1 Introduction, objectives and thesis outline

Biotechnological process development includes the screening of microorganisms and the optimization of cultivation conditions for high-yield bioproduction. Biological reaction kinetics, growth behaviour and product formation are dependent on several physicochemical parameters, e.g., temperature, *pH* and nutrient availability (Krull et al., 2016). There is a high demand for cost-effective, parallel and multi-parametric automated methods for the high-throughput screening (*HTS*) of bioprocesses (Zanzotto et al., 2004). Microprocess engineered bioreactor systems have precise control of the microenvironment and allow the collection of desired information for biotechnological process development that is relevant to the production scale (Hegab et al., 2013; Marques and Szita, 2017). Miniaturised bioreactors with a working volume below 1000 µL are known as microbioreactors (*MBRs*). *MBRs* owing to their small size enable serial processing and analysis and, furthermore, can achieve massive parallelisation through efficient miniaturised integrated sensors and multiplexing. (Perozziello et al., 2012; Zhang et al., 2007). For cultivations, the simultaneous detection of several analytical parameters is mandatory for comprehensive process information, making online analysis here essential since the small reaction volumes of *MBRs* exclude elaborate sampling.

*MBR*s have many advantages, but it is important to keep in mind that they could also present some drawbacks related to laminar flow conditions that are common in microfluidics. First of all, in reaction chamber volumes from ten to a few hundred microliters, where the dimensions are too large for diffusion to be effective and mixing by convection is slow because of a lack of turbulence. In this case, it becomes difficult to mix the cultivation broth rapidly. Here, diffusion alone is not sufficient for rapid mixing in *MBRs* (Karnik, 2015). Secondly, it is crucial to provide an efficient oxygen supply to satisfy the oxygen uptake rate (*OUR*). Due to the low oxygen solubility in aqueous cultivation media and the high oxygen consumption from aerobic bioprocesses, the supply of oxygen characterized by the oxygen transfer rate (*OTR*) to microorganisms is the most important transport process and may lead to mass transfer limitations.

To overcome these challenges, a microbubble column-bioreactor (*µBC*) concept for biotechnological research has been developed and characterized. Bubble columns are reactors in which a discontinuous gas phase in the form of bubbles moves relative to a continuous liquid phase. The stream of bubbles enables sufficient aeration and at the same time ensures the homogenization of the cultivation broth. The buoyancy of the bubbles and the momentum exchange between air and liquid promote unsteady liquid flows, which are essential for mixing of the liquid phase. Thus, the main advantages of using bubble columns instead of other multiphase reactors are higher mass and heat transfer rates and less maintenance due to the absence of moving parts.

Previous research was performed within the research unit FOR 856 *Microsystems for particulate life science products* (*mikroPART,* 2011-2014) at TU Braunschweig with a *µBC* for aerobic cultivation processes of the model organism *Saccharomyces cerevisiae* (Krull and Peterat, 2016; Peterat et al., 2014). The *µBC* developed in the *mikroPART*-project consisted of two components: a glass substrate and a patterned polydimethylsiloxane (*PDMS*) chip, which was fabricated by soft lithography technology. To prevent adhesion of cells and air bubbles on the hydrophobic *PDMS* reactor walls, the *µBC* had to be additionally hydrophilized.

However, the aim of the present PhD thesis is focused on the *µBC* redesign with a reaction volume of 60 µL made exclusively of borosilicate glass, to overcome the existing challenges of working with *PDMS*, and to minimize poorly mixed reactor regions. Using borosilicate glass as manufacturing material avoided the hydrophilization step, and at the same time, it maintained the optical transparency and biocompatible properties of *PDMS*.

The 60 µL *µBC* will be characterized in detail for the mixing and mass transfer performances. Here, the influence of the superficial gas velocity on the volumetric liquid-phase mass transfer coefficient will be shown as well as the influence on other mass transport-related parameters, e.g., gas hold-up, Sauter mean bubble diameter, bubble rise velocity, superficial liquid velocity, volumetric power input, and mixing time. Additionally, a simplified computational fluid dynamic (*CFD*) model will be developed as a complement to the experimental research. The *CFD* model served as

a supporting numerical tool to estimate the fluid dynamics inside the redesigned *MBR*.

To further develop the *µBC* concept, a second *MBR* will be presented in this thesis. It is a custom-made microbioreactor, slightly bigger (550 µL) than the borosilicate glass *MBR*, and it is made of polystyrene, facilitating the manipulation for the sensor integration. The integration of miniaturised optical and electrochemical sensors will allow the real-time and online monitoring of the main cultivation process parameters. As a demonstration, example batch cultivations of *Saccharomyces cerevisiae* will be performed. Validation through these batch cultivations proved the long-term functionality of the sensors and reactor and established that process variable evolution could be observed over time.

In detail the aim of the study is:

- The design and development of a *MBR* for biotechnological research,
- the characterization of the *MBR* and investigation of the mixing performance and mass transfer characteristics,
- the development of a simplified computational fluid dynamic model as a complement to the experimental research,
- the integration of miniaturised sensors for optical density, dissolved oxygen, *pH* and glucose in the *MBR* for the real-time online monitoring of bioprocess variables,
- the validation of the *MBR* and proof of the functionality of the sensors with a cultivation of a model microorganism,
- the application of the *MBR* to determine reaction kinetics of other microorganisms and to biocatalysis research.

The thesis consists of eight chapters structured as follows:

Introduction and main goals of the thesis (chapter 1), introduction to microfluidics and to *MBR*s (chapter 2). It presents the state of the art of *MBR*s, highlighting those focused in bacteria cultivations, the challenge of sensor integration and the characterization of *MBR*s. Chapter 2 gives an overview to bacterial cultivations

presenting the main operation modes of bioreactors and the used strains, and finally it presents the application of heterogeneous biocatalysis into *MBR*s. Chapter 3 describes the materials and methods used in the whole work. This includes the design of two *MBR*s, the strains and enzymes utilized, and the followed methods to characterize the developed *MBR*s, to cultivate and perform biotransformations into them, and the description of the integrated miniaturised sensors. Chapter 4 will show the characterization of a glass-based *MBR*. Here, a detailed engineering characterization including the mixing performance and oxygen mass transfer is given. Additional *CFD* simulations are performed to understand better the behaviour of the fluids in the *MBR*. The system is validated by a batch cultivation of the model organism *S. cerevisiae*. Chapter 5 is focused on the sensor integration for the online monitoring of process variables. While the borosilicate *µBC* includes just the sensors for optical density (*OD*) and dissolved oxygen (*DO*), the integration of sensor spots for *pH* and *DO* and of a glucose biosensor is performed in a second developed *MBR*. The *MBR* and the sensors are validated with a *S. cerevisiae* cultivation. Two main *MBR* application examples will be shown in chapter 6: i) the cultivation in batch and continuous mode of the microorganism *Staphylococcus carnosus* and ii) the biotransformation in batch an continuous mode in a microfluidised bed bioreactor. Chapter 7 gives the main conclusions of the thesis and the outlook for future works. The work concludes with the bibliography (chapter 8).

2 Theoretical background

2.1 State of the art of microfluidics and microbioreactors

Research in the field of microfluidics has its origin in the 1970s with the increasing development of microtechnical production processes, but it was not until the 1990s that it became a relevant research topic, especially with the contributions of Manz and Harrison (Harrison et al., 1992; Manz et al., 1990). Microfluidics deals with the behaviour, precise control and manipulation of fluids that are geometrically constrained to a small, typically sub-millimetre scale. Its main application is the design of devices capable of performing fluid manipulations with very low volumes. It has properties that make it attractive as practical and feasible tool in biotechnology areas. In terms of microfluidics, the main forces that operate on fluids and influence its behaviour are diffusion, convection, inertial, viscous, and interfacial forces. But concerning the fluid dynamic conditions in microfluidics, diffusion is the dominant mass transfer phenomenon due to miniaturised channels. Furthermore, in microfluidic channels, the typically high surface area to volume ratio introduces surface tension and surface wettability. The knowledge of physics and fluid dynamics concepts, as well as several possibilities of material and geometry configurations are important aspects for developing new microfluidic designs with proper applications (Oliveira et al., 2016).

Bioprocesses involve the use of microorganisms or their constituent parts such as enzymes, as a catalyst for producing valuable substances such as recombinant proteins, drugs or bio-based chemicals. Conventional methods for bioprocess development use extensive experiments on laboratory scale to select the best cell metabolism conditions to improve the productivity. These screening processes are not easy to parallelise, require time for experimentation and chemical analysis, and a large number of samples, which leads to the generation of waste. Consequently there is a great need for high-throughput devices that allow rapid and reliable bioprocess development, for instance microbioreactors (*MBR*s). *MBR*s are miniaturised bioreactors with a working volume below 1000 µL (Kirk and Szita, 2013). The origins of *MBR*s lay in the miniaturised total chemical analysis system (*TAS*), also known as lab-on-a-chip (*LoC*). During the early 2000s, there were several

contributions in the field of miniaturised reactors (Betts et al., 2006; Doig et al., 2005a; Doig et al., 2005b; Kostov et al., 2001; Lamping et al., 2003; Puskeiler et al., 2005). *MBR*s were introduced later by the Jensen group (Szita et al., 2005; Zanzotto et al., 2004; Zhang et al., 2006) and have been developed as a screening tool for bacterial and mammalian/ human cell cultivation systems, especially for biotechnological, pharmaceutical and medical process development and optimization (Krull and Peterat, 2016; Schäpper et al., 2010). The main research foci include the feasibility of online analytic integration and strain-dependent biological reaction kinetic parameter measurement.

The main advantages of *MBR*s are the minimization of space, reagents, their easy parallelization that together with the integration of analytical tools makes them very interesting devices to develop bioprocesses with *HTS* potential (Krull et al., 2016; Marques and Szita, 2017). *MBR*s are suitable tools for screening applications, e.g., pharmacokinetics, drug delivery or metabolic flux studies, where expensive and only limited amounts of agents are used, or when screening and analysis of dangerous substances (e.g., gas fermentations) is intended. Therefore, the employment of *MBRs* represents a significant step to accelerate bioprocess research, optimising the cultivation conditions and biocatalytic processes, and consequently increasing productivity at large scale.

The scalability of the *MBR* to the macroscale and *vice versa* is reproduced and validated in several cases, showing their potential to provide much of the data and functionality that a large bioreactor system makes available while offering the advantages of high-throughput processing (*HTP*) in terms of costs, space, and time (Krull et al., 2016). Other advantages that *MBR* offer are automation and standardization of the processes (less manual work), high velocity and resolution of analysis with high information content, high degree of parallelisation, rapid process optimization, portability, ease of manipulation, and minute samples.

*MBR*s have many advantages, but it is important to keep in mind that they could also present some drawbacks related to laminar flow conditions that are common in microfluidics. In reaction chamber volumes from ten to a few hundred microliters, where the dimensions are too large for diffusion to be effective and mixing by

convection is slow because of a lack of turbulence, it becomes difficult to homogenize the cultivation broth rapidly. Rapid and efficient mixing is a fundamental requirement for proper distribution and suspension of the substrate and microorganisms during growth cultivation to ensure a uniform process performance and the quality and reproducibility of the measurements. In macroscale systems, the effect of inertia is often significant, resulting in large Reynolds numbers and turbulence, which can be beneficial for mixing. However, due to the small dimensions, microfluidic flows are characterized by low Reynolds numbers, typically in the range of 0.01 – 100, and the effects of inertia are often negligible (Karnik, 2015). Turbulence is therefore typically not encountered, and mass transport is therefore diffusion-limited.

The high impact of *MBRs* on biotechnology and medical applications is reflected in the number of publications involving *MBR* studies of bacteria, yeast, human and mammalian cells, and biocatalysis over the last 30 years are shown in Fig. 2–1. The search was conducted using the web Scopus from Elsevier, and the number of publications corresponds to the amount of accumulated publications over time and the symbol (*) took into account permutations of the keyword. The results of the literature search revealed the increasingly important role of *MBRs* in biotechnological research.

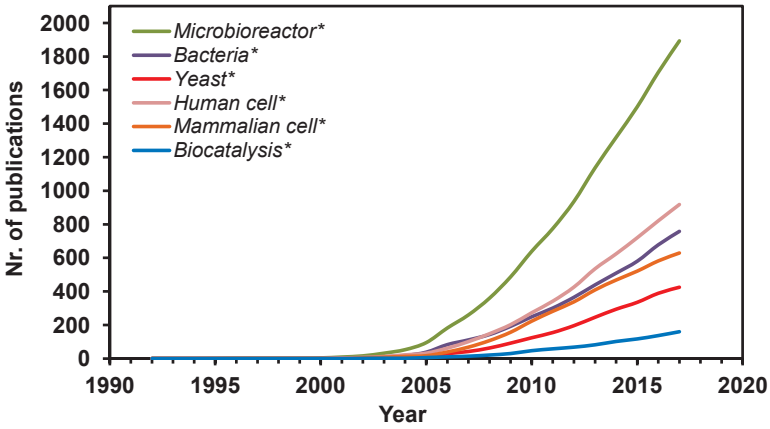

Fig. 2–1 Number of microbioreactor publications over the last 30 years concerning studies with “Microbioreactor*” as a search term along with: “Bacteria*”, “Yeast*”, “Human cell*”, “Mammalian cell*,” and “Biocatalysis*”.

2.1.1 State of the art of microbioreactors for bacterial and yeast cell cultivations

The main focus of this work is the development of *MBRs* for bacterial and yeast cell cultivations. Current research in this field is very active, proof of which is the constant publication of reviews concerning its state of the art. Schäpper et al. (Schäpper et al., 2009) reviewed *MBRs* for cultivation in suspension, focusing on their fabrication materials, mass and heat transfer issues, sensing and control details, and industrial applicability. Marques et al. (Marques et al., 2010) reviewed the criteria for scaling-up cultivation/ bioconversion processes from *MBR* to lab-scale. Gernaey et al. (Gernaey et al., 2012) provided an updated view on the status of *MBRs*, identifying critical needs and issues for furthering the successful development of *MBR* monitoring and control. Hegab et al. (Hegab et al., 2013) gave an overview of *MBR* fabrication techniques as well as their operation and control. Kirk and Szita (Kirk and Szita, 2013) examined oxygen transfer in miniaturised (milliliter-ranged) bioreactors and microliter-ranged *MBR*s and showed comparable characteristics to bench-, pilot-, and production-scale systems. Lattermann and Büchs (Lattermann and Büchs, 2015) reviewed miniaturised bioreactors and *MBR* development, highlighting the mass transfer and power input characterization, optical monitoring, and automation of fed-batch screening systems. Krull et al. (Krull et al., 2016) reviewed the *MBR* platforms used for bacterial and mammalian/ human cell cultivation biotechnology and process development, focusing on the fabrication material, mixing and aeration methods, and implemented sensors of the most relevant *MBRs* in the literature. Oliveira et al. (Oliveira et al., 2016) introduced the possibilities of microfluidics to be applied in the field of industrial biotechnology, presenting the principal definitions and fundamental aspects of microfluidic parameters to better understand advanced approaches. Ladner et al. (Ladner et al., 2017) reviewed the miniaturised bioreactors and *MBR* designed to obtain deeper insight on the level of microbial physiology, single-cell micro-cultivation devices to study the impact of microbial phenotypic heterogeneity on bioprocesses. And finally, Marques and Szita (Marques and Szita, 2017) recently published a review in which the main focus was the development of microfluidic devices for the production of small molecules, therapeutic proteins, and cells.

Besides all the reviews presented here, the main *MBRs* for *HTP* applications to screen bacterial and yeast cell cultivations and to optimize their growth and production in planktonic suspension are summarised in Tab. 2–1. Apart from these prototypes developed in research groups, commercial miniaturised systems in the mL-range with integrated sensors have been also developed like the automated *ambr*-system for cell line cultivation (Sartorius, Göttingen, Germany, working volume of 10 - 15 mL, equipped with *pH* and *DO* sensors) and the microtiter plates (*MTPs*) BioLector (m2p-labs GmbH, Baesweiler, Germany, 0.8 - 2.4 mL equipped with sensors for *OD*, *pH*, *DO*, and fluorescence).

Tab. 2–1 Overview of the developed microbioreactor systems: Bacterial and yeast cell cultivations. Adapted from Krull et al., 2016.

Reference	Type	Mode of operation	Volume	Organism	Application	Material	Mixing	Aeration	Implemented sensors
Yamamoto et al., 2002	Micro-reactor array	Batch	8 chambers x 125 nL	*Escherichia coli*	*HTP* cell-free protein synthesis	*PDMS* and glass		Passive aeration via diffusion	T control chip, intensity of *GFP* and BFP fluorescence
Maharbiz et al., 2003	*MBR* array	Batch	250 µL	*Escherichia coli*	Electrolytic gas generation as a key component for aeration in a miniaturised bioreactor array	Plastic micro-plate strips, commercial printed circuit board, gold wire, silicone membrane	Mixed via external shaker	Passive aeration via diffusion	*OD* (LEDs) and silicon photodiodes (PDB- C154SM; Photonic Detectors, Simi Valley, USA), *pH* (ISFET sensor chip, Sentron Europe), T control via buried thick film thermistors and heaters under each well
Peng and Li, 2004	3D flow controlled microchip	Continuous fresh medium	15 µm deep channels with 15 µm radius	*Saccharomyces cerevisiae*	Cell scanning, and single-cell fluorescent measurement on a single yeast cell, (applicable to mammalian cells)	Glass	3D flow (driven by electric potentials)	Passive aeration through perfusion	Flow field (Optical microscopy, inverted microscope (Nikon TE 300) with a dual-image module (Nikon))
Zanzotto et al., 2004	Membrane-aerated *MBR*	Batch	5 and 50 µL	*Escherichia coli*	Reproduce growth kinetics, observed in bench-scale volumes, in *MBR*	*PDMS* and glass	Diffusion	Passive aeration via diffusion	*DO* (PSt3, PreSens), *pH* (HP2A, PreSens), *OD* (orange LED, Epitex L600- 10V, 600 nm), LEDs and photo- detectors (PDA-55, Thorlabs)
Balagaddé et al., 2005	Micro-chemostat	Continuous	6 x 16 nL	*Escherichia coli*	Monitor the prgrammed behaviour of bacterial populations during long cultivation time	*PDMS*	Diffusion	Passive aeration via diffusion/ influx of fresh medium	Optical microscopy (Nikon TE 2000, Heinze)
Groisman et al., 2005	Micro-chemostat in an array	Continuous	340 µL chambers 100 µm x 70-200 µm x 6 µm	*Candida albicans, E. coli, S. cerevisiae, Staphylococcus epidermidis, Pseudomonas aeruginosa*	Grow cell colonies to high density starting from one cell, and monitor them for extended time at a single-cell resolution	*PDMS* and glass	Through the influx of fresh medium	Passive aeration via diffusion	Colony growth (series of fluorescence images), T (infrared camera)
Szita et al., 2005	Multi-plexed *MBR*	Batch	150 µL	*Escherichia coli*	Demonstrate the reproducible performance of the multiplexed system	*PMMA* and *PDMS*	Magnetic spin bar	Passive aeration via diffusion	Cell density and morphology with single-cell resolution (Optical microscopy, Nikon TE 2000, Heinze)
Zhang et al., 2005	*MBR* with integrated optical sensors	Batch	150 µL	*Escherichia coli, Saccharomyces cerevisiae*	Reproducible growth kinetics observed in conventional experiments	*PMMA* and *PDMS*	Ring-shaped magnetic stir bar	Passive aeration via diffusion	DO (PSt3, PreSens), *pH* (HP2A, PreSens), OD (LED, Epitex L600-10V, 600 nm), LEDs and photodetectors (PDA-55, Thorlabs)