivations. *Biotechnol. Bioeng.* **98**:1017–1028.

Islam RS, Tisi D, Levy MS, Lye GJ. 2008. Scale-up of *Escherichia coli* growth and recombinant protein expression conditions from microwell to laboratory and pilot scale based on matched k La. *Biotechnol. Bioeng.* **99**:1128–1139.

Jaitzig J, Li J, Süssmuth R, Neubauer P. 2014. Reconstituted biosynthesis of the cyclodepsipeptide antibiotic valinomycin in the heterologous host *Escherichia coli*:2–3.

Jänicke G, Sauter C, Bux R, Haas J. 2007. Characterisation of Shake Flasks for Cultivation of Animal Cell Cultures. *Cell Technol. Cell Prod.*:727–731.

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

John GT, 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. *J. Dairy Res.* **70**:327–333.

Johnson IS. 1983. Human insulin from recombinant DNA technology. *Science* **219**:632–637.

Käß F, Prasad A, Tillack J, Moch M, Giese H, Büchs J, Wiechert W, Oldiges M. 2014. Rapid assessment of oxygen transfer impact for Corynebacterium glutamicum. *Bioprocess Biosyst. Eng.* **37**:2567–2577.

Kempf H, Kropp C, Olmer R, Martin U, Zweigerdt R. 2015. Cardiac differentiation of human pluripotent stem cells in scalable suspension culture. *Nat. Protoc.* **10**:1345–1361.

Kensy F, Engelbrecht C, Büchs J. 2009a. Scale-up from microtiter plate to laboratory fermenter: evaluation by online monitoring techniques of growth and protein

expression in *Escherichia coli* and *Hansenula polymorpha* fermentations. *Microb. Cell Fact.* **8**:68.

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

Kim BJ, Diao J, Shuler ML. 2012. Mini-scale bioprocessing systems for highly parallel animal cell cultures. *Biotechnol. Prog.* **28**:595–607.

King RD, Whelan KE, Jones FM, Reiser PGK, Bryant CH, Muggleton SH, Kell DB, Oliver SG. 2004. Functional genomic hypothesis generation and experimentation. *Science (80-.).* **427**:247–252.

Klein CJL, Rasmussen JJ, Rønnow B, Olsson L, Nielsen J. 1999. Investigation of the impact of MIG1 and MIG2 on the physiology of *Saccharomyces cerevisiae*. *J. Biotechnol.* **68**:197–212.

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

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.

Kocharin K, Nielsen J. 2013. Specific growth rate and substrate dependent polyhydroxybutyrate production in *Saccharomyces cerevisiae*. *AMB Express* **3**:18.

Krause M, Neubauer A, Neubauer P. 2016. 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.* **15**:110.

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. *Microb. Cell Fact.* **9**:11.

Kumar S, Wittmann C, Heinzle E. 2004. Minibioreactors. *Biotechnol. Lett.* **26**:1–10.

Kunze M, Roth S, Gartz E, Büchs J. 2014. Pitfalls in optical on-line monitoring for high-throughput screening of microbial systems. *Microb. Cell Fact.* **13**:53.

Ladd Effio C, Baumann P, Weigel C, Vormittag P, Middelberg A, Hubbuch J. 2016. High-throughput process development of an alternative platform for the production of virus-like particles in *Escherichia coli*. *J. Biotechnol.* **219**:7–19.

Lang C, Looman a. C. 1995. Efficient expression and secretion of *Aspergillus niger* RH5344 polygalacturonase in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **44**:147–156.

Lang C, Göllnitz C, Popovic M, Stahl U. 1997. Optimization of fungal polygalacturonase synthesis by *Saccharomyces cerevisiae* in fed-batch culture. *Chem. Eng. J.* **65**:219–226.

Li B-H, Hou B-C, Yu W-T, Lu X-B, Yang C-W. 2017. Applications of artificial intelligence in intelligent manufacturing: a review. *Front Inf. Technol Electron Eng* **18**:86–96.

Li J, Jaitzig J, Hillig F, Süssmuth R, Neubauer P. 2014. Enhanced production of the nonribosomal peptide antibiotic valinomycin in *Escherichia coli* through small-scale high cell density fed-batch cultivation. *Appl. Microbiol. Biotechnol.* **98**:591–601.

Lin Z, Cai Z. 2009. Cell lysis methods for high-throughput screening or miniaturized assays. *Biotechnol. J.* **4**:210–215.

Liotta LA, Petricoin EF. 2003. The promise of proteomics. *Clin. Adv. Hematol. Oncol.* Vol. 1 460-462p.

Listwan P, Pédelacq JD, Lockard M, Bell C, Terwilliger TC, Waldo GS. 2010. The optimization of in vitro high-throughput chemical lysis of *Escherichia coli*. Application to ACP domain of the polyketide synthase ppsC from *Mycobacterium tuberculosis*. *J. Struct. Funct. Genomics* **11**:41–49.

Liu Z, Hou J, Martínez JL, Petranovic D, Nielsen J. 2013. Correlation of cell growth and heterologous protein production by *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **97**:8955–8962.

Long Q, Liu X, Yang Y, Li L, Harvey L, McNeil B, Bai Z. 2014. The development and

application of high throughput cultivation technology in bioprocess development. *J. Biotechnol.* **192**:323–338.

Lübbe C, Demain AL, Bergman K. 1986. Use of controlled-release polymer to feed ammonium to *Streptomyces clavuligerus* cephalosporin fermentations in shake flasks. *Appl. Microbiol. Biotechnol.* **23**:411–411.

Lutz MW, Menius JA, Choi TD, Laskody RG, Domanico PL, Goetz AS, Saussy DL. 1996. Experimental design for high-throughput screening. *Drug Discov. Today* **1**:277–286.

Maier U, Büchs J. 2001. Characterisation of the gas-liquid mass transfer in shaking bioreactors. *Biochem. Eng. J.* **7**:99–106.

Maier U, Losen M, Büchs J. 2004. Advances in understanding and modeling the gas-liquid mass transfer in shake flasks. *Biochem. Eng. J.* **17**:155–167.

Malo N, Hanley JA, Cerquozzi S, Pelletier J, Nadon R. 2006. Statistical practice in high-throughput screening data analysis. *Nat. Biotechnol.* **24**:167–175.

Mandenius C, Brundin A. 2008. Review : Biocatalysts and Bioreactor Design Optimization, Bioprocess Methodology, Using Design-of-experiments. *Biotechnol Progr* **24**:1191–1203.

Markert S, Joeris K. 2017. Establishment of a fully automated microtiter plate-based system for suspension cell culture and its application for enhanced process optimization. *Biotechnol. Bioeng.* **114**:113–121.

Marques MPC, Cabral JMS, Fernandes P. 2010. Bioprocess scale-up: Quest for the parameters to be used as criterion to move from microreactors to lab-scale. *J. Chem. Technol. Biotechnol.* **85**:1184–1198.

Marteijn RCL, Jurrius O, Dhont J, De Gooijer CD, Tramper J, Martens DE. 2003. Optimization of a feed medium for fed-batch culture of insect cells using a genetic algorithm. *Biotechnol. Bioeng.* **81**:269–278.

Matos CFRO, Robinson C, Alanen HI, Prus P, Uchida Y, Ruddock LW, Freedman RB, Keshavarz-Moore E. 2014. Efficient export of prefolded, disulfide-bonded recombinant proteins to the periplasm by the Tat pathway in *Escherichia coli* CyDisCo strains. *Biotechnol. Prog.* **30**:281–290.

Mayr LM, Bojanic D. 2009. Novel trends in high-throughput screening. *Curr. Opin. Pharmacol.* **9**:580–588.

Mercier SM, Diepenbroek B, Wijffels RH, Streefland M. 2014. Multivariate PAT solutions for biopharmaceutical cultivation: Current progress and limitations. *Trends Biotechnol.* **32**:329–336.

Micheletti M, Barrett T, Doig SD, Baganz F, Levy MS, Woodley JM, Lye GJ. 2006. Fluid mixing in shaken bioreactors: Implications for scale-up predictions from microlitre-scale microbial and mammalian cell cultures. *Chem. Eng. Sci.* **61**:2939–2949.

Moses S, Manahan M. 2012. Assessment of AMBR as a model for high-throughput cell culture process development strategy. *Adv. Biosci. Biotechnol.* **3**:918–927.

Mozzetti V, Grattepanche F, Moine D, Berger B, Rezzonico E, Meile L, Arigoni F, Lacroix C. 2010. New method for selection of hydrogen peroxide adapted bifidobacteria cells using continuous culture and immobilized cell technology. *Microb. Cell Fact.* **9**:60.

Nahku R, Valgepea K, Lahtvee P-J, Erm S, Abner K, Adamberg K, Vilu R. 2010. Specific growth rate dependent transcriptome profiling of *Escherichia coli* K12 MG1655 in accelerostat cultures. *J. Biotechnol.* **145**:60–65.

Nakayama GR. 1998. Microplate assays for high-throughput screening. *Curr. Opin. Drug Discov. Devel.* **1**:85–91.

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

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

Nguyen V, Hatahet F, Salo KE, Enlund E, Zhang C, Ruddock LW. 2011. Pre-expression of a sulfhydryl oxidase significantly increases the yields of eukaryotic disulfide bond containing proteins expressed in the cytoplasm of *E. coli*. *Microb. Cell Fact.* **10**:1.

Nickel DB, Cruz-Bournazou MN, Wilms T, Neubauer P, Knepper A. 2017. Online

bioprocess data generation, analysis, and optimization for parallel fed-batch fermentations in milliliter scale. *Eng. Life Sci.* **17**:1195–1201.

Nienow AW, Rielly CD, Brosnan K, Bargh N, Lee K, Coopman K, Hewitt CJ. 2013. The physical characterisation of a microscale parallel bioreactor platform with an industrial CHO cell line expressing an IgG4. *Biochem. Eng. J.* **76**:25–36.

Noorman H. 2011. An industrial perspective on bioreactor scale-down: What we can learn from combined large-scale bioprocess and model fluid studies. *Biotechnol. J.* **6**:934–943.

Nowicki MW, Blackburn EA, McNae IW, Wear MA. 2015. A streamlined, automated protocol for the production of milligram quantities of untagged recombinant rat lactate dehydrogenase A using ÄKTAxpress™. *PLoS One* **10**:1–13.

Olson BJSC, Markwell J. 2007. Assays for determination of protein concentration. *Curr. Protoc. Protein Sci.* **Chapter 3**:Unit 3.4.

Paalme T, Elken R, Vilu R, Korhola M. 1997. Growth efficiency of *Saccharomyces cerevisiae* on glucose/ethanol media with a smooth change in the dilution rate (A-stat). *Enzyme Microb. Technol.* **20**:174–181.

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

Panula-Perälä J, Vasala A, Karhunen J, Ojamo H, Neubauer P, Mursula A. 2014. Small-scale slow glucose feed cultivation of *Pichia pastoris* without repression of AOX1 promoter: Towards high throughput cultivations. *Bioprocess Biosyst. Eng.* **37**:1261–1269.

Parekh S, Vinci V a, Strobel RJ. 2000. Improvement of microbial strains and fermentation processes. *Appl. Microbiol. Biotechnol.* **54**:287–301.

Pierce JJ, Turner C, Keshavarz-Moore E, Dunnill P. 1997. Factors determining more efficient large-scale release of a periplasmic enzyme from *E. coli* using lysozyme. *J. Biotechnol.* **58**:1–11.

Pilarek M, Brand E, Hillig F, Krause M, Neubauer P. 2013. Enhanced plasmid production in miniaturized high-cell-density cultures of Escherichia coli supported

with perfluorinated oxygen carrier. *Bioprocess Biosyst. Eng.* **36**:1079–1086.

Porro D, Sauer M, Branduardi P, Mattanovich D. 2005. Recombinant Protein Production in Yeasts. *Mol. Biotechnol.* **31**:245–259.

Puskeiler R, Kaufmann K, Weuster-Botz D. 2005a. Development, parallelization, and automation of a gas-inducing milliliter-scale bioreactor for high-throughput bioprocess design (HTBD). *Biotechnol. Bioeng.* **89**:512–523.

Puskeiler R, Kusterer A, John GT, Weuster-Botz D. 2005b. Miniature bioreactors for automated high-throughput bioprocess design (HTBD): reproducibility of parallel fed-batch cultivations with *Escherichia coli*. *Biotechnol. Appl. Biochem.* **42**:227–235.

Rathore AS. 2009. Roadmap for implementation of quality by design (QbD) for biotechnology products. *Trends Biotechnol.* **27**:546–553.

Rebnegger C, Graf AB, Valli M, Steiger MG, Gasser B, Maurer M, Mattanovich D. 2014. In Pichia pastoris, growth rate regulates protein synthesis and secretion, mating and stress response. *Biotechnol. J.* **9**:511–525.

Rohe P, Venkanna D, Kleine B, Freudl R, Oldiges M. 2012. An automated workflow for enhancing microbial bioprocess optimization on a novel microbioreactor platform. *Microb. Cell Fact.* **11**:144. Microbial Cell Factories.

Samorski M, Müller-Newen G, Büchs J. 2005. Quasi-continuous combined scattered light and fluorescence measurements: A novel measurement technique for shaken microtiter plates. *Biotechnol. Bioeng.* **92**:61–68.

Sang YL, Lee DY, Tae YK. 2005. Systems biotechnology for strain improvement. *Trends Biotechnol.* **23**:349–358.

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 Res.* **10**:83–92.

Schiefelbein S, Fröhlich A, John GT, Beutler F, Wittmann C, Becker J. 2013. Oxygen supply in disposable shake-flasks: Prediction of oxygen transfer rate, oxygen saturation and maximum cell concentration during aerobic growth. *Biotechnol.*

Lett. **35**:1223–1230.

Schmid I, Aschoff J. 2016. A scalable software framework for data integration in bioprocess development. *Eng. Life Sci.* **17**:1159–1165.

Schmideder A, Hensler S, Lang M, Stratmann A, Giesecke U, Weuster-Botz D. 2016. High-cell-density cultivation and recombinant protein production with *Komagataella pastoris* in stirred-tank bioreactors from milliliter to cubic meter scale. *Process Biochem.* **51**:177–184.

Schmideder A, Severin TS, Cremer JH, Weuster-Botz D. 2015. A novel milliliter-scale chemostat system for parallel cultivation of microorganisms in stirred-tank bioreactors. *J. Biotechnol.* **210**:19–24.

Schmidt-Hager J, Ude C, Findeis M, John GT, Scheper T, Beutel S. 2014. Noninvasive online biomass detector system for cultivation in shake flasks. *Eng. Life Sci.* **14**:467–476.

Schneider K, Schü tz V, John GT, Heinzle E. 2010. Optical device for parallel online measurement of dissolved oxygen and pH in shake flask cultures. *Bioprocess Biosyst. Eng.* **33**:541–547.

Shu P, Johnson MJ. 1948. Citric Acid Production by Submerged Fermentation with *Aspergillus niger*. *Ind. Eng. Chem.* **40**:1202–1205.

Sieben M, Giese H, Grosch J-H, Kauffmann K, Büchs J. 2016. Permeability of currently available microtiter plate sealing tapes fail to fulfil the requirements for aerobic microbial cultivation. *Biotechnol. J.*:1–36.

Šiurkus J, Neubauer P. 2011a. Heterologous production of active ribonuclease inhibitor in Escherichia coli by redox state control and chaperonin coexpression. *Microb. Cell Fact.* **10**:65.

Šiurkus J, Neubauer P. 2011b. Reducing conditions are the key for efficient production of active ribonuclease inhibitor in Escherichia coli. *Microb. Cell Fact.* **10**:31.

Siurkus J, Panula-Perälä J, Horn U, Kraft M, Rimseliene R, Neubauer P. 2010. Novel approach of high cell density recombinant bioprocess development: optimisation and scale-up from microliter to pilot scales while maintaining the fed-batch cultivation mode of *E. coli* cultures. *Microb. Cell Fact.* **9**:35.

Van Sluis C Der, Westerink BH, Dijkstal MM, Castelein SJ, Van Boxtel a. JB, Giuseppin MLF, Tramper J, Wijffels RH. 2001. Estimation of steady-state culture characteristics during acceleration-stats with yeasts. *Biotechnol. Bioeng.* **75**:267–275.

de Smet MJ, Kingma J, Witholt B. 1978. The effect of toluene on the structure and permeability of the outer and cytoplasmic membranes of *Escherichia coli*. *Biochim. Biophys. Acta* **506**:64–80.

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**:76–85.

Sparkes A, Aubrey W, Byrne E, Clare A, Khan MN, Liakata M, Markham M, Rowland J, Soldatova LN, Whelan KE, Young M, King RD. 2010. Towards Robot Scientists for autonomous scientific discovery. *Autom. Exp.* **2**:1.

Stöckmann C, Losen M, Dahlems U, Knocke C, Gellissen G, Büchs J. 2003. Effect of oxygen supply on passaging, stabilising and screening of recombinant *Hansenula polymorpha* production strains in test tube cultures. *FEMS Yeast Res.* **4**:195–205.

von Stosch M, Hamelink J-M, Oliveira R. 2016. Hybrid modeling as a QbD/PAT tool in process development: an industrial *E. coli* case study. *Bioprocess Biosyst. Eng.* **39**:773–784.

Sun J, Chew L. 2012. Rapid Fermentation Process Development and Optimization. *Eng. Complex Phenotypes Ind. Strains*:133–168.

Suresh S, Srivastava VC, Mishra IM. 2009. Techniques for oxygen transfer measurement in bioreactors: A review. *J. Chem. Technol. Biotechnol.* **84**:1091–1103.

Szeker K, Niemitalo O, Casteleijn MG, Juffer AH, Neubauer P. 2011. High-temperature cultivation and 5′ mRNA optimization are key factors for the efficient overexpression of thermostable Deinococcus geothermalis purine nucleoside phosphorylase in Escherichia coli. *J. Biotechnol.* **156**:268–274.

Takàtsy G. 1955. The use of spiral loops in serological and virological micro-methods. *Acta Microbiol. Acad. Sci. Hung.* **3**:191–202.

Takors R, Wiechert W, Weuster-Botz D. 1997. Experimental design for the identification of macrokinetic models and model discrimination. *Biotechnol. Bioeng.* **56**:564–576.

Tang YJ, Laidlaw D, Gani K, Keasling JD. 2006. Evaluation of the effects of various culture conditions on Cr(VI) reduction by *Shewanella oneidensis* MR-1 in a novel high-throughput mini-bioreactor. *Biotechnol. Bioeng.* **95**:176–184.

Tolosa L, Kostov Y, Harms P, Rao G. 2002. Noninvasive measurement of dissolved oxygen in shake flasks. *Biotechnol. Bioeng.* **80**:594–597.

Tyrrell EA, Macodnald RE, Gerhardt P. 1958. Biphasic system for growing bacteria in concentrated culture. *J. Bacteriol.* **75**:1–4.

Ude C, Hentrop T, Lindner P, Lücking TH, Scheper T, Beutel S. 2015. New perspectives in shake flask pH control using a 3D-printed control unit based on pH online measurement. *Sensors Actuators, B Chem.* **221**:1035–1043.

Ukkonen K, Vasala A, Ojamo H, Neubauer P. 2011. High-yield production of biologically active recombinant protein in shake flask culture by combination of enzyme-based glucose delivery and increased oxygen transfer. *Microb. Cell Fact.* **10**:107.

Ukkonen K, Veijola J, Vasala A, Neubauer P. 2013. Effect of culture medium, host strain and oxygen transfer on recombinant Fab antibody fragment yield and leakage to medium in shaken E. coli cultures. *Microb. Cell Fact.* **12**:73.

Unthan S, Radek A, Wiechert W, Oldiges M, Noack S. 2015. Bioprocess automation on a Mini Pilot Plant enables fast quantitative microbial phenotyping. *Microb. Cell Fact.* **14**:216.

Vaara M. 1992. Agents that increase the permeability of the outer membrane. *Microbiol. Rev.* **56**:395–411.

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

Veglio F, Beolchini F, Ubaldini S. 1998. Empirical models for oxygen mass transfer: A comparison between shake flask and lab-scale fermentor and application to manganiferous ore bioleaching. *Process Biochem.* **33**:367–376.

Veselá AB, Rucká L, Kaplan O, Pelantová H, Nešvera J, Pátek M, Martínková L. 2016. Bringing nitrilase sequences from databases to life: the search for novel substrate specificities with a focus on dinitriles. *Appl. Microbiol. Biotechnol.* **100**:2193–2202.

Vester A, Hans M, Hohmann HP, Weuster-Botz D. 2009. Discrimination of riboflavin producing *Bacillus subtilis* strains based on their fed-batch process performances on a millilitre scale. *Appl. Microbiol. Biotechnol.* **84**:71–76.

Viaggi D, Mantino F, Mazzocchi M, Moro D, Stefani G. 2012. From Agricultural to Bio-based Economics? Context, State of the Art and Challenges. *Bio-based Appl. Econ.* **1**:3–11.

Vincentelli R, Cimino A, Geerlof A, Kubo A, Satou Y, Cambillau C. 2011. High-throughput protein expression screening and purification in *Escherichia coli*. *Methods* **55**:65–72.

Vos T, de la Torre Cortés P, van Gulik WM, Pronk JT, Daran-Lapujade P. 2015. Growth-rate dependency of de novo resveratrol production in chemostat cultures of an engineered *Saccharomyces cerevisiae* strain. *Microb. Cell Fact.* **14**:133.

Weber W, Fussenegger M. 2011. Emerging biomedical applications of synthetic biology. *Nat. Rev. Genet.* **13**:21.

Weiss A, Delproposto J, Giroux CN. 2004. High-throughput phenotypic profiling of gene-environment interactions by quantitative growth curve analysis in *Saccharomyces cerevisiae*. *Anal. Biochem.* **327**:23–34.

Wellenbeck W, Mampel J, Naumer C, Knepper A, Neubauer P. 2017. Fast-track development of a lactase production process with Kluyveromyces lactis by a progressive parameter-control workflow. *Eng. Life Sci.* **17**:1185–1194.

Welsh JP, Rauscher M a., Bao H, Meissner S, Han I, Linden TO, Pollard JM. 2015. Domain antibody downstream process optimization: High-throughput strategy and analytical methods. *Eng. Life Sci.* **16**:133–142. http://onlinelibrary.wiley.com/doi/10.1002/elsc.201400255/abstract.

Weuster-Botz D, Hekmat D, Puskeiler R, Franco-Lara E. 2006. Enabling technologies: Fermentation and downstream processing. *Adv. Biochem. Eng. Biotechnol.*

105:205–247.

Weuster-Botz D, Stevens S, Hawrylenko A. 2002. Parallel-operated stirred-columns for microbial process development. *Biochem. Eng. J.* **11**:69–72.

Wilming A, Bähr C, Kamerke C, Büchs J. 2014. Fed-batch operation in special microtiter plates: A new method for screening under production conditions. *J. Ind. Microbiol. Biotechnol.* **41**:513–525.

Wittmann C, Kim HM, John G, Heinzle E. 2003. Characterization and application of an optical sensor for quantification of dissolved O 2 in shake-flasks. *Biotechnol. Lett.* **25**:377–380.

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

Xu L, Li S, Ren C, Cai Z, Lin Z. 2006. Heat-inducible autolytic vector for high-throughput screening. *Biotechniques* **41**:319–323.

Xu P, Clark C, Ryder T, Sparks C, Zhou J, Wang M, Russell R, Scott C. 2017. Characterization of TAP Ambr 250 disposable bioreactors, as a reliable scale-down model for biologics process development. *Biotechnol. Prog.* **33**:478–489.

Zarschler K, Witecy S, Kapplusch F, Foerster C, Stephan H. 2013. High-yield production of functional soluble single-domain antibodies in the cytoplasm of *Escherichia coli*. *Microb. Cell Fact.* **12**:97.

Zhao F, Yu J. 2001. L-asparaginase release from *Escherichia coli* cells with K2HPO4 and Triton X100. *Biotechnol. Prog.* **17**:490–494.

Zimmermann HF, Anderlei T, Büchs J, Binder M. 2006. Oxygen limitation is a pitfall during screening for industrial strains. *Appl. Microbiol. Biotechnol.* **72**:1157–1160.

Zimmermann HF, Rieth J. 2006. A Fully Automated Robotic System for High Throughput Fermentation. *JALA - J. Assoc. Lab. Autom.* **11**:134–137.

List of presentations at international conferences

Glauche F, Knepper A, Theuer L,Heiser M, Wollny F, Bigesse S, Neubauer A, Arain S, John GT, Aschoff J, Stehlik B, Schmidt I, Violet N, King R, Goelling D, Raab A, Kiesewetter G, Nolte R, Neubauer P. **Consistent high throughput bioprocess development under process relevant conditions** 3rd BioProScale Symposium, Berlin, Germany, 2 April 2014

Glauche F, Knepper A, Theuer L, Heiser M, Wollny F,Bigesse S,Neubauer A,Arain S, John GT, Aschoff J,Stehlik B,Schmidt I,Violet N,King R, Goelling D, Raab A, Kiesewetter G, Nolte R, Neubauer P. **Automated development of recombinant bioprocesses - from vision to mission**. 16. European Congress of Biotechnology, Edinburgh, UK. 13-16 July 2014.

Glauche F, Neubauer P**. Scale-down, parallel bioreacors and cultivation strategies for accelerated bioprocess development.** BioProduction Congress, Dublin, UK. 21 October 2016.