Scale-Down of oxygen supply in bioprocess development with *Corynebacterium glutamicum*

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Paper II[2]:

Käß, F. Junne, S. Neubauer, P. Wiechert, W. & Oldiges, M. **Process inhomogeneity leads to rapid side product turnover in cultivation of *Corynebacterium glutamicum***, *Microb. Cell Fact.* **13,** 6 (2014).

Paper III[3]:

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List of presentations at international conferences

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ACHEMA 2012, Frankfurt (presentation): "**Microtiter scale determination of growth and productivity in *Corynebacterium glutamicum***", F. Käß, A. Prasad, W. Wiechert, M. Oldiges

BioProScale 2012, Berlin (presentation): "**Effects of oxygen transfer limitation and inhomogeneous supply in *Corynebacterium glutamicum***", F. Käß, M. Oldiges

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1 Abstract

Corynebacterium glutamicum has large-scale industrial applications in the production of amino acids, *e.g.* L-lysine, and serves as a platform organism for new products. Challenges arise because production scales for biological bulk products often reach reactor working volumes of several hundred cubic meters, while strain evaluation and process development is based on lab scale assessments. Oxygen supply is a frequent driver of scaling complications. This dissertation introduces scale-down methods for oxygen transfer assessments, and demonstrates them on *C. glutamicum* ATCC13032 (wildtype) and DM1933 (L-lysine producer).

The first topic of this work is the development of an oxygen transfer screening in a down-scaled microtiter plate format. The method is demonstrated by characterizing the impact of oxygen supply limitation on process yields, side product secretion, and growth behavior in a batch process. High process robustness against oxygen supply limitation is observed, and optimal supply conditions are identified. The second topic is the application of established two-compartment scale-down setups for analysis of inhomogeneous oxygen supply in fed-batch environments. The studies demonstrate a remarkable robustness of *C. glutamicum* against oscillating oxygen and substrate availability, and expose some of the mechanisms which underlie the metabolic flexibility, *e.g.* intermediate side product formation/reabsorption between reactor zones. Multi-omics analysis is performed to gain a deeper understanding of the physiological properties behind inhomogeneity resistance.

Application of the presented methods has demonstrated that *C. glutamicum* is highly adjusted to oxygen transfer limitation in homogeneous and inhomogeneous process environments, which is an asset for industrial commodity bioprocess development. Going forward, a model workflow is proposed to apply the established scale-down methods for other aerobic

bioprocess developments, which could improve the resulting process performance and minimize risks for failure during scale-up.

Kurzzusammenfassung

Corynebacterium glutamicum wird zur industriellen Aminosäureproduktion eingesetzt, z.B. für L-Lysin, und dient als Platform-Organismus für weitere Produkte. Bioprozesse können einen Maßstab von mehreren hundert Kubikmetern erreichen. Den Ausgangspunkt bilden jedoch Versuche im Labormaßstab, was zu Komplikationen führen kann. Einer der maßgeblichen Faktoren ist Sauerstoffversorgung. Diese Dissertation führt Methoden zur Maßstabsverkleinerung für die Untersuchung von Sauerstoffversorgung ein, und demonstriert sie an den Stämmen *C. glutamicum* ATCC13032 (Wildtyp) und DM1933 (L-Lysin Produzent).

Das erste Element dieser Arbeit ist die Entwicklung eines Sauerstofftransfer-Screenings im maßstabsverkleinerten Mikrotiterplattenformat. Es erfolgt eine Charakterisierung des Einflusses von Sauerstofflimitation auf Prozessausbeuten, Nebenproduktbildung und Wachstumsverhalten im Batch-Prozess. Eine hohe Prozess-Robustheit gegen Sauerstofflimitation wird festgestellt, und optimale Versorgungsbedingungen werden identifiziert. Als zweites Thema werden etablierte Zweikompartiment-Reaktoren für Maßstabsverkleinerung inhomogener Sauerstoff- und Substratversorgung in Fed-Batch Prozessen angewendet. Im Ergebnis zeigt *C. glutamicum* eine hohe Robustheit bei oszillierender Sauerstoff- und Substratverfügbarkeit. Zugrundeliegende Mechanismen werden offengelegt, z.B. Bildung und Wiederaufnahme von Nebenprodukten in einzelnen Reaktorzonen. Multi-Omics Analysen liefern dazu tiefere Einblicke in die Grundlagen dieser Robustheit.

Die präsentierten Methoden zeigen, dass *C. glutamicum* stark an Sauerstofflimitation und inhomogene Prozessbedingungen angepasst ist. Dies stellt einen Vorteil für die Anwendung in Bioprozessen von großem Maßstab dar. Für die Zukunft wird ein Modellprozess zur Verbesserung aerober Bioprozessentwicklung vorgeschlagen, der Maßstabsverkleinerungen einschließt. Dies kann zur Verbesserung der Performance neuentwickelter Bioprozesse führen, und die Risiken für Fehlschläge bei der Maßstabsvergrößerung minimieren.

2 Introduction

2.1 Motivation

Industrial bioprocess development gets more important with every new demand for a chemical, pharmaceutical or nutrition product that arises within the world market. One of many driving factors is that industrial biotechnology needs to replace existing petrochemical process chains, because regenerative sources will eventually be without alternative. Higher biocompatibility of the substrates is another of many reasons why commodity chemicals from biotechnological origin are often preferred- not only in the field of food, feed and pharmaceuticals. However, bioprocess development has some unique risks which have to be mitigated in order to create and maintain reliable process chains.

One challenge is that using biological entities for production requires ambient, controlled reaction environments, because correct operation of the cell factory's enzymatic machinery is bound to physical and chemical constraints. Furthermore, carbon fluxes along metabolic networks can easily be redirected if a necessary compound gets limiting or regulation is triggered, so that futile reactions reduce the product yield if there is an environmental disturbance. Therefore, bioprocesses are hard to optimize. It is necessary to find the best strategies for identification of ideal bioprocess conditions, and to match the biological demands with technical feasibility during process development.

One of the most critical parameters for many bioprocesses is oxygen supply, because the low solubility and high metabolic uptake of molecular oxygen can make precise in-process-control very demanding. Failing bioprocess development is often caused by neglected oxygen supply requirements during research, development, and transfer to final process scale. This thesis is an approach for comprehensive consideration of oxygen supply requirements during bioprocess development, and aims to supply the necessary tools to deal with its impact in a typical aerobic development chain. The organism which is

assessed, *C. glutamicum*, and the established techniques should serve as an example how to improve aerobic bioprocess development. The discussion aims to point out the scope, validity, limitations, and combined added value for the presented methods. It also aims to point out the potential for transfer of the practical conclusions to new studies which consider oxygen supply impact during bioprocess development.

2.2 Background: challenges in bioprocess development

Typical strategies that are applied in the development of bioprocesses should be considered as a context for the presented results.

2.2.1 Common strategies

Biological systems express complex and highly regulated behaviors, which are activated by multiple external triggers. In the ideal case, biological systems for industrial application are adjusted to technically feasible bioreactor conditions, which can be one of the targets for rational strain design. However, due to biological complexity there is always the potential for unwanted metabolic reactions, which can have negative impact on growth or productivity in a bioprocess. Avoiding unwanted reactions means avoiding the respective trigger conditions. Therefore, physical and chemical control of reaction environments is necessary, and suitable parameter ranges must be defined for every bioprocess. Process development can be regarded as the pursuit of fixed, suitably characterized process parameter ranges, which should be determined as easily and quickly as possible. This ultimately results in a process validation with a subsequent control strategy.

A major complication for designing optimal processes is that bioprocess environments often change throughout the development phases. System properties like process scale or reactor type must be adjusted based on economic constraints, leading to repeated transfers between development stages. This means that not all environmental process parameters can be maintained as constants, even if sophisticated transfer strategies are applied. Depending on the robustness of the biological entity, any of these changes may pose a threat to process performance. Industrial biotechnology has devised strategies how to deal with this development challenge. The applied strategy is set according to the target application.

High-margin/ high-regulation fields, *e.g.* the pharmaceutical industry, try to keep the degree of process changes after initial development as low as possible. The intention is to minimize the risk of performance loss, while maximizing the development speed for earliest possible process validation. This usually happens at the expense of process efficiency, because optimization is limited to the early research phase, and scaling effects are neglected. A corresponding method to this strategy is to apply numbering-up instead of scaling-up[4], *i.e.* increasing the number of small-scale production units instead of increasing the production unit size. The roller bottle technology for mammalian cell culture[5] is a typical example for this strategy, which has a high prevalence even though cell carrier technologies have long been established.

In contrast, low-margin/ medium regulation fields, *e.g.* commodity chemicals and feed products, aim to reach the ideal economy of scale. The main risk is that the process may not be competitive in the final reactor system, which means that the price of substrates and energy may exceed the profit margin of the product. Therefore, maximal process efficiency is targeted through repeated optimization steps during development, *e.g.* during the transfer from shake-flasks in the initial development stage to stirred tanks of several hundred cubic meters in the commercial stage. This requires repeated bioprocess transfers with multiple adaptations in the reaction environments, and constitutes one of the most challenging tasks in bioprocess engineering.

2.2.2 Reaching ideal economy of scale

The main challenges of large-scale, low-margin bioprocess development are the transfer from initial process-related data acquisition (*e.g.* picking the most promising mutant strain from a screening) to lab scale process development, and the transfer from lab to production scale. These steps introduce various changes to the bioprocess which might act as harmful triggers for biological regulation. They are therefore often separated into multiple steps with separate optimization cycles. In the following, pitfalls for this strategy from the process

development perspective are highlighted. The chosen example corresponds to a large-scale fermentation optimization with a product-secreting microorganism, which is a typical challenge faced in industrial amino acid production (*e.g.* L-lysine). Development works in initial strain design and downstream treatment are assumed to be the starting and end point of development, respectively, and are therefore not part of the assessment:

In the beginning of a development chain, a number of potential production strain candidates have been generated that show promising metabolic traits. Strains may have been isolated by selection for successful target gene transfer (*e.g.* antibiotic resistance), or by more sophisticated approaches, for example product-specific induction of fluorescence with intracellular biosensors [6]. This is followed by a screening procedure in order to select the most suitable strains.

Screening is typically understood as the process of comparing large pools of strains or cultivation parameters under simplified process conditions. The target of screening is to narrow down the options for subsequent bioprocess optimization, which takes up more resources and should only be performed with the most promising strains or conditions. Systems for screening are usually small-scaled, *e.g.* shaken microtiter cultivation or shake flasks, due to the high throughput of strain generation procedures and vast parameter spaces like media composition. Screenings are performed based on a limited set of indirect performance parameters, *e.g.* fluorescence or sufficient viability to indicate target gene transfer. More sophisticated approaches may provide first quantitative performance information, *e.g.* product yield per substrate. Ideally, the screening conditions anticipate elements of the later production scale, *e.g.* industrially relevant media preparations or culture times. Eventually, the most promising strain candidates or conditions are identified. This is the usual output of a screening stage, which is then used as a basis to proceed with process optimization.

After screenings, potential production strain candidates have to be compared based on their performance under feasible target process conditions. Typical

biological traits for comparison can be productivity, viability, robustness against long culture times, susceptibility to contaminants, or other general criteria for the desired route of further process development. Several traits may also be combined into more complex target criteria, *e.g.* space-time-yield in a target reactor system. Process optimization requires a controlled process environment, *e.g.* a stirred tank reactor. Also, the reactor system will typically show similarity to commercial scale reactors, keeping in mind that the subsequent steps of process development should be kept as simple as possible. This means that the strain candidates are compared in a high number of typically Design-of-Experiments (DoE)-based stirred-tank reactor cultivations with the respective analytics. The result will be a balanced ranking of the producer strain candidates under optimal conditions, and facilitate identification of the best-suited strain to proceed in the process development chain.

At the end of the lab-scale optimizations, the fixed bioprocess with best-performing strain and optimized process conditions will be subjected to incremental increases in process scale. These scale-up steps are usually twofold to tenfold, depending on the expected robustness of the bioprocess. Scale-up criteria are selected based on the available systematic knowledge about the biological entity. Example criteria can be similarity of dissolved oxygen or power input per volume in case of critical oxygen dependency. The aim of the scale-up is to maintain the desired productivity through repeated optimization. Necessary adaptations in cultivation parameters are typically made whenever a loss of productivity is observed, or when technical or commercial restrictions cannot be avoided.

Application of the described process development strategy means that the target of the development phases changes in three stages. Initial screenings or preliminary studies aim to pick only the most promising strains or parameter settings from a high amount of candidates. Lab-scale development optimizes the process as far as possible. Subsequent scale-up aims to maintain productivity from the optimized process over repeated transfer steps. The

advantage of this development strategy is that only high-performing bioprocesses can reach the final reactor stage, because all others will be rejected in previous stages. The disadvantage is that the changing targets of the development phases can lead to a disconnection between the initial strain selection and the commercial scale process requirements, so that ideal strains or conditions are wrongly discarded before the final development stage. For example, this could mean that a strain that has ideal robustness against unavoidable stress factors of the industrial scale never reaches large reactors, because it is discarded when inferior product yield is observed under screening conditions. A strain like this might be the optimal candidate for the final reactor scale, but would never pass initial stages of process development. Therefore, it is highly advisable to refine the typical development strategy with more consistent approaches that are aimed at the final commercial production conditions. This thesis is an approach for such a refinement (see 2.4).

2.3 Background: metabolism of *C. glutamicum*

C. glutamicum is a highly characterized industrial microorganism with a broad range of applied production strains for many commodity products, which is most thoroughly documented in the recent "Handbook of *Corynebacterium glutamicum*"[7]. In the following, several relevant properties for the presented studies are summarized.

2.3.1 Respiration and substrate spectrum

Metabolic properties of *C. glutamicum* and other members of the genus *Corynebacterium* have been investigated extensively in the past due to their relevance in industry and medicine. This comprises a broad knowledge about the respiratory chain, which was summed up by Bott and Niebisch[8]. There is a broad spectrum of substrates which can be metabolized[9], although the industrially relevant media preparations are mainly based on sucrose from cane or starch hydrolysates as carbon source[7].

C. glutamicum is an aerobic respiratory organism. It utilizes both substrate level phosphorylation and oxidative phosphorylation as a source of energy for growth and specific biosyntheses. Oxidative phosphorylation provides the surplus of metabolic energy and is therefore crucial for growth. There is also anaerobic or micro-aerobic potential for biotransformations, in which *C. glutamicum* can be used as a catalyst. However, this is a less common application, because the growth and self-maintenance of the organism is one of the most attractive features for large-scale industrial application. This means that availability of oxygen is a prerequisite for sustainable cultures that rely on active growth or maintenance metabolism. Concerning oxygen metabolism, there is a high degree of flexibility in the branched breathing chain of *C. glutamicum*, which likely provides options for adjusting to different levels of oxygen availability. The fundamentals of these oxygen-dependent properties of *C. glutamicum* have been summed up in a publication of Bott and Niebisch in 2003[8].

There is a wide range of substrates which can be oxidized by *C. glutamicum* in order to facilitate electron transfer to reducing equivalents, which facilitates the maintenance of metabolic function in many biochemical environments. Several of these substrates are also known side-products of the organism, which get secreted – and in some cases reabsorbed - most pronouncedly under non-ideal growth conditions. The most prominent example of side product secretion occurs under oxygen supply limitation, when organic acids are secreted as side products, *e.g.* described for L-lysine production by Ensari and Lim[10]. The substrate spectrum of *C. glutamicum* also contains a much wider scope of sugars, acids and alcohols, which is facilitated by a comparatively versatile set of enzymes for uptake and breakdown of these compounds[7]. The combination of a broad substrate scope and versatile oxygen-dependent metabolism allows a high flexibility of application in bioprocesses. In order to avoid energy loss through side product generation, most industrial applications of *C. glutamicum* are centered on fed-batch processes with limited carbon source. At homogeneous conditions, this ensures that metabolic activity is limited, so that oxygen supply limitation and side product generation can be avoided.

2.3.2 Metabolic stress

There are several kinds of stress which are relevant for industrial microorganisms, most prominently in large-scale process environments. These can get particularly critical in inhomogeneous cultivation systems, where individual cells face rapidly changing, *i.e.* oscillating stress conditions. The most process-relevant stress factors for microorganisms with a highly active metabolism are hyperosmotic stress and oxygen or substrate supply limitation, because these are directly related to feed zone conditions in industrial substrate-limited environments. *C. glutamicum* production strains are comparatively well-adjusted to osmotic stress in a reasonable range, as was characterized for an L-lysine process by Guillouet *et al.*[11]. On the other hand, oxygen and substrate supply limitation has direct impact on the substrate

conversion and biomass yield, *i.e.* increases the energy demand for maintenance metabolism[7].

There are also other kinds of metabolic stress, *e.g.* shear stress or pressure gradients, against which *C. glutamicum* does not seem to be susceptible in a bioprocess-relevant range. Acidity of medium can also play a role: Follmann *et al.* report that growth is negatively influenced below pH 6[12]. Carbon dioxide saturation can be considered as another potential stress factor, because some metabolic reactions rely on sufficient availability, while elevated levels of CO_2 can also have other regulation effects[13]. Except for oxygen and substrate limitation, all these stress factors can be considered as relatively unproblematic in typical production conditions, which are characterized by controlled reaction environments in actively agitated reactors.

2.3.3 Production of L-lysine

The current annual production of L-lysine by mutants of *C. glutamicum* is in the range of 2.2 million tons, which makes it one of the leading products generated by biotechnological means[14]. L-lysine is an essential amino acid for feed applications, and L-lysine producing *C. glutamicum* strains have been established as early as 1956[15,16] with continuous improvements in strains and processes ever since then. The high competitiveness of the organism for amino acid production has to do with its secretion capacity for amino acids, as well as highly efficient central metabolic pathways. L-lysine is synthesized from the tricarboxylic acid (TCA) cycle intermediate product oxaloacetate and various precursors ($NADPH+H^+$, pyruvate, glutamate/NH_4^+) through a sequence of enzymatic reactions, starting with L-aspartate[7]. Robust strains have been constructed with various modifications, one of which is the non-auxotrophic *C. glutamicum* DM1933[17] that is applied in this work. The modifications of this strain comprise several deletions, duplications and new insertions which facilitate overproduction and secretion of L-lysine (Δ*pck pyc*(P458S) *hom*(V59A), 2 copies of *lysC*(T311I), 2 copies of *asd*, 2 copies of *dapA*, 2 copies of *dapB*, 2 copies of *ddh*, 2 copies of *lysA*, 2 copies of *lysE* derived

from Wildtype *C. glutamicum*). The strain has comparatively robust growth and production behavior and can be regarded as a model system for currently applied industrial mutants.

2.4 Structure of this work

Applying a scale-down approach means decreasing the size of a system for gaining a benefit over the original scale. In process engineering, this usually has the target to save time and resources by obtaining process performance data without having to run full-scale experiments. This thesis introduces two scale-down elements for oxygen supply assessment into bioprocess development. Both are performed with *C. glutamicum* ATCC13032 (wildtype) as a proof-of-concept for the method itself, and with the L-lysine producer *C. glutamicum* DM1933 as an application example for an industrially relevant process.

First, oxygen transfer assessment is implemented into a screening workflow for increased throughput[1]. This facilitates more thorough assessment of the oxygen supply impact as a basis for further process development. This new scale-down methodology for a process parameter assessment is discussed in section 4.1. Second, the inhomogeneity of large-scale oxygen supply is brought to the lab-scale in two compartment reactors. Two manuscripts contain a general assessment of the wildtype strain[2] and a more detailed analysis of the L-lysine process[3]. This provides more information about the process robustness and scalability. The inhomogeneity scale-down is discussed in section 4.2.

Subsequently, the combined scope of results is discussed in section 4.3. The conclusion for integration of the presented scale-down elements into bioprocess development workflows is drawn. As an outlook, the section contains a proposed model workflow for consistent consideration of oxygen supply requirements throughout the development phases.

3 Results

3.1 List of publications with authors' contributions

Paper I[1]: Rapid assessment of oxygen transfer impact for *C. glutamicum*

Käß F, Prasad A, Tillack J, Moch M, Giese H, Büchs J, Wiechert W, Oldiges M.

Authors' contributions: FK designed the experiments, developed and validated the experimental methods and prepared the manuscript. AP and FK performed and evaluated the experiments. JT developed the data processing for volume-dependent calibration in MATLAB. MM and HG conducted analytical process development and performed tests. JB is the scientific supervisor in development of the applied RAMOS analytics. MO and WW initiated the project. MO is the principal investigator, supported with conception and manuscript preparation. All authors read and approved the final manuscript.

Paper II[2]: Process inhomogeneity leads to rapid side product turnover in cultivation of *Corynebacterium glutamicum*

Käß F, Junne S, Neubauer P, Wiechert W, Oldiges M.

Authors' contributions: FK designed the experiments, developed and validated the experimental methods and prepared the manuscript. FK and SJ performed and evaluated the experiments. PN and SJ provided the previously published two-compartment reactor setup, helped with conception of experimental methods, and participated in conduction of experiments. MO and WW initiated the project. MO is the principal investigator, supported with conception and manuscript preparation. All authors read and approved the final manuscript.

Paper III[3]: Assessment of robustness against dissolved oxygen/substrate oscillations for *C. glutamicum* DM1933 in two-compartment bioreactor

Käß F, Hariskos I, Michel A, Brandt HJ, Spann R, Junne S, Wiechert W, Neubauer P, Oldiges M.

Authors' contributions: FK designed the experiments, developed and validated the experimental methods and prepared the manuscript. IH, RS and FK performed and evaluated the experiments. AM conducted the transcriptome analysis. HJB designed and built the two-compartment reactor which was used for most cultivations. PN and SJ provided the previously published two-compartment reactor setup for reference, which the described reactor is based on, helped with conception of experimental methods, and participated in conduction of experiments. MO and WW initiated the project. MO is the principal investigator, supported with conception and manuscript preparation. All authors read and approved the final manuscript.

3.2 Scale-down of oxygen supply assessment into a screening system

Method development

Paper I[1] starts with an assessment of the importance of oxygen supply in bioprocess development. It is emphasized that each commercial bioprocess development step has unique requirements and characteristics in oxygen supply. Shaken bioreactor systems form the typical setup of early strain and process characterization. The rationale of using the BioLector® as a development platform for fast oxygen supply assessment is presented as a combination of analytic versatility and easy bioprocess scale-up. Also, the system has a broad range of oxygen transfer capacities that can be reached, with a very high peak value of over 100 $mmol \cdot L^{-1} \cdot h^{-1}$ that spans the feasible transfer rates for stirred tank cultivation. The high degree of parallelization in bioprocess conduction and analysis means that scale-down of bioprocess lab assessments into the BioLector® is a promising approach. The target of the development in the presented study is therefore to set up and test the assessment of oxygen supply impact in the BioLector® system by characterizing oxyen supply impact on *C. glutamicum* as a model organism for industrially relevant conditions. This should provide speed, data abundance and easy scale-up potential. Main challenges are identified in the necessary online analytics that have to be adjusted to said purpose.

The functionality of the oxygen transfer screening approach is detailed in Supplementary Fig. 6[1]. Utilized elements are the regulation capacity of headspace oxygen content in the BioLector® and the availability of unpublished characterization data for flowerplate cultivation geometries at various filling volumes (sulfite oxidation-based determination of maximum oxygen transfer capacity (OTR_{max}), m2p-Labs Baesweiler). By using HENRY's law. the oxygen transfer capacity is adjusted with a combination of headspace oxygen control and filling volume changes.

Experimental design was performed as a pattern of batch cultivations that spans feasible oxygen supply ranges. Cultivation conditions were set as a batch process at fixed agitation. Samples were prepared by a liquid handling station, which improves reproducibility due to standardized sample preparation times (Methods section[1]). Replicate samples for offline measurement were pooled to achieve sufficient volume for HPLC-based analytics of side products. Reference cultivations in a MicroTiter Plate Respiration Activity Monitoring System (MTP-RAMOS) were performed at identical cultivation conditions as in the BioLector® (flowerplate geometry, agitation type, culture inoculation density and media[1]).

Data processing & method verification

The main challenge for the study was identified in the analytical codependency for online analytics of *C. glutamicum* cultures (see Method section and Supplementary Fig. 7[1]). The cultivation at changing filling volume is not a typical application form of the BioLector®, which explains why this unusual analytical behavior has not been reported previously. Risk mitigation is presented as a MATLAB calibration procedure for backscatter-based biomass determination. Development of this technique was achieved by non-linear interpolation of the parameter space biomass - filling volume- backscatter from reference measurements. The underlying plots of the interpolated function are depicted in Fig.1[1]. Stationary biomass samples were measured in the BioLector® as reference for the interpolation. Accuracy of the biomass determination was evaluated as sufficient for a screening application, although very low biomass and filling volume carry an increased risk of measurement inaccuracy (Fig.1[1]). It is speculated that this may be caused by increased surface reflection at low turbidity. As a result, cultivation experiments in the conducted screening were performed with multiple replicates to minimize measurement errors.

Method verification was performed by comparing oxygen-limited BioLector® cultivations to similar process conditions of filling volume and headspace oxygen content in MTP-RAMOS. Both systems returned similar oxygen

supply limitation times when compared on the basis of OTR (MTP-RAMOS) and DO (BioLector®) (Fig. 2B[1])... Oxygen-limited growth behavior was observed in clear dependence on the volume-adjusted maximum oxygen transfer capacity (OTR_{max}) (Fig. 2A[1]). Distinct peak values of oxygen transfer capacity could be identified for each assessed filling volume. The observed values for OTR_{max} in MTP-RAMOS suggest a systematic deviation to BioLector® characterization data, which were obtained by sulfite oxidation reference experiments (Fig. 2A[1]).

It is concluded that oxygen-limited cultivation of *C. glutamicum* in the BioLector® yields characteristic plateau-shaped oxygen transfer conditions with distinct OTR_{max} for each filling volume, which is the crucial requirement for conduction of the oxygen transfer screening.

Metabolic analyses

The screening was performed by scattering cultivations with distinct OTR_{max} in the range from 0.8 to 35 $mmol \cdot L^{-1} \cdot h^{-1}$. Application of highly limited oxygen transfer conditions showed the dependency of growth rate and biomass yield on OTR_{max}, which is clearly demonstrated by growth curve comparison in a single flowerplate with variation in filling volumes for *C. glutamicum* wildtype (Fig.3A and 3B[1]). The growth curves follow an approximately linear profile after initial batch growth, meaning that there is a decreasing growth rate during the linear phase, and that biomass increase is roughly proportional to the oxygen that is supplied to the culture. Consequently, lower OTR_{max} leads to extended linear growth phases, and to increased process time until the stationary phase is reached. Optode data facilitates the identification of the relationship between growth and oxygen supply limitation (Fig. 3A[1]), which shows a delay of several hours between the full consumption of dissolved oxygen ($OTR = OTR_{max}$) and the change of growth characteristics from a near exponential to a linear profile. Sampling of the culture supernatant at synchronized timepoints shows the increasing degree of organic acid side product secretion with oxygen-limited growth (Fig. 3C[1]). The identified side products lactate, succinate and acetate are formed in a seemingly conserved

sequence and later reabsorbed when carbon source becomes limiting (Fig. 3D[1]).

Secretion of the side products is necessary to maintain redox balance in an environment of oxygen limitation, where NAD^+ is regenerated through enzymes as lactate dehydrogenase instead of oxidative phosphorylation. Later reabsorption of organic acids as carbon source means that lower quality substrate is used for growth, which in turn can lead to irregular growth patterns, which is observed for very low oxygen transfer capacities. In summary, metabolic energy is clearly reduced by the lack of oxidative phosphorylation, which is assumed to be the driver of decreased growth at oxygen supply limitation. The biomass yields can be plotted over OTR_{max} , which gives the most striking visualization of the detrimental effect of oxygen supply limitation for *C. glutamicum* wildtype (Figure 1, not shown in publication). Biomass yield roughly follows a saturation curve, meaning that oxygen supply limitation has an increasingly detrimental effect below a threshold value of ca. 12 $mmol \cdot L^{-1} \cdot h^{-1}$ for the wildtype strain.

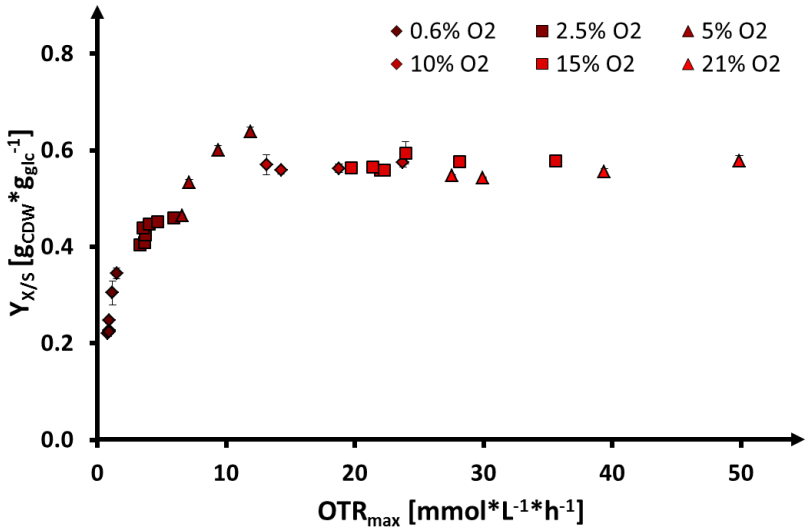

Figure 1: biomass yield on substrate for *C. glutamicum* ATCC13032 (wildtype) as a function of maximum oxygen transfer in batch process, as determined in newly developed oxygen transfer screening procedure (BioLector®)

After analysis of wildtype behavior, the screening was applied to generate a broad set of process performance data for the L-lysine producer strain *C. glutamicum* DM1933 (Fig. 4A, Fig. 4B, Fig. 5A and Fig. 5B[1]). One result was that the optimum OTR_{max} lies above 22.8 mmol·L^{-1}·h^{-1}, which results in batch growth kinetics that are unaffected by phases of oxygen transfer limitation (Fig. 4A[1]). In contrast, the sufficient OTR_{max} for maximum biomass yield and product yield is in the range of 15 mmol·L^{-1}·h^{-1}, meaning that the intermediate range has a slowing effect on growth without loss of yield. The relationship between OTR_{max} and process yield is consistent for both biomass and product (Fig. 5A and Fig. 5B[1]). Biomass yield roughly follows a saturation curve, meaning that oxygen supply limitation has an increasingly detrimental effect below the threshold value of 15 mmol·L^{-1}·h^{-1}. Product yield is largely unaffected by OTR_{max}. An exception to this behavior can be found in the product formation at very low oxygen transfer, where a slight increase in L-lysine formation was observed. This growth decoupling of product formation is in accordance with findings of Neuner *et al.*[18]. In this case, it demonstrates how a performance screening may serve to identify new

bioprocess working points and optimization potential. However, it should be noted that the respective product yield was obtained after a highly prolonged culture time, because low OTR lead to a six fold higher duration until full carbon consumption. It is therefore unlikely that the newly identified L-lysine production optimum is a desirable target for large-scale application due to drastically reduced space-time yield.

Scale-Up into lab-scale stirred tank

Some of the cultivations that were performed in Paper I[1] were also assessed in a laboratory scale (5 L) stirred tank reactor. The target was a direct scale-up of OTR_{max} conditions in order to verify if process performance could be reproduced. The scale-up was challenged by the identified variation in OTR_{max} data between sulfite oxidation (BioLector® characterization data) and balance-based OTR_{max} (RAMOS), because the most informative process performance effects that were identified in the screening occurred at extremely low oxygen supply. These conditions could not be verified in MTP-RAMOS before scale-up because of time and resource constraints. Therefore, the scale-up was based exclusively on the sulfite oxidation reference data which were used as a basis for the screening. Low oxygen transfer in the stirred tank was achieved through lowering the gassing rate and stirring speed. Figure **2** and Figure **3** show that although a qualitatively similar behavior was observed concerning product yields at variation of OTR_{max}, there is still a high deviation between the actual process yields.

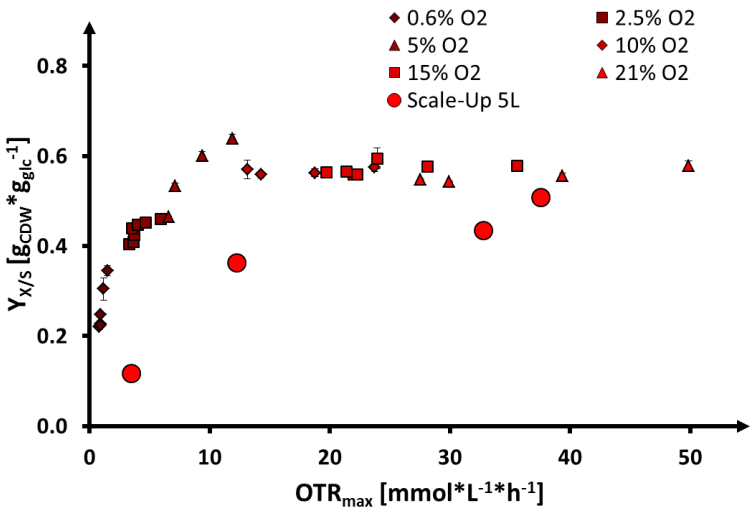

Figure 2: 5 L stirred-tank cultivation biomass yield on substrate (large circles) for *C. glutamicum* ATCC13032 (wildtype) as a function of maximum oxygen transfer capacity, comparison to data from newly developed oxygen transfer screening procedure (BioLector, see Figure 1)

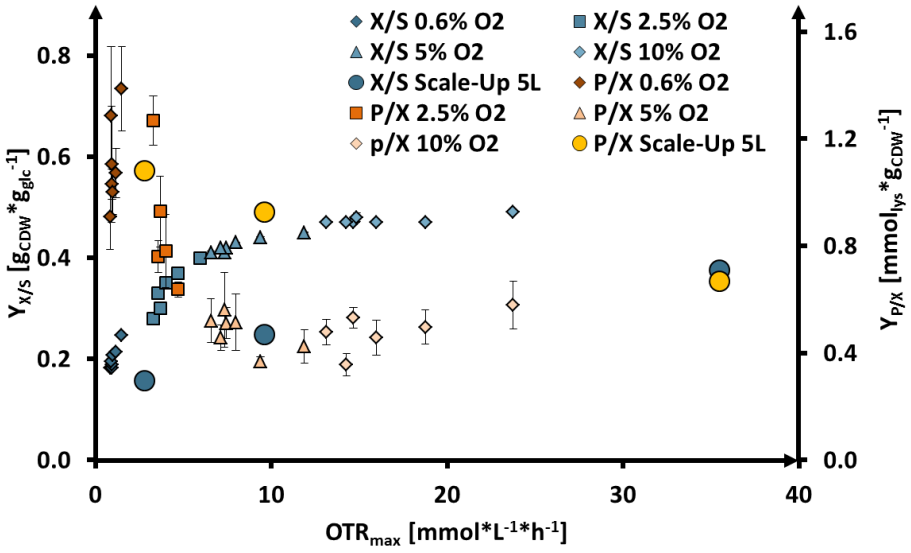

Figure 3: 5 L stirred-tank cultivation biomass yield on substrate (blue circles) and product yield on biomass (yellow circles) for *C. glutamicum* DM1933 (L-lysine producer) as a function of maximum oxygen transfer capacity, comparison to data from newly developed oxygen transfer screening procedure (BioLector, see Fig. 5[1])

It can be expected that the main influencing factor that causes the deviation in yields is indeed the method of OTR_{max} determination. The systematic

underestimation of OTR_{max} from balance-based approaches in comparison to sulfite oxidation has been demonstrated in Fig. 2[1]. This phenomenon may have an even higher impact in cultivations with very low OTR_{max}, which would mean that limitation in the BioLector® is less strong than OTR_{max} data from sulfite oxidation would suggest. This would explain the quantitatively different, yet qualitatively similar behavior that was observed in the uncorrected OTR_{max}-based comparison of BioLector® and stirred tank reactor.

3.3 Scale-down of oxygen transfer inhomogeneity into lab-scale

Method development

Paper II[2] and Paper III[3] introduce *C. glutamicum* cultivation in two-compartment reactors with stirred tank and plug flow reactor elements (STR-PFR) for analysis of process inhomogeneity in oxygen and substrate supply. Fed-batch is the cultivation mode of choice because of its high industrial relevance and susceptibility to mixing challenges. Paper II[2] is the first published application of STR-PFR reactors for *C. glutamicum* wildtype. Paper III[3] is a focused metabolic robustness assessment of the L-lysine producer strain *C. glutamicum* DM1933. Both studies share their general approach, in which STR-PFR cultivation at variable plug flow residence time is compared to homogeneous reference cultivations (stirred tank reactor). Oxygen supply limitation is achieved in the unaerated plug flow elements due to high metabolic turnover rates, whereas the stirred tank compartment is kept at aerobic conditions with sufficient dissolved oxygen at all times. Paper II[2] has the focus of characterizing basic metabolic phenomena that occur under inhomogeneous oxygen/substrate supply in a batch/fed-batch environment of *C. glutamicum* wildtype. It has a broad process scope that takes into account batch and fed-batch phases with a smaller emphasis of process yields. In contrast, Paper III[3] is a focused assessment of metabolic robustness that applies multi-omics analyses in an exponential fed-batch process. It is designed for targeted comparison of various process characteristics with the L-lysine producing *C. glutamicum* DM1933 in multiple replicates, including homogeneous reference cultivation in pilot scale. Process yields and reproducibility are the central elements of this second study, which applies highly extended exposure to oxygen supply limitation in the plug flow element for robustness assessment.

Reactor characterization

Defined process inhomogeneity was achieved by using two-compartment reactor systems with characterized backmixing behavior of the plug flow elements. Extrusion experiments[2] and tracer pulse-based determination of residence time distribution[3] were used to determine the mean residence time at defined pump circulation settings, and to verify plug flow behavior. The reactor for the wildtype study (Fig. 1[2]) is a specialized setup with the potential for gassing the plug flow element through static mixers, while the newly constructed unit for the L-lysine producer study (Fig.1[3]) was designed with the target of generating extended residence times in the plug flow compartment. Paper III[3] has an increased degree of biological process characterization, with studies on the speed of dissolved oxygen depletion (Fig. 2B[3]) and required biomass for reaching oxygen supply limitation (Results section[3]).

PFR-monitoring: immediate responses to step change conditions

Paper II[2] is based on a process with two phases: the batch and fed-batch phase (Fig.2[2]). In both phases, oxygen supply depletion leads to immediate limitation, as was demonstrated by the lack of dissolved oxygen and the reduced oxygen uptake in the plug flow compartment (Fig.3[2]). A concomitant lactate secretion was identified (Fig.4B[2]) that can be traced along the plug flow reactor, which is accompanied by a moderate pH drop (Fig.4A[2]). The extent of carbon fraction turnover during the fed-batch phase reaches very high extents, with the majority of glucose-carbon being converted into extracellular lactate within an oxygen-limited residence time of *ca.* 87 seconds (Fig.5[2]). The shorter plug flow residence time of 45 seconds has similar effects to a weaker extent (Fig.5A[2]). Glucose uptake of the wildtype was increased compared to aerobic conditions (Results section[2]). Determination of pool sizes for intracellular adenosine phosphates (AMP, ADP, ATP) and redox cofactors ($NAD(P)^+/NAD(P)H+H^+$) did not show any immediate response to oxygen supply limitation (Fig.6[2]). It is concluded that the regeneration of NAD^+ through lactate formation and excretion is the balancing factor that allows *C. glutamicum* wildtype to rapidly prevent any redox imbalance that might

occur under oxygen supply limitation. This way, substrate uptake and conversion are not negatively affected by changes in the redox levels (*e.g.* no inhibition of glyceraldehyde-3-phosphate dehydrogenase through unfavorable $NAD^+/NADH+H^+$ ratio).

PaperIII[3] contains a more drastic challenge of robustness against inhomogeneity of oxygen and substrate supply for the L-lysine producer strain. Acidification and side product secretion were observed at oxygen supply limitation, with the identified side products lactate, succinate and pyruvate (Fig.5[3]). Pyruvate was the only side product that did not follow the rapid secretion and reabsorption behavior, which had previously been observed for lactate in the wildtype study[2]. Although the residence time under oxygen supply limitation was extended to *ca.* three minutes, the extent of carbon fraction turnover from glucose into side products was around 25% maximum. Part of this effect may have been caused by lower biomass concentration in the L-lysine producer study, which decreases the speed at which dissolved oxygen becomes limiting. Acidification (Fig. 4[3]) was observed to be roughly proportional to biomass and feed amount. Substrate uptake was identified to continue at oxygen supply limitation, with a comparable rate to the maximum uptake capacity (see below). This indicates the robustness of the producer strain. Metabolic consequences of substrate excess/ oxygen supply depletion could be identified as an increase in cellular energy charge (Fig. 7[3]), although the robustness identified in redox cofactors of the wildtype study was also observed for DM1933.

Culture monitoring: robustness against process inhomogeneity

Paper II[2] does not identify changes in the growth behavior of *C. glutamicum* wildtype in response to plug flow residence times of up to 87 seconds, although the database for comparison is limited due to the small amount of cultivation replicates. Net respiration rates remained similar at homogeneous reference and inhomogeneous STR-PFR cultivation during the fed-batch phase (Fig.3[2]), which demonstrates a redistribution of oxygen uptake from limited to the unlimited zones of the reactor. In the batch phase, however, the oxygen

uptake rate of the inhomogeneous culture is depleted by roughly the volume fraction of the PFR in the two compartment system, which demonstrates that partial oxygen supply limitation cannot be compensated under batch conditions (Fig.3[2]). In spite of pH effects during oxygen supply limitation (Fig.4[2]), base requirement for the cultivations was not increased, which suggests reversibility of the acidification along with the metabolization of organic acids in aerobic zones. No net accumulation of organic acids was observed, which means that the inhomogeneous process is in fact a mixed substrate situation of primary carbon source and the intermediately formed side product lactate. In conclusion, the fed-batch mode is assumed to be a superior working condition for processes with *C. glutamicum* that are subject to process inhomogeneity of oxygen and substrate supply. It is deduced that the microbial organism has a native adaptation to rapidly changing microenvironments, because the reversible switch to fermentative metabolism does not impair growth.

Paper III[3] was performed with exponential fed-batch, and therefore has a very reproducible and stable process configuration that is aimed at identifying changes in metabolic maintenance demands and process yield changes. It does not contain a comparison to batch phase conditions. Nevertheless, the zonal redistribution of oxygen uptake in the fed-batch is clearly observable for a full cultivation period in Fig.3[3]. As in the wildtype study, the producer strain is capable of compensating partial oxygen supply limitation through increased breathing activity in the aerobic bulk zone. Targeted analysis of the maximum substrate uptake capacity (Fig. 6[3]) shows that oxygen/substrate inhomogeneity does not affect the functionality of the cell's substrate uptake system and metabolization speed. Instead, a maximum substrate uptake capacity of *ca.* 0.95 $g \cdot g^{-1} \cdot h^{-1}$ is maintained throughout the various degrees of inhomogeneity that were assessed in the study. This is an indication for similarity in enzyme availability and regulation. In a multi-omics approach, a set point after extended exposure to inhomogeneous cultivation conditions was compared to homogeneous reference cultures in a targeted proteome- and transcriptome-assessment for enzymes of the central metabolic pathways (Fig. 8[3]).

Regulation in response to process inhomogeneity could not be detected for any of the targeted enzymes. This shows that the reversible switch to fermentative pathways is exclusively mediated on the enzyme activity level (*e.g.* allosteric control, enzyme activities), instead of requiring specific regulation responses on transcriptome or proteome level. Finally, the strongest robustness indicator is sustained by the overall similar process yields for L-lysine secretion and biomass growth for the various inhomogeneity degrees that were assessed in the study (Fig. 9^3).

4 Discussion

4.1 Scale-down of oxygen supply assessment into a screening system

Screening for oxygen supply demands during early process development stages allows characterization of ideal net supply and robustness against limitation. This constitutes a scale-down for a parameter assessment that would otherwise be performed at later process development stages with significantly higher resource constraints. As outlined in the results section, the methodology in Paper I[1] offers an improvement for screening setups whenever the degree of oxygen supply is a critical parameter for process efficiency. In addition to the discussion of the results in the original manuscript, there are two elements that should be elaborated further: the technical quality and limitations of the screening method itself, and the quality and limitations of the metabolic dataset that was generated for bioprocesses with *C. glutamicum*.

4.1.1 *Evaluation of oxygen supply screening method*

Two key assets are the basis of the presented parameter screening for oxygen supply: first, the newly established principle of adjusting surface-to-volume ratio that results in variation of oxygen transfer conditions within microtiter plates. Second, the interface for transferring specific oxygen supply properties between reactor systems, that facilitates to use screening data as a basis for further process development. Both elements must be critically evaluated for further application of the method.

As one influencing factor, potential side-effects of changing filling volumes in BioLector® cultivation must be excluded for obtaining valid results. Due to the consistency of the obtained data, it is assumed that all analytical side-effects were identified and mitigated in the presented study. The main new development is the multi-parameter calibration based on backscatter, volume and biomass, which makes it possible to compare parallel cultivations of

different filling volume. This calibration is a vital requirement with respect to determination of valid biomass specific data, *e.g.* yield or product formation at different oxygen transfer capacity. Therefore, there was no alternative to its implementation. The quality of the obtained biomass data can be assessed based on the variances that were observed during the method characterization and screenings. The degree of accuracy is in a range below 5% standard deviation above 2 $g \cdot L^{-1}$ of cell dry weight for triplicate measurements, which is acceptably low for comparative yield determination. Error rates are higher at low biomass concentration and filling volume, which can be explained by physical factors related to the measuring principle, *e.g.* increased surface reflections at thinner liquid coverage of Flowerplate base foil. These irregularities are factored in by the multi-parameter calibration, which therefore forms a reliable enhancement of the BioLector® cultivation system.

It is vital for the screening method that specific oxygen-transfer results can be used as a basis for process development in other reactor systems. However, the initial study design was based on a sulfite-oxidation system[19], for which concerns about the precision have been raised[20]. Also, the sulfite oxidation system belongs to the class of capacity-based systems for oxygen transfer determination, which is prone to systematic errors due to its dependency on reference experiments. Therefore, validation of the oxygen transfer properties was performed with a balance-based system for oxygen transfer determination. Balance-based systems, *e.g.* the respiration activity monitoring system (RAMOS) and exhaust-gas analysis, are the most reliable option for process transfers due to their online determination of actual oxygen transfer rates. In particular, RAMOS has been proven as a reliable alternative to exhaust-gas analysis of active *C. glutamicum* cultures[21]. Unfortunately, balance-based systems are also hard to implement or miniaturize into systems of higher cultivation throughput that are most suitable for screenings. The recently introduced microtiter-compatible MTP-RAMOS system forms a compromise: it can be applied for microtiter plates, although oxygen transfer can only be determined for the whole microtiter plate[22]. In the presented study, the capacity-based oxygen transfer control was applied for the screening, and then

verified with the respective RAMOS system. This combination provides the most reasonable trade-off between high throughput and reliability of results. This way, maintaining precise oxygen transfer control during scale-up to subsequent process stages is feasible, and the strength of balance-based determination of oxygen transfer can be accessed as a reference standard for large-scale bioprocess development early on.

4.1.2 Evaluation of oxygen supply screening results

The biological properties of *C. glutamicum* that were identified during the screening directly influenced the quality of the obtained characterization dataset. It was demonstrated that *C. glutamicum* has a remarkably high tolerance against very low levels of oxygen supply in its biomass and product yields. The metabolic behavior during oxygen supply limitation was successfully characterized, but the most relevant impacts on process yields were observed at the lowest possible supply settings of the chosen study design, *i.e.* below an oxygen transfer capacity of 8 $mmol \cdot L^{-1} \cdot h^{-1}$. This means that most of the obtained results can be expressed as a robustness threshold, while only a small fraction of the screened settings yielded deeper insights beyond the robustness of the aerobic phenotype. The resolution of the screening results could therefore be increased if respective means for stronger oxygen supply limitation were taken. This could be achieved by a number of design changes, *e.g.* increase of initial batch carbon source or decrease of agitation. Due to the systematic approach of the screening, both means would make it necessary to devise a new study and screening layout.

The high tolerance for oxygen supply limitation also calls more attention to the behavior at anaerobic conditions. Analysis of metabolism without oxygen supply was not in the focus of this study. The reason for this is that anaerobic conditions mark an extreme point without considerable growth or maintenance for the aerobic *C. glutamicum*. They have little relevance for bioprocesses that rely on regeneration of biomass, which is the typical setting of amino acid production, and were therefore neglected in the presented study. However, it is

generally possible to include anaerobic conditions into an oxygen supply screening approach with anaerobic BioLector® technology, which has been established recently[23]. Microaerobic conditions, on the other hand, have successfully been analyzed at the lower oxygen transfer settings of the presented screening data. Further adjustment of the process conditions could be applied for reducing the oxygen transfer rate (see previous paragraph), and could easily be screened with more resolution in a follow-up study.

As the screening has shown, there is a broad range within aerobic to partially limited oxygen supply levels which neither influences the biomass nor product yield. Quick yield determination based on oxygen transfer conditions is the key strength of the screening method, so that the robustness threshold values are the main result of the screening. However, dissolved oxygen profiles were also determined for all wildtype cultivations as a supplement to the yield data. Their main purpose was the verification of oxygen supply limitation, and respective raw data were not presented in the manuscript. It was observed that the dissolved oxygen measurements were more prone to technical malfunctions than in other BioLector® setups, which may have been a result of insufficiently covered base foil at agitated conditions and low filling volume. However, even with this quality concern, correlation of a major fraction of the dissolved oxygen data with growth and side product secretion was possible. It can be considered as a positive quality attribute for the screening method that in-process data are available for dissolved oxygen, so that calculation of oxygen transfer rates is principally possible. However, the system may yet require some modifications if quantitative data become necessary for future studies.

As was summarized in the publication, the generated results do not contain fundamentally new information about the metabolic pathways of *C. glutamicum*, because its aerobic and anaerobic pathways have been characterized extensively in the past[8]. The optimum of L-lysine productivity is in the fully aerobic range, which confirms studies that were performed with other producer strains[11,24]. An interesting element of the results is the fact that

growth rate is not directly affected when dissolved oxygen levels are fully depleted, which can be deduced from the dissolved oxygen signals to the respective growth curves. There seems to be a metabolic buffering potential, which is connected to increased side product turnover rates. Only after this intermediate phase has passed, there is a reduction in growth. This strongly suggests that metabolic energy supply is in fact a very dynamic and flexible system that facilitates sufficient output for anabolism even at changing environmental conditions.

An intrinsic quality of all obtained screening results is the transferability into other reactor systems (see 4.1.1), which is heavily influenced by the applied organism and cultivation medium. Therefore, the presented study contains a combination of a simple oxygen transfer algorithm based on sulfite oxidation for screening design in the BioLector®, and a precise kinetic study in a balance-based reactor system (RAMOS) for reference. Oxygen-limited growth was confirmed by RAMOS with characteristic kinetics of exponential-plateau-stationary succession in oxygen uptake rates. This combination provides the ease and flexibility that is necessary to screen a parameter range together with the sound characterization of oxygen transfer behavior over cultivation time. The biological properties of any given microorganism make it indispensable to put some effort into finding a suitable strategy for transfer between reactor systems. In this case, the transfer constant determined with the RAMOS system can serve as a gateway to other reactor systems that rely on balance-based approaches to oxygen transfer monitoring.

It is the main strength of the oxygen transfer screening that the replicate extent and resolution of the generated dataset on oxygen supply impact is unique in the field of microbial bioprocess characterization. The ease of performing parallel cultivations with a full range of applicable supply conditions cannot be matched with stirred tank investigation. Alternative reactor systems with higher throughput so far either lacked the potential for changing the oxygen transfer rate from unit to unit, or could not be characterized sufficiently to ensure relevance of specific conditions for larger reactors. Although the

presented screening is yet limited to a rather simple batch process, the potential for incorporating more advanced reactor conditions increases with recent progress in process miniaturization, which will be discussed in section 4.3.

As was presented in the Results section, several screened cultivation conditions were subjected to a series of scale-up experiments to 5 liter stirred-tank cultivation (data not shown in publication). This was intended as a quality check and extension for the presented oxygen transfer screening data. Qualitatively, these experiments provided similar results as the screening. However, several challenges emphasized the importance of proper interface design (*i.e.* scale-up criteria) when dealing with very low oxygen supply conditions. As had previously turned out during the screening, the most metabolically relevant range of oxygen supply lies in the region of oxygen transfer capacity $OTR_{max} < 8$ $mM*h^{-1}$, where growth and side product accumulation characteristics are heavily dependent on oxygen supply. This range was also subject to technical limitations in precise control of oxygen supply in the stirred-tank setup, which is why the exact matching of the oxygen transfer capacity in the BioLector® and the stirred tank was hard to achieve. Both systems were in their lowest feasible range of gassing and, in the case of the stirred tank, agitation, and secondary effects like decreased gas exchange (stirred tank) may have had an influence on the respective characterization data. Transferring high oxygen transfer conditions, on the other hand, was entirely unproblematic, but also without phenotypic changes being associated with the magnitude of oxygen supply. Therefore, selected scale-up of screened oxygen-limited batch cultivations into 5 liter lab-scale stirred tank provided only a limited set of useful data that generally confirmed the screening results. Verification of highly oxygen-limited scale-up for scientific purposes would require to apply the MTP-RAMOS reference, as has been described above, which was not possible in the timeframe of the presented study. However, this step could easily be included into future experimental designs when the magnitude of suitable oxygen transfer settings can be estimated with more precision.

4.2 Scale-Down of oxygen transfer inhomogeneity into lab-scale

Scale-down of metabolic stress factors in inhomogeneous process environments has been established over a long period of time and proceeds to offer new insights into metabolic behavior under industrially relevant conditions, as has been summarized in recent perspective publications[25–27]. Paper II[2] and Paper III[3] are applications of an established inhomogeneity scale-down technique for *C. glutamicum*: cultivation in two-compartment stirred tank/ plug flow reactors (TCR or STR-PFR) for simulation of large-scale fed-batch situations. They provide a systematic description of metabolic robustness under inhomogeneous oxygen and substrate supply. In the following sections, the results will be evaluated in their relevance for industrial application, and in their quality and limitations compared to other inhomogeneity scale-down sources.

4.2.1 Evaluation of relevance for industrial application

The study designs for this work are based on the assumption that parallel oscillation of oxygen and substrate availability is a crucial influencing factor for large-scale bioprocesses. This stress factor, among others, is widely accepted in literature as one of the inhomogeneity-related causes for failure in large-scale process development[25–27]. For organisms with high metabolic activity, scaling complications of oxygen and substrate supply inhomogeneity are especially likely to occur. The reason is that these substrates have very high metabolic turnover in active bacterial cultures compared to characteristic mixing times of large-scale reactors[27]. As a rule of thumb, oxygen depletion can take seconds at substrate saturation, while gradient depletion through mixing is in the minute range[25]. Fed-batch cultivation, which is applied in most industrial processes with *C. glutamicum*, intensifies this challenge due to the local addition of concentrated substrates. Of course, there are also other factors besides oxygen and substrate supply which are subject to process

inhomogeneity in typical large-scale bioprocesses: for example temperature, pressure, shear forces, or concentration of dissolved gasses. However, these other inhomogeneities are typically less demanding for mixing power, and can therefore be neglected in comparison to oxygen and substrate supply. Additionally, many influencing factors of large-scale bioprocesses are not related to inhomogeneity, *e.g.* culture times and media preparations. These scaling influences that are not related to inhomogeneity can usually be included into lab-scale assessment with small experimental effort, *e.g.* by adjusting preculture conditions or using industrial substrates. Therefore, it can be concluded that the assessed scale-down topic is highly relevant for industrial purposes, and can be applied as a simplified robustness indicator for simplified assessment of complex bioprocess scaling challenges.

Industrial relevance of inhomogeneity scale-down assessment is heavily dependent on the choice of the applied reactor system, in this case the STR-PFR. There are many alternative scale-down systems available which can be applied for subjecting microbial cell cultures to oscillating environments[26]. For example, mixing time increase can also be reached by disc installation inside a stirred tank[28] or STR-STR reactors[29]. The main advantage of STR-PFR systems is that they can distinguish between time-dependent metabolism during the course of oscillations, and overall inhomogeneity effects on an inhomogeneous culture. The relatively narrow PFR residence time distribution does not necessarily improve the simulation quality compared to other designs, *e.g.* STR-STR, as will be discussed below. However, sampling the PFR part of the reactor yields quantitative data about turnover of metabolites under step-change conditions. This allows studying the reasons for metabolic robustness or susceptibility against oscillations, rather than just providing information about the degree of robustness. Therefore, the choice of STR-PFR as scale-down reactor system is a compromise between the quality of metabolic characterization and quality of large-scale inhomogeneity simulation.

The quality of prediction for the presented studies, *i.e.* whether the obtained results could be reproduced under industrial process conditions, is restricted by

the lack of relevant industrial reference data. In order to provide precise scalability results for a bioprocess, it is important that the design of a scale-down reactor is based on a valid process model for the target large-scale system. In principle, the STR-PFR with feed injection into the unaerated PFR is designed to simulate the mixed bulk and feed zone of a top-fed industrial bioreactor. Fed-batch processes are the typical setup for highly active bacterial processes in large-scale, with *C. glutamicum* amino acid production being no exception[30]. An example for a model-based scale-down study of this particular process situation is detailed in Lapin *et al.*[31], who applied mechanistic and fluid-phase models to distinguish between reactor zones of high and low substrate availability for an *E. coli* process. Theoretical background on scale-down design has also been described by Delvigne *et al.*[32], who used stochastic modelling for STR-PFR dimensioning based on large scale mixing characteristics. However, simulation quality of any scale-down study depends on large-scale mixing characterization for existing or simulated production plants in the target scale. This can be achieved by computational fluid dynamics (CFD) models of a planned reactor system. Based on the availability of these large-scale data, the adjustment and characterization of respective mixing properties in the scale-down reactor is a precondition for adequate simulation. Suitable procedures for mixing characterization of reactor compartments with continuous circulation are outlined in Levenspiel[33] for calculating Bodenstein-numbers (Bo) in plug flow compartments based on the axial dispersion model, which is highly relevant for STR-PFR setups. It is the main drawback for the presented studies that there is currently no published characterization for state-of-the-art industrial stirred tank reactors, which are applied for amino acid production with *C. glutamicum*. Large-scale mixing data for realistic reactor systems, which reach stirred tank volumes of *ca.* 500 m³[30] up to 750 m³[37], could also not be obtained from industrial partners due to corporate confidentiality. Therefore, the presented studies were set up as a worst-case approach for an estimated inhomogeneity assessment. Residence times and volume proportions of the two compartment reactors were chosen in a resembling range to previous studies with *B. subtilis*[34], *E. coli*[35] and

S. cerevisiae[36] to facilitate robustness comparison between the biological systems. Transferability of the results towards industrial application is therefore limited to the qualitative description of metabolism at inhomogeneous conditions, so that later simulations for target applications can principally be matched with the available data. In spite of the lack of a specific target reactor, the applied scale-down systems were fully characterized in their mixing properties to facilitate their incorporation into suitable large-sale simulations.

4.2.2 Evaluation of inhomogeneity scale-down results

The presented manuscripts provide extensive data about STR-PFR inhomogeneity scale-down cultivation for *C. glutamicum*, with a sequential assessment of general metabolic behavior in a wildtype strain[2] and detailed robustness-assessment for a producer strain of L-lysine[3]. The manuscripts complement one another due to their shared inhomogeneity scale-down approach, although strategy and focus are different except for the general setup. In the following, contents of both manuscripts are summarized and compared to literature.

The wildtype-paper[2] comprises analysis of batch and linear feed conditions. It is a broad approach for description of process inhomogeneity effects that compares anaerobic circulation times of 45 and 87 seconds to a homogeneous reference culture. The study was conducted as a basic assessment of metabolism at inhomogeneous process conditions, and therefore contained a limited set of analytics and cultivation replicates. This provided insight into intracellular metabolism and side product circulation between the aerobic and oxygen-limited zones of the STR-PFR. The study identified comparable growth, similar pool sizes of adenosine phosphates and reducing equivalents, and constant pH shifts during anaerobic holding time for the inhomogeneous processes. A comparison of batch versus feed phase was made for oxygen uptake redistribution, with the feed phase showing similar breathing activity between STR-PFR and homogeneous reference cultivation. The batch phase

showed reduced breathing activity in the inhomogeneous system, and was therefore assumed to be less robust against inhomogeneity. The main result of the paper is the identification of the changed carbon metabolism with futile cycling of side products into extracellular space and back, the resulting mixed-substrate growth, and general information about robustness compared to other organisms that were assessed in reactor systems with similar scale-down settings.

The second scale-down study with the producer strain for L-lysine was partly based on the robustness results of the wildtype study, because it contains an emphasis on more analytical depth and increased process inhomogeneity. A focus on extended exponential fed-batch phases was chosen for robustness assessment, after initial analysis in the wildtype study had proven that oxygen uptake redistribution only occurs in a substrate-limited environment, whereas batch growth inhomogeneity leads to reduced breathing activity. Residence times of up to 180 seconds were assessed in the oxygen-limited PFR. This extreme exposure to the substrate excess/ oxygen limitation environment is in the range of mixing times that can be expected in stirred tank reactor systems of above 30,000 liters[37], and exceeds the conditions that were applied in the available inhomogeneity scale-down literature with comparable STR-PFR systems. An exponential feed profile was applied, which generates more stable substrate-limited process conditions over cultivation time than the linear feed in the wildtype study. This improves sensitivity for the detection of changes in growth parameters caused by inhomogeneity. Also, several cultivation replicates and redundant reactors were applied for each condition which improves precision and variance detection for the study results. Analytics of intracellular adenosine phosphates and reducing equivalents yielded comparable results to the wildtype study, even at highly increased inhomogeneity, which is a strong indicator for metabolic robustness. Targeted peptide quantification and maximum substrate uptake capacity were added to the analytic spectrum as indicators for proteome and enzymatic robustness against inhomogeneity. The L-lysine product yield was similarly unchanged and robust against high degrees of inhomogeneity. The pH shift which was

observed for the wildtype also occurred with the producer strain, with the extent of pH decrease correlating to biomass and feed profile. The futile conversion of substrate into extracellular side products was also identified for the producer strain, although to a smaller extent than in the wildtype (up to 16% carbon fraction turnover, *cf.*[2]).

The common conclusion of both manuscripts is that *C. glutamicum* possesses high robustness against oscillating oxygen and substrate supply in a fed-batch environment. Integration of multiple analytical methods provided more insight into the nature of process inhomogeneity in oxygen and substrate supply. Repeated exposure to oxygen supply limitation does not trigger any uncommon regulation pathways, at least not in the assessed anaerobic circulation times in the minute range. The phenomenon of side product secretion and reabsorption has been analyzed in detail and can be assumed to be a direct, yet often unrecognized side effect of process inhomogeneity for *C. glutamicum*. The presented results are a strong indication that the additionally required cross-membrane transport steps of the side product lactate do not have a negative impact on metabolism and growth. The main function of the reversible transport mechanism appears to be the maintenance of fast substrate uptake and conversion at oxygen supply limitation, when respiratory chain action is impaired. Under these conditions, NAD^+ is regenerated by the conversion of pyruvate into lactate through the action of lactate dehydrogenase. Previously, several studies have focused on adaptation processes and metabolic challenges in growth on lactate, which can now be assessed against the present study results that imply mixed substrate growth in inhomogeneous environments. Literature shows that the activity of several central metabolic enzymes is regulated under conditions of growth on lactate: phosphoenolpyruvate-synthethase is upregulated by a factor of two to three[38], glucose-6-phosphate-dehydrogenase and 6-phosphogluconate-dehydrogenase are downregulated[39–41], pyruvate carboxylase is upregulated by a factor of three[42,43], and phosphoenolpyruvate carboxykinase is upregulated by a factor of two[44]. Furthermore, protein expression is changed under oxygen deprivation conditions: for example, increased lactate dehydrogenase expression was

shown by Inui *et al.*[45]. Neither of these regulation effects could be identified in the presented studies on the level of transcriptome or proteome, which illustrates the metabolic robustness threshold of *C. glutamicum* against inhomogeneity of oxygen and substrate supply. This is also a strong indication for robustness against the mixed-substrate conditions of organic acid and sugar, which are a direct consequence of inhomogeneous oxygen and substrate supply. Furthermore, the number of cultivation replicates and the two analyzed strains is a big advantage of the performed studies, because it provides an increased security that the observed metabolic behavior is reproducible. This quality attribute is important for the characterization of a robustness behavior, which otherwise harbors the risk that small metabolic changes might be overlooked as typical batch-to-batch variance.

The presented study results of Paper II[2] and Paper III[3] must be assessed against the past and recent observations of other groups, who have chosen different approaches for scale-down of industrial *C. glutamicum* mixing properties. There are currently three peer-reviewed sources that describe comparable studies in compartmented reactors. The first one by Schilling *et al.*[28] applies disc installations in a stirred tank with an auxotrophic L-lysine producer strain, which induces mixing time increase in the minute range for postulated simulation of a 10 m^3 stirred tank cultivation. Growth and critical enzyme activities are negatively affected by this degree of inhomogeneity. Due to the strain's auxotrophy, the focus of the study shifts from oxygen and carbon source inhomogeneity to the distribution of the limiting amino acid. Therefore, the fact that a negative impact of inhomogeneity is only observed in the case of Schilling *et al.*, and not in the presented studies, can most likely be attributed to the special properties of the applied biological system. Due to selection pressure for efficient stress response during the previous evolution of *C. glutamicum*, non-auxotrophic strains are more robust against temporary oxygen or substrate depletion, while an auxotrophic strain lacks adequate regulatory responses for oscillating availability of the essential amino acid. Furthermore, the reactor design which was applied by Schilling *et al.* creates a complicated residence time distribution for individual cells within the

insufficiently mixed reactor zones. While reduced back-mixing in a STR-PFR defines a narrow time range for exposure to harsh growth conditions, Schilling's reactor may have areas resembling dead zones that can capture cells over extended timespans. Due to a lack of position-specific reactor characterization data, these dead zones may generate unknown degrees of oxygen supply limitation with availability of the limiting amino acid and substrate supply, which could generate increased stress compared to non-auxotrophic strains. Therefore, it is highly probable that Schilling's observation of decreased robustness against inhomogeneous cultivation was caused by the biological and reactor type difference, and that a comparison to the results of the presented studies is therefore not applicable.

Two more inhomogeneity scale-down papers were published recently after the presented manuscripts, which utilize three compartment reactors: Lemoine *et al.*[46] apply defined substrate depletion/excess along with oxygen supply limitation in two coupled PFRs, thus simulating distinct zones in a bottom-fed industrial stirred tank. A producer strain of L-lysine was used for cultivation. Lemoine observes similarities in the cellular energy charge that resemble results of the presented manuscripts, but also reports enhanced organic acid accumulation and reduced product yield in scale-down cultivations. This result is surprising because the extent of anaerobic residence time applied in Lemoine is lower than in one of the presented manuscripts[3]. Also, this effect is similarly observed without substrate depletion, *i.e.* if only one PFR is applied. Due to the similarity of the setup to the presented studies, this different observation can most likely be attributed to subtle differences in the biological system. The observed metabolite pool and nucleotide data support that *C. glutamicum* DM1800 might well be more susceptible to process inhomogeneity than DM1933. On the other hand, Buchholz *et al.*[47] apply an STR cascade of dissolved gas gradients concerning CO_2/HCO_3^- with industrially relevant residence times in the minute range. Buchholz reports overall process robustness against inhomogeneity of CO_2, although several responses concerning transcription are identified. This complements the findings of the presented studies in the sense that quantifiable perturbations in

CO_2 levels are not a concern for process scale robustness, and thus confirms the assumptions made in section 2.3.2.

A comparison with inhomogeneity scale-down analysis of other biological systems points out the uniqueness of the identified robustness against process inhomogeneity in oxygen and substrate supply. Starting from early analysis of *S. cerevisiae*[36] and *P. chrysogenum*[48], many industrial organisms and strains have been subjected to respective scale-down studies. Perhaps the best comparison to the presented studies can be drawn with the extensive *E. coli* characterization data, many of which have been summed up by Enfors *et al.*[49]. From differences in transcription[50] to substrate uptake capacity[29] and side product accumulation /reabsorption with reduced biomass yield[51], various changes in metabolism have been described in response to oxygen and substrate supply inhomogeneity with the help of scale-down reactors. The overall effect of process inhomogeneity on bioprocesses is detrimental to product and biomass yield, with very few exceptions (*e.g.* increased *E. coli* cell viability at inhomogeneous process conditions reported by Hewitt *et al.*[52]). Judging from these results, *C. glutamicum* can be considered as a positive example of robustness against inhomogeneous process conditions in oxygen and substrate supply. This means that in order to generate visible changes in process outcome, harsh process conditions are required, which are beyond the extent of large-scale stirred tank mixing times. In the presented studies, a reasonable maximum of holding times in the range of previous studies[31,34,36,49] has been applied. The fact that the metabolic robustness of *C. glutamicum* exceeds these challenging environments means that there may be an unused design space for cultivation under inhomogeneous conditions of oxygen and substrate availability in *C. glutamicum*. This constitutes a positive example for passing the scale-down test of a specific worst-case challenge.

4.3 Conclusion & outlook

4.3.1 Oxygen supply screening

Development of the screening method for metabolic impact of oxygen transfer conditions was successful. The organism *C. glutamicum* is very robust against oxygen transfer limitations, which led to an unexpectedly intense stress test for the screening method: the detected changes in biomass and product yield of the presented study were close to the technically feasible limit of adjustable oxygen transfer conditions. Side product detection has shown that data about metabolic states during oxygen supply limitation can be generated in parallel with a performance screening. Therefore, the new screening tool can replace stirred tank investigation of bioprocess susceptibility against oxygen supply limitation with the more powerful parallel assessment in microtiter plate format. The gained systematic knowledge about incremental changes on a model process provides a new dimension to the current metabolic understanding of oxygen transfer limitation. The resulting database contains individually monitored cultivations that cover the full range from aerobic to highly oxygen-limited growth. It can be used to verify hypotheses and determine the set points at which physical or chemical triggers change metabolic flow profiles. The resolution of the screening system is sufficient for identification of metabolic turning points and cause-and-effect chains through mapping of all applicable supply situations. The methodology is ready for implementation into mini-plant process development workflows.

It has to be considered that the presented approach for screening oxygen supply as a process parameter requires more experimental effort than most established screening applications, *e.g.* for strains or media composition. This mainly corresponds to the need to establish a multi-parameter calibration and system transfer reference for validation of oxygen transfer over cultivation time. Thus, the initial preparation time for the screening is quite long. However, the presented approach provides unprecedented speed and replicate availability once the system has been established. Furthermore, the integration

of oxygen supply as another complex process parameter is a big improvement for bioprocess miniaturization. Although the presented study was a batch growth setup, there are autofeed alternatives[53] and microfluidic solutions[54] on the rise. Also, screening for oxygen transfer demands fits into place with several current developments in the field of bioprocess miniaturization. There are many successful application examples of the BioLector®, bioREACTOR® 48 and other miniaturized, yet highly controlled parallel reactor systems. Implementation of sophisticated process chains into the milli- to microliter scale has become a viable alternative to the previous approaches with untested strains and conditions, which have led to many development failures in the past. From a Design of Experiments (DoE) perspective, every new parameter that can be investigated in early phases means less time and resource demand during the later stages of process development. Thus, a screening for the effects of variations in oxygen transfer on the metabolism of prokaryotic cell cultures was long overdue. Implementation into fully miniaturized process development chains, *e.g.* enhanced BioLector® setups[55], can improve the quality of results from the initial process design phase to new dimensions.

4.3.2 Scale-down of process inhomogeneity

Application of the STR-PFR scale-down method for analysis of inhomogeneous oxygen and substrate supply was successfully performed in a worst-case assessment of metabolic robustness. This has provided versatile new data to previously available studies of inhomogeneity scale-down for *C. glutamicum*. The combination of state-of-the-art analytical methods with established bioprocess systems has provided a comprehensive picture of metabolic robustness against inhomogeneity. Increasing the anaerobic holding times beyond usually analyzed extents has shown that *C. glutamicum* has remarkable traits of flexibility in changing microenvironments, which is marked by oscillation of metabolic flows. The combined analysis of short-time reversible effects and robustness assessment through process yields has provided a step into the direction of systems biology for inhomogeneous

cultures, and a deeper understanding and characterization of process dynamics in an industrial context. Most pronouncedly, the scale-down experiments have provided new information concerning the extent of dynamic redistribution of carbon flow in inhomogeneous cultures with the subsequent formation of local sub-populations that differ in their metabolic activity.

It can be concluded that adjustments for improved inhomogeneity robustness on the level of metabolic engineering are not required for current production strains of *C. glutamicum*.

4.3.3 Outlook

This work is an appeal for early consideration of oxygen supply requirements throughout all phases of bioprocess development in an industrial context. The newly developed oxygen transfer screening and application of the inhomogeneity scale-down can be applied in a comprehensive process development chain. This could constitute a more efficient methodology for aerobic bioprocess development, whenever the dependency of process-outcome on oxygen supply is critical. The proposed workflow can be depicted as follows (see Figure 4):

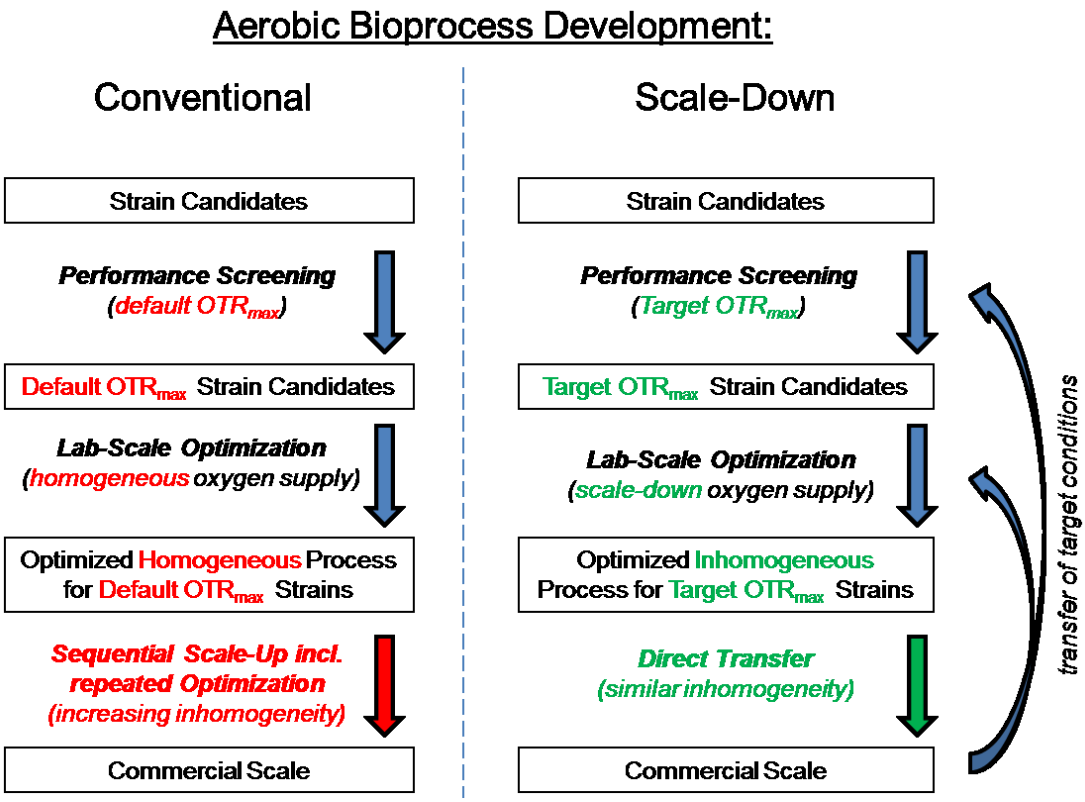

Figure 4: workflow comprehensive consideration of oxygen supply requirements during bioprocess development, OTR_{max} = maximum oxygen transfer capacity, Default OTR_{max} = arbitrary constant supply condition during screening, Target OTR_{max} = realistic supply condition which is feasible for commercial scale

In the proposed development workflow, all oxygen supply elements of bioprocesses are optimized in early development phases, and later transferred into industrial scale application. This serves to prevent avoidable performance loss. The critical step is that respective target conditions must be transferred from the industrial reality to the respective process development phase at which they become relevant. This means in particular that strain selection in oxygen supply screening must be based on the achievable oxygen transfer rates in industrial-scale reactors. Inhomogeneity scale-down for robustness assessment must be the direct consequence once suitable strains have been identified. This perspective on bioprocess development transfers the most crucial development challenges into the reactor systems that can most easily be operated, and thereby minimizes the possible sources of error during the cost-intensive final development steps. The workflow is particularly well-suited for

implementation into a DoE- based assessment of new producer strain candidates, with oxygen supply being one of the influencing factors for performance indicators during screening, and inhomogeneity being a worst-case or qualitative assessment parameter during lab-scale assessment. Resources could be saved both due to more efficient resulting processes and less futile process development steps for failing process transfers. Speed and high resolution during screening, as well as deeper process understanding of inhomogeneity is the key to avoid unnecessary scale-up failures due to neglected oxygen supply demands.

In conclusion, industrial bioprocess development could benefit from comprehensive consideration of oxygen supply requirements. Necessary elements of a proposed workflow have been illustrated for the example of *C. glutamicum* bioprocesses in this thesis. By implementing the presented workflow, resulting bioprocesses could become more competitive during their development process. This strategy could be applied for the benefit of aerobic bioprocess development in general, thus mitigating one key complexity in bioprocess engineering.

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