Online Monitoring in Continuously Shaken Microtiter Plates for Scalable Upstream Bioprocessing

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Abstract

Online Monitoring in Continuously Shaken Microtiter Plates for Scalable Upstream Bioprocessing

This thesis is focusing on the applicability of microtiter plates as platform for high-throughput experimentation in bioprocess development. Therefore, three main aspects have been studied in this thesis: 1) characterization of mass transfer in microtiter plates (MTPs), 2) development of a new online-monitoring technique for detecting the most relevant fermentation parameters, 3) proof of scalabilty from microtiter plates to stirred tank fermenters. The oxygen mass transfer into microtiter plates was characterized with the sulfite oxidation method in 24-, 48- and 96-well MTPs. On the one hand, the results pointed out that the achieved maximum oxygen transfer capacities (OTR_{max}) of 0.039 and 0.052 mol/L/h (k_La= 250 and 300 1/h) for round 24- and 96-well MTPs could be limiting for most of microbial fermentations. On the other hand, round 48-well MTPs provided very high OTR_{max} up to 0.28 mol/L/h (k_La= 1600 1/h) with the drawback that these high values could only be achieved at very high shaking frequencies (1400 1/min) and very small filling volumes (300 µL) which were not very suitable for online monitoring in the microwells and for further offline sample analysis. Therefore, also the 48-well Flowerplate was characterized in oxygen mass transfer resulting in high OTR_{max} values of 0.14 mol/L/h (k_La= 800 1/h) at acceptable filling volumes (500 µL) for online detection and further offline sample analysis. Furthermore, a new online monitoring technique for the online detection of all relevant fermentation parameters such as biomass and fluorescent protein concentrations as well as *pH* and dissolved oxygen tension (*DOT*) by optodes in continuously shaken MTPs - in the meantime commercialized under the trade name BioLector - was developed and validated. This technique was approved in several examples of microbial fermentations with *Eschericha coli* and the yeast *Hansenula polymorpha* as model organisms. It could be demonstrated that online biomass detection is possible up to biomass concentrations of 50 g/L cell dry weight due to a linear correlation between scattered light intensities and cell dry weight in the mentioned range. The scalability of microtiter plate fermentations to standard stirred tank fermenters was proven with *Eschericha coli* and the yeast *Hansenula polymorpha.* A comparison of fermentations in 200 µL in MTPs with fermentations in 1.4 L stirred tank fermenter depicted the same fermentation kinetics and fermentation times as well as absolute concentrations proved by online biomass and protein expression of green fluorescent protein (*GFP*) as model protein in both scales. The combination of the online monitoring technique with the recently developed Flowerplate finally demonstrated the broad applicability of this technology in upstream bioprocessing providing all relevant fermentation parameters online at elevated oxygen transfer conditions. This technology fulfills all requirements of an ideal microbioreactor system by featuring high-throughput, high information content and scalability to standard stirred tank fermenters. The application of this new technology in bioprocess R&D could dramatically facilitate and accelerate clone screening and fermentation development by providing more relevant bioprocess information at microscale.

Preface

This thesis was conducted at the RWTH Aachen University, AVT-Biochemical Engineering from January 2002 to April 2006 and later completed at the spin-off of that department, m2p-labs GmbH.

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Funke M, Diederichs S, Kensy F, Müller C, Büchs J: **The baffled microtiter plate: increased oxygen transfer and improved online monitoring in small scale fermentations**. *Biotechnol Bioeng* 2009, **103**(6):1118-1128.

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

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Symbols

a	specific mass transfer area (1/m)
CDW	cell dry weight (g/L)
C_L	oxygen concentration in the bulk liquid (mol/L)
C_{O2}^*	oxygen concentration at the interface (mol/L)
$C_{Na_2SO_3}$	sodium sulfite concentration (mol/L)
CTR	carbon dioxide transfer rate (mol/L/h)
d	well diameter (mm)
d_O	orbital shaking diameter (mm)
D_{O2}	oxygen diffusion coefficient (m^2/h)
dpH	slope of the pH calibration with optodes (-)
E_{chem}	chemical enhancement factor (-)
f	proportionality factor (-)
Fr_a	axial Froude number (-)
FU	fluorescence unit (-)
Ha	Hatta number (-)
He	Henry constant (L*bar/mol)
I_{em}	*emission intensity (FU) or (-)*
I_R	ratio of emission intensities at different excitation wavelengths (FU_1/FU_2)
$I_{R,max}$	maximum ratio of emission intensities (FU_1/FU_2)
$I_{R,min}$	maximum ratio of emission intensities (FU_1/FU_2)
k_1	first-order reaction constant (1/h)
k_2	second-order reaction constant (L/mol/h)
k_n	n^{th}-order reaction constant ($L^{n-1}/mol^{n-1}/h$)
k_La	volumetric mass transfer coefficient (1/h)
k_L	mass transfer coefficient (m/h)
L_{O2}	oxygen solubility (mol/L/bar)
n	reaction order in oxygen (-)
n	shaking frequency (1/min)
n_{crit}	critical shaking frequency (1/min or 1/min)
N	picture number (-)
OD	optical density (-)
OTR	oxygen transfer rate (mol/L/h)

OTR_{enh}	oxygen transfer rate in the chemically enhanced reaction regime (mol/L/h)
OTR_{max}	maximum oxygen transfer capacity (mol/L/h)
OUR	*oxygen uptake rate (mol/L/h)*
p_G	oxygen partial pressure in the gas phase (bar)
pH	pH value (-)
pH_0	pH value of the inflection point of the calibration curve (-)
Ph	Phase number (-)
pKa	negative logarithmic measure of the acid dissociation constant (-)
p_R	reactor pressure (bar)
P_V	volumetric productivity (g/L/h) or (FU/h)
R^2	coefficient of determination value (-)
Re_f	liquid film Reynolds number (-)
RQ	respiratory quotient (mol CO_2/mol O_2)
Δt	time interval between two fotos (min)
T	temperature (°C)
$\dot{V}_G$	norm gas flow rate (NL/min)
V_L	filling volume (L)
V_m	molar gas volume (L)
y_{O2}	mole fraction of oxygen in the gas phase (mol/mol)
$y_{O2,cal}$	mole fraction of oxygen from the calibration gas
y_{CO2}	mole fraction of carbon dioxide in the gas phase (mol/mol)
$Y_{P/X}$	specific yield product to biomass (g product/g biomass)

Greek Symbols

α	mass transfer coefficient due to chemical reaction (m/h)
β	physical mass transfer coefficient (m/h) dynamic viscosity of the fluid (mPa·s) liquid density (kg/L)
σ	surface tension (N/m)

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1 Introduction and Objectives

Biotechnology is one of the strongest emerging technologies in the 21st century. Increasingly more industrial applications and products involve or stem from biocatalysts. Besides biopharmaceuticals that make up the greatest fraction of biotechnological products (with sales of € 4.4 billion in 2008 only in Germany = 16% of all pharmaceuticals, Michl and Heinemann, 2009), fine chemicals and biofuels are also gaining industrial market significance (Schmid et al., 2001, Hatti-Kaul et al., 2007). Most of these emerging products are produced in microbial or cell culture fermentations.

In addition, genetic engineering has generated new research tools to manipulate cells in order to produce new biomolecules. Today, the field of directed evolution, systems biology and synthetic biology paves the way towards bottom up design of new bioprocesses with improved productivity (McDaniel and Weiss, 2005). Here, cloning technologies are applied that generally increase the diversity of genes and the resulting clones. These technologies intrinsically lead to the processing of an increasingly large number of different samples. Moreover, the ability to design new metabolic pathways in microorganisms has made bioprocesses a highly competitive alternative to chemical production processes. Consequently, a huge number of bioprocesses are evolving to replace or improve chemical production processes. Thus, very fast and efficient tools are necessary to develop theses bioprocesses. Furthermore, the chemical and pharmaceutical industries are pressured by market forces to develop new products as fast as possible to reduce time to market. Limited patent lifetimes of new pharmaceutical entities force these companies to launch new products early on the market to skim the greatest profits. Here again, new research tools are necessary to facilitate bioprocess R&D (Figure 1.1).

These requirements in biotechnology have led to high-throughput screening (HTS) technologies. Invented in 1951, microtiter plates (MTPs) have been primarily developed to satisfy the demand for high-throughput in pharmaceutical, chemical and basic research (Sever, 1962). The throughput of these simple test reactor arrays has been continuously increased by concentrating more wells on the same footprint.

MTPs have meanwhile evolved from 6, 12, 24, 48, 96, 384, 1536 up to 3456 wells per MTP. Mainly applied in drug discovery, MTPs today are the standard for high-throughput experimentation.

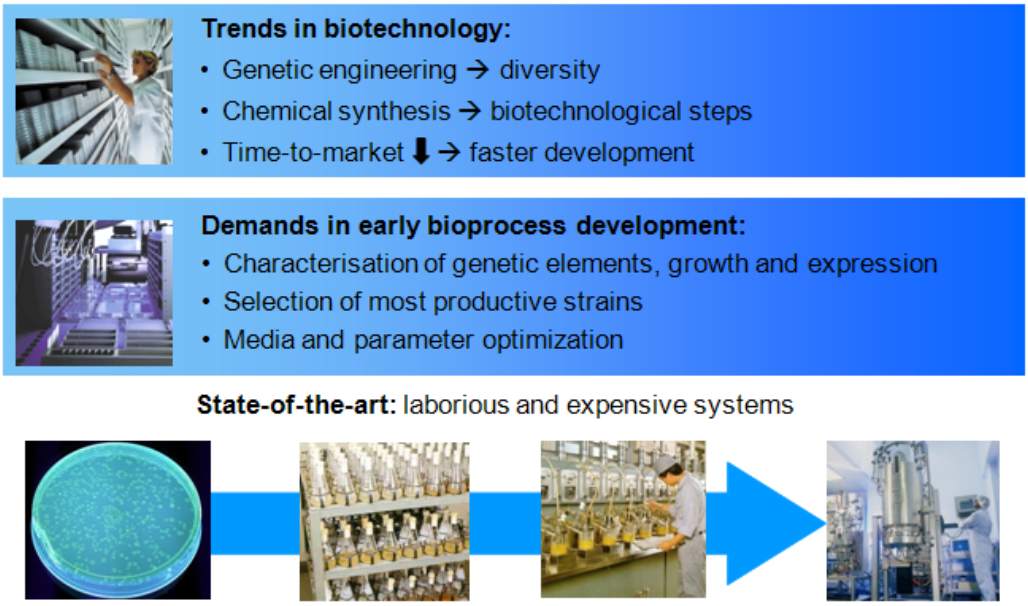


Figure 1.1: Trends and demands in biotechnology.

In bioprocess development, however decades have passed without any changes in the standard laboratory workhorses - **shakes flasks** and **stirred tank bioreactors**. Each system has the following features which have ensured their long survival in the laboratory:

Table 1.1: The characteristic features of the standard laboratory workhorses

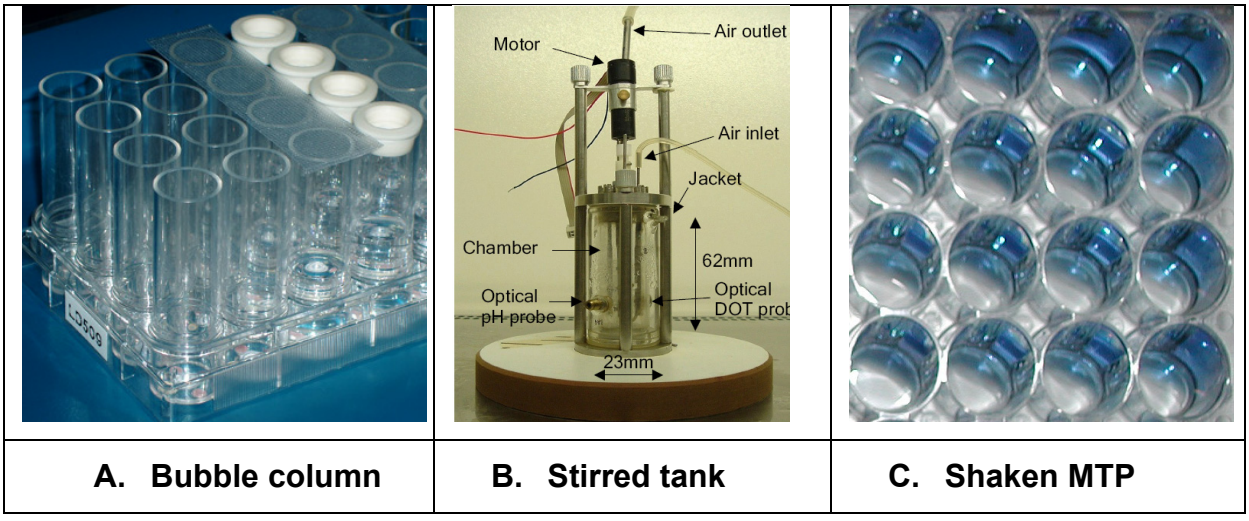
Shake flask	small, easy to handle and inexpensive
Stirred tank bioreactor	standardized, well characterized, equipped with standard sensors (*pH*, *DOT*, *T*), the same reactor design as in certified production processes

In the late 1990s, the first sensor-equipped shake flasks were developed (Weuster-Botz et al., 2001). These flasks contained standard *pH*- and *DOT*-electrodes and could be connected to feeding lines for fedbatch fermentation experiments. A system of 16 equipped shake flasks was the maximum of parallelization at this scale (20 mL–300 mL). Subsequently, this system was commercialized by the company DASGIP in

1997 (Jülich) and later complemented by sparged bubble columns and, more recently, by magnetic stirred bar bioreactors working on a subliter scale.

Another advanced and instrumented shake flask system was developed by Anderlei and Büchs (2001). This Respiration Activity Monitoring System (RAMOS) applied an oxygen and a pressure sensor in the head space of a shake flask to measure oxygen transfer rates (*OTR*), carbon dioxide transfer rates (*CTR*) and the respiratory quotient (*RQ*) in up to eight parallel shake flasks on an orbital shaker (Anderlei et al., 2004). Normal working volumes ranged from 10–50 mL in 250 mL shake flasks depending on the oxygen demand of the applied cells.

Driven by the industrial demand for higher throughput in fermentation and the pressure for faster development times in the pharmaceutical industry, new microbioreactor (MBR) concepts were initiated in several universities worldwide since 2000. Besides the increase in throughput and the reduction in reactor size, the characterization of engineering parameters and the integration of sensors in the microbioreactors played a dominant role in their development. These MBR concepts could be generally classified in three different categories: bubble columns, stirred tank reactors and shaken MTP reactor systems (Figure 1.2).



A. Bubble column	B. Stirred tank	C. Shaken MTP

Figure 1.2: Classification of microbioreactor concepts.

The bubble column concept was first reported by Doig et al. (2005). This group applied the bubble aeration in a square 48-well MTP having a 2 mL filling volume.

Whereas this sparging concept was advantageous in that the system could be operated statically without shaking and stirring, the rising bubbles caused strong foaming problems at this small scale which could only be solved by adding anti-foaming agents. Tackling this problem, the group determined volumetric mass transfer coefficients (k_La) values ranging from 30 1/h to 324 1/h. However, it was difficult to integrate online sensors into these bubble columns. Another bubble column concept using additional orbital shaking was commercialized under the name of µ24 by MicroReactor Technologies Inc. (Mountain View, CA, USA) and marketed by Applikon Inc. (Foster City, CA, USA, Figure 1.2A). For this shaken 24-well bubble column, uneven aeration rates over the plate were reported (Chen et al., 2009) and, thus, only k_La values for non-sparged operation conditions were reported in literature (Isett et al., 2007). The achieved k_La values lay at 33-56 1/h for the simple shaking operation with volumes of 4-6 mL at a shaking frequency of 500-800 1/min. The authors also cited maximum k_La values of 624 1/h, when the columns were sparged with pure oxygen.

This µ24 technology possesses integrated *pH*- and *DOT*-sensors. These sensors are optodes which are basically dots of immobilized fluorescent dyes at the bottom of each well. These dyes are sensitive either to *pH* or *DOT*. These optodes are read out on the bottom of the 24-well plate by underlying specific light-emitting diodes (LEDs) and detecting photodiodes. The µ24 technology has been successfully applied in batch fermentations of *Escherichia coli, Saccharomyces cerevisiae*, *Pichia pastoris and Chinese Hamster Ovary (CHO)* cells (Isett et al., 2007, Chen et al., 2009).

In principle, the stirred tank MBR concepts are mostly similar to stirred tank production bioreactors because of their same mechanical mixing principle. Their miniaturization, however, is challenging due to the need for high precision in fabrication of small vessels, stirrers and baffles. One concept which keeps the same stirrer design (6-blade rushton turbine) for both the small scale and the large scale has been presented by Betts et al. (2006, Figure 1.2B). The authors report k_La values of up to 480 1/h and short mixing times of 4.8 s, both at 7000 1/min. Their microfermenter could successfully grow various organisms such as *Escherichia coli (E. coli)* and the filamentous bacteria *Saccharopolyspora erythraea*. Studied only in a

prototype state, this MBR has not been further developed. Again here, *pH*- and *DOT*-optodes are integrated in the reactor which thus requires individual fiber optics for each individual sensor and reactor.

Another stirred MBR was developed by Puskeiler et al. (2005). This group designed a gas-inducing impeller which provided k_La values of up to 1600 1/h with 8 mL filling volumes at 2300 1/min. The whole system consisted of 48 parallel reactors in a reactor block, which could be tempered individually. Integrated into a liquid-handling robot, the system could be operated successfully in fedbatch mode with *E. coli* (Puskeiler et al., 2005) and *B. subtilis* (Knorr et al., 2006). Here, *pH*- and *DOT*-optodes were immobilized on the bottom of each reactor. The reactors are generally operated with 8-12 mL filling volume. Six sensor bars with LEDs and photodiodes for 8 parallel reactors were applied to detect all optode signals from the 48 reactors. The system was able to detect optical densities (*OD*) by taking samples with the robot and, thus, analyzing these samples in a separate MTP reader after sample dilution. This system has not been marketed as yet.

Moreover, the Klavs Jensen´s group at MIT (USA) designed another stirred MBR. This reactor type was produced by microfabrication techniques using such materials as PMMA and PDMS. *DOT*, *pH* and *OD* could be measured in this MBR by applying optodes and by measuring cell absorbance, respectively. The aeration resulted from air diffusion through a covering PDMS membrane and liquid mixing was realized by magnetic stirrers. This group reported maximum k_La values of 75 1/h with 150 µL filling volume and showed parallel fermentations of *E. coli* in the MBR and in a Sixfors laboratory fermenter with a filling volume of 500 mL (Infors, CH), but mostly under oxygen-limited conditions (Szita et al., 2005). Whereas the parallelization of reactors in this MBR could be easily realized by the applied microfabrication methods, the measurement optics and the stirring motor for each reactor here were difficult to integrate and required high investments.

In general, shaken microtiter plates are probably the simplest microbioreactors applied in biotechnology. Intensive research has been conducted in characterizing engineering parameters in shaken microtiter plates in recent years (Hermann et al., 2003, Duetz et al., 2000, John et al., 2003, Doig et al., 2005b). Moderate k_La values

of maximum 175 1/h for standard round 96-well MTPs with 200 µL filling volume have been found (Hermann et al., 2003) indicating the limited application range of these plate types for microbial fermentations.

Typically, process information can be acquired in microtiter plates by applying the aforementioned optodes. These optodes are currently available for *pH*, dissolved oxygen tension (*DOT*), dissolved carbon dioxide tension (DCT) and temperature (*T*). Commercially, microtiter plates with integrated *pH*- or *DOT*-optodes are available for standard 6-well, 24-well and 96-well MTPs from Precision Sensing GmbH (Regensburg, Germany).

Another means to better understand bioprocesses in microwells is applying common microplate readers or the BioScreen C reader (Oy Growth Curves Ab Ltd., Finnland). Normally, optical densities can be detected via transmission measurements, but this approach requires very thin culture densities. Furthermore, the gas-permeable membrane covering the MTP, which is necessary for monoseptic operation, may interfere with these measurements. The linear range of *OD* measurements lie in the range of 0.1-0.3 and, therefore, *OD* measurements are limited to diluted samples and are not very useful in bioprocess development where higher biomass concentrations (> 5 g/L cell dry weight (*CDW*) ~ *OD*= 10-20) are normally desired.

Moreover, fluorescence measurements in MTPs on microplate readers are very common and widely applied in biotechnology. These measurements normally make use of applied back-scattering of the emitted fluorescence of a sample and, hence, neither depend on path lengths nor on the covering membranes as transmission measurements do. One important drawback of fluorescence and the aforementioned *OD* measurements is the interrupted shaking during the measurement and, thus, the resulting interruption of oxygen mass transfer into the culture broth and probable sedimentation of cells during the measurement period.

1.1 Objectives of this work

Based on the intrinsic high-throughput capacity of microtiter plates, these types of reactors are to be investigated with respect to their applicability for high-throughput fermentations.

The objectives of this thesis are:

- Mass transfer characterization of operation conditions in shaken microtiter plates;
- Development of an optical online monitoring technique in shaken microtiter plates to extract more process information from parallel fermentations on a microscale; and
- Proof of scalability from microtiter plates to stirred tank bioreactors.

2 Oxygen Transfer Phenomena in 48-Well Microtiter Plates: Determination by Optical Monitoring of Sulfite Oxidation and Verification by Real-Time Measurement during Microbial Growth

Abstract: Oxygen limitation is one of the most frequent problems associated with the application of shaking bioreactors. The gas-liquid oxygen transfer properties of shaken 48-well microtiter plates (MTP) were analyzed at different filling volumes, shaking diameters and shaking frequencies. On the one hand an optical method based on sulfite oxidation was used as a chemical model system to determine the maximum oxygen transfer capacity (OTR_{max}). On the other hand the Respiration Activity Monitoring System (RAMOS) was applied for online measurement of the oxygen transfer rate (*OTR)* during growth of the methylotropic yeast *Hansenula polymorpha*. A proportionality factor between the OTR_{max} of the biological system and the OTR_{max} of the chemical system were indicated from these data, offering the possibility to transform the whole set of chemical data to biologically relevant conditions. The results exposed “out of phase” shaking conditions at a shaking diameter of 1 mm, which were confirmed by theoretical consideration with the Phase number (*Ph*). At larger shaking diameters (2-50 mm) the oxygen transfer rate in microtiter plates shaken at high frequencies reached values of up to 0.28 mol/L/h corresponding to a volumetric mass transfer coefficient k_La of 1600 1/h. The specific mass transfer area (*a*) increases exponentially with the shaking frequency up to values of 2400 1/m. In contrary, the mass transfer coefficient (k_L) is constant at a level of about 0.15 m/h over a wide range of shaking frequencies and shaking diameters. Although, at high shaking frequencies, when the complete liquid volume forms a thin film on the cylindric wall of the well, the mass transfer coefficient (k_L) increases linearly to values of up to 0.76 m/h. Essentially, the present investigation demonstrates that the 48-well plate outperforms the 96-well MTP and shake flasks at widely used operating conditions with respect to oxygen supply. The 48-well plates emerged therefore as an excellent alternative for microbial cultivation and expression studies combining the advantages of both the high throughput 96-well MTP and the classical shaken Erlenmeyer flask.

2.1 Introduction

Shaken small-scale bioreactors are undoubtedly most frequently used for fermentations, because they are simple to operate and inexpensive to conduct, allowing many cultures to be run simultaneously. The lower limit for small-scale fermentations has historically been defined by requirements such as ease of reproducible and aseptic operations and for reliable sampling and sufficient broth volume for sample analysis (Hilton, 1999). With the introduction of the first microtitrator system by Gyula Takatsy in 1950 (Sever, 1962) the miniaturization of liquid handling and analytical methods started, and some fermentations have similarly been miniaturized (Fung and Kraft, 1968 and 1969; Wilkins and Walker, 1975). An entire industry has developed around automated liquid handling and biochemical analysis in microtiter plates, especially the 96-well MTP. In addition, 48-, 24-, 12-, and 6-well plates are now available with the same standard footprint. The microtiter plate format-based industry has increased its precision and accuracy, thus, automation of liquid handling and diverse reactions in microtiter plate format were realized. Millions of tests are nowadays running in these formats. Drug discovery in particular is accelerating the development of automated test systems to screen compound libraries. Microtiter plate formats of 384, 1536 and even more wells became reality. Microtiter plates and liquid handling technologies are now routinely used for the cultivation of microorganisms. They are especially useful when large numbers of strains need to be evaluated simultaneously, and growth is measured at the end of the experiment (Hilton, 1999).

The apparent drawback of microbial growth in MTPs is its limited maximum oxygen transfer capacity (Hermann et al., 2003). Therefore, the use of microtiter plates for the growth and maintenance of microbial strains has been mainly limited to clonal libraries in *Escherichia coli* and yeasts. For this purpose, the oxygen limitation is generally less relevant, since *E. coli* and the majority of yeasts can grow anaerobically (Duetz et al., 2000). Poor growth behavior, however, is observed in microtiter plates during the cultivation of fastidious aerobes – e.g. *Cryptococcus neoformans* (Odds et al., 1995) – unless the required oxygen amount is made available. Furthermore, if a high-throughput screening (HTS) or strain development

procedure is aimed at finding a new production strain for an aerobic fermentation (e.g. amino acid or antibiotic production), it is essential to provide the screened microbes with sufficient oxygen. Otherwise, the strain chosen during screening under oxygen limitation might not perform in a well aerated stirred tank fermenter. On the other hand, a strain, which could be a suitable candidate for the desired production process might fail during an oxygen-limited screening procedure (Büchs, 2001). Shaking in 96-, 384-, and 1536-well plates becomes less effective with decreasing vessel size. Capillary forces superimpose and partially impede free diffusion or even forced motion of the molecules (Berg et al., 2001). Surface tension can exert a strong resistant force against the fluid movement in 96-well MTPs resulting in a low mass transfer over a wide range of common shaking frequencies (Hermann et al., 2003). Obviously wider cross section areas of microtiter plate well geometries can reduce the influence of the surface tension on the hydrodynamics and on mass transfer. Therefore 48-well MTPs were evaluated as a compromise between the shake flasks and 96-well MTPs.

This publication presents a complete characterization of the gas-liquid mass transfer in 48-well MTPs. The maximum oxygen transfer capacity (OTR_{max}), the volumetric mass transfer coefficient (k_La), the specific mass transfer area (a) and the mass transfer coeffient (k_L) were determined with a chemical model system (sulfite oxidation) for a wide range of operating conditions: 1) shaking frequencies between 1-2000 1/min, 2) shaking diameters 1-50 mm, and 3) filling volumes between 300-600 µL. Finally, a new method for the measurement of the *OTR* in a biological culture over the whole microtiter plate was applied, offering the possibility to correlate biological and chemical maximum oxygen transfer capacities (OTR_{max}).

2.2 Theory

2.2.1 Mass transfer with chemical reaction

For the characterization of the gas-liquid mass transfer it has for a long time been common practice to use well defined chemical reaction systems (Cooper et al., 1944). The sulfite oxidation is a very frequently applied system when studing the oxygen mass transfer in various kinds of bioreactors. The disadvantages of this

method for bubble aerated bioreactors are well known (Nordkvist et al., 2003). Compared to biological fermentation systems, it leads to quite different absolute values of the oxygen transfer rate and to sometimes different dependencies on the operating conditions (Maier et al., 2001). This is due to high ionic strength and, therefore, the completely inhibited coalescence of the bubbles (Keitel and Onken, 1982). On the other hand, in surface aerated bioreactors like unbaffled shaken bioreactors, coalescence phenomena do not occure. The oxygen transfer rate from measurements using the sulfite method can then be directly recalculated to biological systems by means of a proportionality factor (*f*) (Duetz et al., 2000; Hermann et al., 2003; Maier et al., 2004).

Recently, a new optical method has been developed for the characterization of small scale reactors where no *DOT*-electrodes or exhaust gas analysis can be applied (Hermann et al., 2001). The sulfite becomes oxydized to the more acidic sulfate, which leads to a drop of the *pH* at the end of the reaction. This moment can be monitored using an adequate *pH*-indicator. This method has been successfully applied to determine the OTR_{max} in 96-well plates (Hermann et al., 2003) and in large shaken vessels (Kato et al., 2004).

The theoretical background of the gas-liquid mass transfer with chemical reaction was recently described in detail (Hermann et al., 2003, Maier et al., 2004). Based on the theory of absorption accompanied by an (m,n)-order chemical reaction and the assumption of the film model, Reith (1973) has derived an approximation of the exact solution of the stationary mass balance for the absorption process:

$$OTR = \sqrt{\underbrace{\frac{2}{n+1} \cdot k_n \cdot (C_{O2}^*)^{n-1} \cdot D_{O2}}_{\alpha} + \underbrace{k_L^{\,2}}_{\beta}} \cdot a \cdot (C_{O2}^* - C_L) \qquad (2.1)$$

The expression under the square root can be regarded as a reaction term α and a physical mass transfer term β, as shown in Eq. [2.1]. The square root of these terms is defined as the dimensionless Hatta number (*Ha)*, according to Eq. [2.2]:

$$Ha = \frac{reaction\ rate}{mass\ transfer\ rate} = \sqrt{\frac{\alpha}{\beta}} = \frac{\sqrt{\frac{2}{n+1} \cdot k_n \cdot (C_{O2}^*)^{n-1} \cdot D_{O2}}}{k_L} \qquad (2.2)$$

This dimensionless number indicates different reaction regimes. At Hatta numbers *Ha* < 0.3, the so called non-enhanced reaction regime is reached, which means that the reaction occurs only in the liquid bulk. The overall reaction rate is thus only limited by the physical mass transfer through the gas-liquid interface and Eq. [2.1] simplifies to:

$$OTR = k_L a \cdot (C_{O2}^* - C_L) \qquad (2.3)$$

At Hatta numbers *Ha* > 3, the so called enhanced reaction regime is reached, which means that the complete reaction occurs only in the liquid film. The overall reaction rate is thus only limited by the reaction in the liquid film. Therefore, this regime can be used to characterize the surface area of the gas-liquid interface. From these results it is possible to derive the specific mass transfer area (a) knowing the applied liquid volume. Conditions where the *Ha* number is >3, can be obtained by accelerating the reaction regime to a second-order reaction by a simple increase of catalyst concentration in the sulfite system. The OTR_{enh} for the enhanced reaction regime of a second order reaction (n=2) can be derived from Eq. [2.1] by neglecting the term β, resulting in:

$$OTR_{enh} = \sqrt{\frac{2}{3} \cdot k_2 \cdot C_{O2}^* \cdot D_{O2}} \cdot a \cdot C_{O2}^* \qquad (2.4)$$

In some cases it is not possible to clearly separate the chemical reaction from the physical absorption process, thus both phenomena have to be considered. The so called transition regime reflects a reaction occuring in the bulk liquid as well as in the liquid film and is expressed by *Ha* numbers between 0.3 to 3.0. The chemical enhancement factor E_{chem} can be introduced to express the influence of the reaction regime on the *OTR*. Introducing Eq. [2.2] in Eq. [2.1] the following equation (Eq. [2.5]) is obtained:

$$OTR = k_L a \cdot \sqrt{1 + Ha^2} \cdot (C_{O2}^* - C_L) \; = k_L a \cdot E_{Chem} \cdot (C_{O2}^* - C_L) \tag{2.5}$$

The maximum oxygen transfer capacity (OTR_{max}) is defined as the oxygen transfer rate, if C_L is zero. For the considered first-order reaction (n=1), the dissolved oxygen concentration in the bulk liquid is not necessarily equal to zero (even in the transition regime), but can be calculated by transforming the mass balance of oxygen to $C_L = OTR/k_1$. The first-order kinetic reaction constant k_1 of 2385 1/h was taken from Herrmann et al. (2003) while applying the same conditions. The value of OTR_{max} can then simply be calculated by:

$$OTR_{max} = \frac{OTR}{E_{chem}} \cdot \frac{C_{O2}^*}{C_{O2}^* - \frac{OTR}{k_1}} \tag{2.6}$$

2.2.2 Description of the "out of phase"-phenomenon by the dimensionless Phase number (Ph)

The hydrodynamics in shaken reactors have to be divided in two general operating conditions, the "in phase" and "out of phase" operating conditions. While in the "in phase" mode the bulk of the liquid within the reactor circulates "in-phase" with the shaking drive, in the "out of phase" mode the bulk of the liquid remains at the base of the reactor and shows only very little relative movement. This "out of phase"-phenomenon was first described by Büchs et al. (2000) for shake flasks. "Out of phase" operating conditions are unfavorable for microbial cultivation because the mass transfer and the power input are reduced. Therefore the dimensionless Phase number (*Ph*) was introduced by Büchs et al. (2000) to describe this phenomenon:

$$Ph = \frac{d_0}{d} \cdot (1 + 3 \cdot log_{10}\, Re_f) \tag{2.7}$$

with the liquid film Reynolds number (Re_f):

$$Re_f = \frac{\rho \cdot (2 \cdot \pi \cdot n)}{\eta} \cdot \frac{d^2}{4} \cdot \left(1 - \sqrt{1 - \frac{4}{\pi} \cdot \left(\frac{V_L^{1/3}}{d} \right)^2} \right)^2 \qquad (2.8)$$

The *Ph* number is defined in analogy to a partially filled, rotating horizontal drum.. The liquid film Reynolds number (Re_f) relates the power of inertia to the power of friction in the liquid film of a hypothetical uniformly distributed liquid inside a rotating horizontal drum (for details refer to Büchs et al., 2000).

Derived from more than a thousand measuring points for Erlenmeyer flasks with a nominal volume of 50-2000 ml, a simple criterion to distinguish between ”in phase” and “out of phase” shaking conditions was formulated (Eq. [2.7]). If the resulting value of Eq. [2.7] is higher than 1.26, the liquid is “in phase”. If the result is lower than 1.26, the liquid is “out of phase”. It should be pointed out that the model is only valid for axial Froude numbers (Fr_a) > 0.4. The method is described with more detail in Büchs et al. (2000). In this work the same criterion was applied to describe the hydrodynamics in shaken MTPs of the 48-well format.

2.2.3 Description of the hydrodynamics by the critical shaking frequency (n_{crit})

Hermann et al. (2003) defined the critical shaking frequency (n_{crit}) as the shaking frequency, when the labor delivered by the centrifugal force of a shaking machine is equal to the surface tension of the liquid medium in a microtiter plate well. At that point the liquid in the microtiter plate starts to enlarge its surface and, thus, the OTR_{max} increases exponentially. From these considerations, Hermann et al. (2003) deduced the following equation for the critical shaking frequency depending on the shaking diameter (d_O) and the physical and geometric parameters (σ, ρ, d, V_L) for 96-well microtiter plates:

$$n_{crit} = \sqrt{\frac{\sigma \cdot d}{4 \cdot \pi \cdot V_L \cdot \rho \cdot d_O}} \qquad (2.9)$$

2.3 Materials and Methods

Two types of especially designed orbital shakers were used: (1) A modified table shaker (ES-W, Kühner AG, Basel, Switzerland) allowing different shaking diameters and shaking frequencies of up to 1000 1/min (Hermann et al., 2001; Hermann et al., 2003), was used for experiments with a shaking diameter of 3, 6, 12.5, 25, and 50 mm. (2) Three separate new custom-made variants of a microtiter plate shaker (Teleshake, H+P Labortechnik AG, Oberschleissheim, Germany) were used. These had fixed shaking diameters of 1, 2, and 3 mm, respectively, and allowed shaking frequencies of up to 2000 1/min. 48-well suspension culture plates (art.-no.: 677 102, Greiner, Frickenhausen, Germany), with a maximum filling volume of 1.7 ml, F-bottom design and manufactured from non-treated polystyrene, were used as reaction and fermentation vessels.

2.3.1 Determination of the Maximum Oxygen Transfer Capacity (OTR_{max}) using optical monitoring of sulfite oxidation

A solution of 0.5 M sodium sulfite (98% purity, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 10^{-7} M cobalt sulfate (Fluka, Neu-Ulm, Germany), 0.012 M Na_2HPO_4/NaH_2PO_4 (Merck, Darmstadt, Germany) phosphate buffer, *pH* 8 adjusted with 2 M sulfuric acid, and $2.4 \cdot 10^{-5}$ M Bromthymol blue (Fluka, Neu-Ulm, Germany) was used. The experiments were conducted at an ambient temperature of about 25 °C. The color shift from blue to yellow of the indicator Bromthymol blue was monitored by a digital camera (Olympus C-3030 Zoom, Olympus Optical Co. Ltd., Tokyo, Japan), that was remotely controlled by a personal computer. Digital pictures were taken at constant time intervals Δt of 5, 10, or 15 min, depending on the estimated total time of the reaction. The *OTR*-value can then be calculated using the number *N* of pictures after which the color shift is completed:

$$OTR = \frac{1}{2} \cdot \frac{C_{Na_2SO_3}}{N \cdot \Delta t} \tag{2.10}$$

For the measurements performed at 6–50 mm shaking diameter an already published experimental setup (Hermann et al., 2001) was used, where solely the CCD camera was replaced by a digital camera (Figure 2.1A). For measurements with the microtiter plate shakers at 1, 2, and 3 mm, however, the whole shaker including the 48-well plate was put under a transparent hood and flushed with humidified air to minimize evaporation during the experiment (Figure 2.1B). To avoid clouding of the transparent box (Figure 2.1), the inside surfaces of the box were treated with a hydrophobic spray, which is normally used for windows of motor-cycle helmets.

The Hatta number (*Ha*), calculated from Eq. [2.2] with the help of the data of the mass transfer coefficient (k_L) described in the following chapter, revealed that the sulfite reaction was conducted in the transition regime ($0.3 < Ha < 3.0$) at shaking frequencies (n) below 1100 1/min. Therefore in the calculation of the OTR_{max} and k_La the chemical enhancement of the sulfite reaction must be taken into account. With the Hatta number (*Ha*) the enhancement factor E_{chem} could be determined to be 1.33 (see Eq. [2.5]). The presented data of the OTR_{max} and k_La for all shaking frequencies (n) up to 1100 1/min were calculated from Eq. [2.6]. At shaking frequencies (n) higher than 1100 1/min the reaction regime was adequate (Ha < 0.3) and E_{chem} was approximately one.

2.3.2 Determination of the specific mass transfer area (*a*) and the mass transfer coefficient (k_L)

The same optical method used for the determination of the OTR_{max} can be applied for the determination of the specific mass transfer area (a). The only difference between the methods is that the catalyst concentration of cobalt has to be increased to a concentration of 10^{-4} M instead of 10^{-7} M to work in the chemically enhanced reaction regime, where $Ha > 3$. Under these conditions the reaction order (n) in oxygen is 2, so that Eq. [2.4] can directly be applied. The reaction constant k_2 was experimentally determined from the average of the four studied volumes (V_L = 300, 400, 500 and 600 µL) under non-shaken conditions and thus known specific mass transfer area (a) ($a = A/V_L$, with A the cross section area of a well). The determined value for k_2 = $9.41 \cdot 10^5$ L/mol/s is in the range of that found by Hermann et al. (2003) and well comparable to those published by Linek and Tvrdik (1971) under similar conditions.

With the known reaction kinetic it is now possible to solve Eq. [2.4] and calculate the specific mass transfer area (*a*), while applying the enhanced reaction method under different operating conditions of the microtiter plate. The required oxygen diffusion coefficient (D_{O2}) in aqueous electrolyte solutions can be calculated from Akita (1981). Under the applied conditions a value of D_{O2} = $1.79 \cdot 10^{-9}$ m^2/s was obtained. The oxygen concentration at the gas-liquid interface C_{O2}* can be calculated from $L_{O2} \cdot p_G$, with the oxygen solubility (L_{O2}) ($8.35 \cdot 10^{-4}$ mol/L/bar at 25°C according to Weisenberger and Schumpe, 1996) and the oxygen partial pressure in air (p_G) (0.2095 bar). As a result of the specific mass transfer area (*a*) and the OTR_{max} under the same operating conditions, it is now possible to resolve Eq. [2.3] for C_L = 0, thus, the k_L-value can be obtained.

2.3.3 Measurement of the Oxygen Transfer Rate (*OTR)* of microbial cultures using a new variant of the Respiration Activity Monitoring System (RAMOS)

In contrast to results from bubble-aerated reactors like stirred tanks (Maier et al., 2001), it has been shown that results obtained by the sulfite oxidation model system in shaken bioreactors can be easily converted to biological culture systems (Maier et al., 2004). The biological and the chemical OTR_{max} differs only by a proportionality factor (*f*) (Duetz et al., 2000, Hermann et al., 2001), which represents the different oxygen solubilities and diffusion coefficients of the applied media.

Recently, a new technology for the online determination of the *OTR* in shake flasks has been introduced and described in detail (Anderlei and Büchs, 2001 and Anderlei et al., 2004). The decrease of oxygen partial pressure in the gas phase of a closed system containing the microbial culture is monitored by an oxygen sensor during the measuring cycle. With this data, the *OTR* is calculated from the slope of the oxygen partial pressure over time. To avoid an oxygen depletion in the gas phase the reaction vessel is repetitively flushed with ambient air (Anderlei and Büchs, 2001). The repeating measuring and flushing intervals are controlled in a way, that the resulting average oxygen partial pressure in the gas phase of the measuring device is equal to the oxygen partial pressure in the head space of a normal shake flask with a sterile barrier made from cotton or similar materials (Mrotzek et al., 2001).

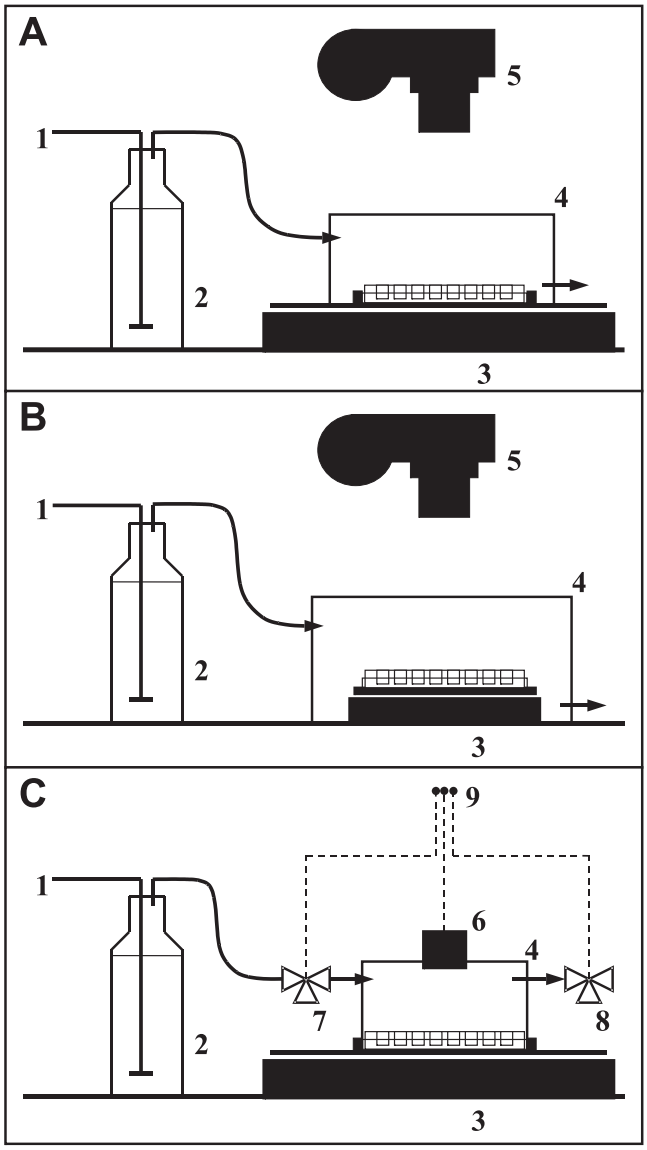


Figure 2.1: Experimental setup of the chemical and biological *OTR* determination.

(A) for optical determination of maximum oxygen transfer capacity (OTR_{max}) in a microtiter plate shaken on machines with 6, 12.5, 25, or 50 mm shaking diameter. (B) for a plate shaken at 1, 2, or 3 mm. (C) for direct determination of the oxygen transfer rate (*OTR*) in a multiwell plate using the RAMOS-technology (principle described in Anderlei et al., 2004).

(1) Air supply; (2) gas humidifier; (3) rotary shaker (black); (4) transparent box providing a controlled gas environment; (5) computer controlled digital camera (black); (6) oxygen sensor (black); (7) inlet valve; (8) outlet valve; (9) data connection to control unit.

In this study, a respiration box containing the microtiter plate was used as a closed measurement system (Figure 2.1C) instead of a modified shake flask. The new RAMOS variant is therefore applied for the online measurement of the *OTR* of a microbial culture as the integral of all wells of one microtiter plate. The aim of this setting is not to distinguish between the different wells. For this measurement all

wells have to be filled with the same medium and inoculum. During these experiments the microtiter plates were sealed with gas permeable adhesive seals (art.-no.: AB-0718, Abgene, Hamburg, Germany). The seals were necessary to avoid culture contamination. On the other hand they reduce evaporation without lowering the *OTR* in microtiter plates (Zimmermann et al., 2003).

For the validation of the obtained mass transfer data from the sulfite oxidation with a biological system, a well established expression system, the methylotrophic yeast *Hansenula polymorpha* (Gellissen, 2000), was used. The inoculation of the microtiter plate culture was always done from a frozen (-70°C) working cell bank (WCB) of *Hansenula polymorpha* wild type CBS4732 in YNB-WCB-medium (50 g/L glycerol, 5 g/L $(NH_4)_2SO_4$ (Fluka, Neu-Ulm, Germany) and 1.4 g/L YNB w/o ammonium sulfate and amino acids (Becton Dickinson, Franklin Lakes, USA), buffered with 100 mM Na_2HPO_4/NaH_2PO_4 (Merck, Darmstadt, Germany) phosphate buffer, at *pH* 6.0). The WCB was harvested from an exponentially growing culture with a final OD_{600nm} of 8.0. If not otherwise specified the main culture was inoculated with 5%-v/v WCB in 2xYNB medium (30 g/L glycerol, 10 g/L $(NH_4)_2SO_4$ and 2.8 g/L YNB w/o ammonium sulfate and amino acids, buffered with 100 mM Na_2HPO_4/NaH_2PO_4 phosphate buffer at *pH* 6.0).

2.4 Results and Discussion

2.4.1 OTR_{max} and k_La of 48-well MTPs

The OTR_{max} was studied over a wide range of different shaking diameters (1, 2, 3, 6, 12.5, 25, and 50 mm) and shaking frequencies (0-2000 1/min) at different filling volumes (300, 400, 500, and 600 µL). The experiments presented here were limited by the maximum possible shaking frequency for the 600 µL filling volume, which ensures no spill out of liquid from the wells. The specific mass transfer coefficient (k_La) is proportional to OTR_{max} and can easily be calculated from Eq. [2.3] for $C_L = 0$. To prove the general comparability of the applied different shaker types, the experiment at a shaking diameter of 3 mm were performed with both shaker types. The resulting data corresponded closely to each other. Therefore, only the data of the microtiter plate shaker are presented in the following figures.

Figure 2.2 shows that at low shaking frequencies the maximum oxygen transfer rate (OTR_{max}) remains at a minimum value, which is characteristic for non shaken conditions at the investigated filling volumes. At higher shaking frequencies an exponential increase of OTR_{max} is observed, a shaking diameter of 1 mm being the exception. The smaller the shaking diameter (d_0), the higher the chosen shaking frequency must be to obtain an increase of the OTR_{max} from non shaken conditions. This biphasic dependency of the OTR_{max} on the shaking frequency is specific to shaken microtiter plates and has already been observed for 96-well MTPs (Hermann et al., 2003). Such behavior is different from the steady increase of OTR_{max} with shaking frequency, which has been observed for unbaffled shaken test tubes (Danielson et al., 2004) and shake flasks (Maier and Büchs, 2001). The critical shaking frequency (n_{crit}), which is defined by a force balance between the labor of the centrifugal force against the surface tension of the liquid media in a microtiter plate well, was calculated referring to Eq. [2.9] and is also presented in Figure 2.2 for the shaking diameters from 2 to 50 mm. The calculated critical shaking frequencies (n_{crit}) agree well with the beginning of the characteristic exponential increase of the OTR_{max}. This proves that the theory presented in Hermann et al. (2003) is also applicable for 48-well MTPs. The resisting surface tension of aqueous liquids located within hydrophobic well walls seem to have the same importance in 48-well MTPs as in 96-well MTPs.

Among the studied shaking diameters presented in Figure 2.2, the d_0 of 1 mm seems to be an exception. Under these conditions the OTR_{max} of the plates is low with all shaking frequencies and any studied filling volume. Instead of an exponentially increasing behavior, the OTR_{max} remains near the baseline for the non shaken conditions over the whole range of shaking frequencies. Additionally, it could clearly be observed during the experiments that the hydrodynamics of the liquid at a d_0 of 1 mm were completely different to the others. Thus, the liquid could not follow the rotating centrifugal field of the shaker. The liquid was not spread over the well walls and was retained at the well bottom during shaking at all shaking frequencies. That means that at a shaking diameter of 1 mm the fluid seemed to be “out of phase”, a phenomenon which was discovered by Büchs et al. (2001) for shake flasks.

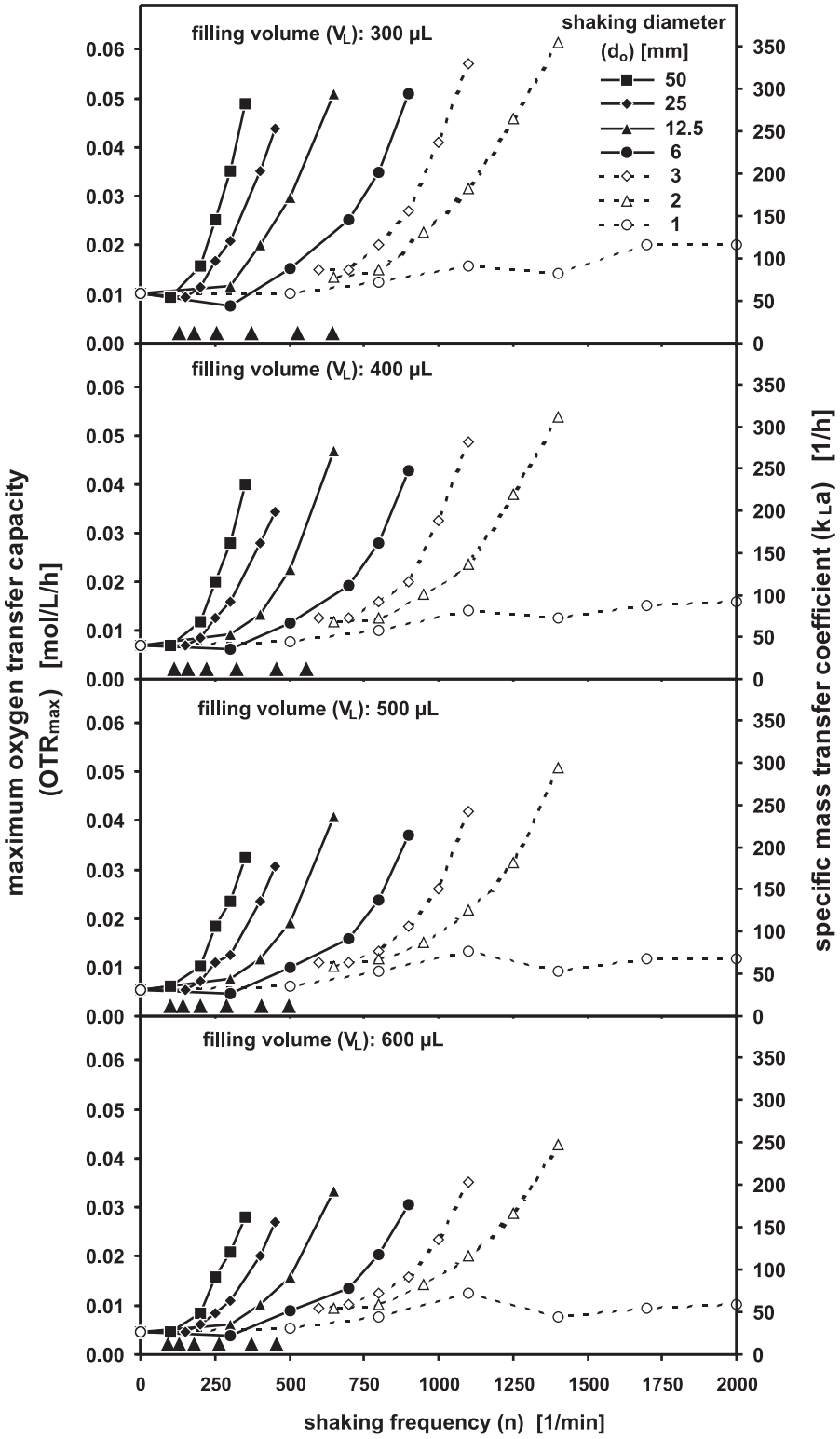


Figure 2.2: Maximum oxygen transfer capacity (OTR_{max}) and specific mass transfer coefficient (k_La) in conventional 48-well microtiter plates at different shaking diameters, shaking frequencies and filling volumes.

Experimental conditions: 25 °C, 0.5 M Na_2SO_3, 10^{-7} M $CoSO_4$, $2.4 \cdot 10^{-5}$ M Bromothymol blue, 0.012 M phosphate buffer, initial *pH* 8; the critical shaking frequencies (n_{crit}) for shaking diameters 2 to 50 mm (in order from right to left) are indicated by (▲) above the abscissa and were calculated corresponding to Eq. [2.9].

Figure 2.2 demonstrates that with the highest applied filling volume (V_L) of 600 µL similar maximum oxygen transfer capacities (OTR_{max}) of about 0.03 mol/L/h were obtained as those published for 96-well MTPs (Hermann et al., 2003). With decreasing V_L the OTR_{max} increases and reaches values of 0.05 to 0.06 mol/L/h with a filling volume of 300 µL. The maximum specific mass transfer coefficient (k_La) that could be reached under the applied conditions was 350 1/h.

2.4.2 Estimation of hydrodynamic regime with the Phase number (*Ph*)

The “out of phase” phenomenon was theoretically studied with the Phase number (*Ph*) (Büchs et al., 2000) for the most sensitive operating conditions of this work, namely for shaking diameters of 1, 2, and 3 mm and the minimum (300 µL) and the maximum (600 µL) filling volumes. The results are presented in Figure 2.3.

Figure 2.3 is divided by the critical *Ph* number of 1.26 in a upper zone of “in phase” and lower zone of “out of phase” operating conditions. Figure 2.3 points out that over the whole shaking frequency range, for the minimum and maximum applied filling volumes, the operating conditions on a rotary shaker with 1 mm shaking diameter are “out of phase”. That corresponds well with the observed results of the OTR_{max}-studies (Figure 2.2), where for all volumes the OTR_{max} in a 48-well MTP shaken at 1 mm could not significantly be increased from the baseline of non shaken plates by increasing the shaking frequency. The theoretically calculated “out of phase” conditions for 2 mm shaking diameter (Figure 2.3) are not relevant, because these conditions are predicted for lower shaking frequencies than the critical shaking frequency (n_{crit}) where the OTR_{max} is still on the baseline. The *Ph* number estimation for a d_0 of 3 mm lies well within the “in phase” region. The exponentially increasing behavior of OTR_{max} over shaking frequencies at $d_0 \geq 3$ mm strongly supports “in phase” operating conditions. As a consequence of these results, the *Ph* number seems to be a very useful tool to design new experiments not only in shake flasks but also in 48-well MTPs. This is particuarly important for researchers working with filamentous organisms or other viscous culture broths, because “out of phase” conditions are more probable at elevated viscosities.

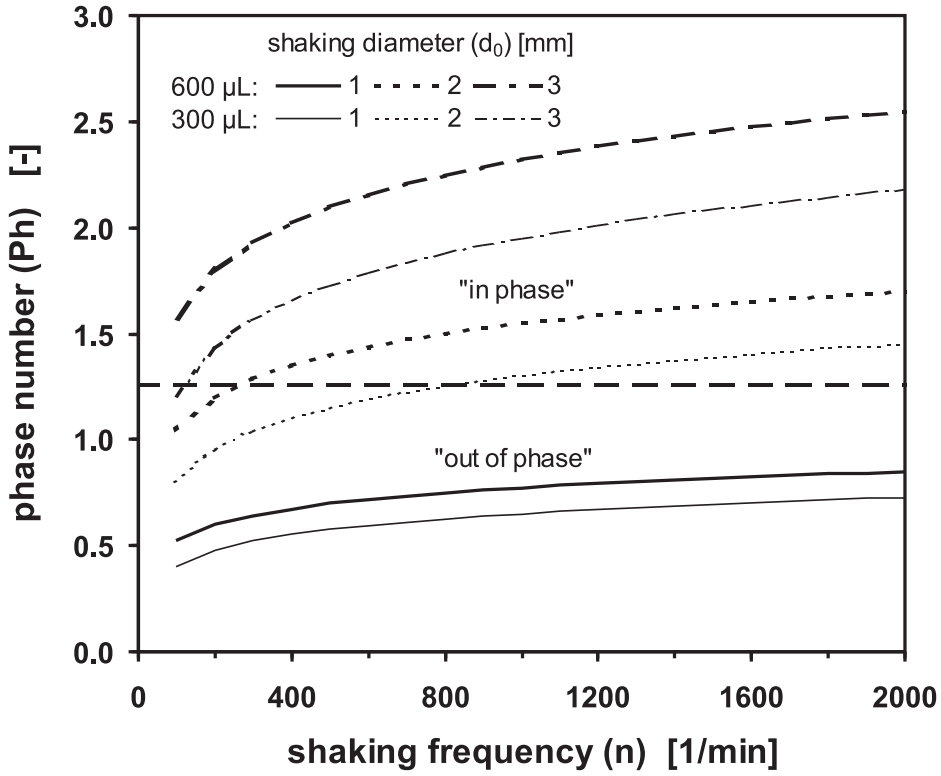


Figure 2.3: Calculated Phase number (*Ph*).

According to Eq. [2.7 and 2.8] of a water like liquid in 48-well microtiter plates for the minimum (300 µL) and maximum (600 µL) filling volumes applied in this work at shaking diameters (d_0) 1, 2, and 3 mm versus the shaking frequency (*n*), the bold dashed line (*Ph*= 1.26) separates the upper region of "in phase" from the lower region of the "out of phase" operating conditions.

2.4.3 Hydrodynamic phenomena observed at high shaking frequencies

The experiments presented in Figure 2.2 were limited by the maximum possible shaking frequency for the 600 µL filling volume, which ensures no spill out of liquid from the wells. It was interesting to study the absolute maximum oxygen transfer capacity (OTR_{max}) for small filling volumes. For these studies, the most frequent shaking diameter in industrial application, 25 mm, was used on the one hand. On the other hand a small shaking diameter of 3 mm was chosen, which is frequently used for microtiter plate shakers and does not provoke problems with respect to "out of phase" shaking conditions. The experiments were conducted with 300 µL and 400 µL filling volume up to shaking frequencies close to the "spill out"-point for each diameter. In Figure 2.4 the same results for 3 and 25 mm as in Figure 2.2 are shown with results at very high shaking frequencies (note different scale). It becomes

obvious that a very high OTR_{max} can be reached at high shaking frequencies. The results however, reveal a large difference between the two shaking diameters. While for a V_L of 300 µL at a shaking diameter of 3 mm an OTR_{max} of 0.28 mol/L/h was reached, a comparatively low OTR_{max} of 0.09 mol/L/h was obtained at a shaking diameter of 25 mm. A specific mass transfer coefficient (k_La) of up to 1600 1/h (3 mm/300 µL) is surprisingly high and lies in the range of industrial fermenters (Linek et al., 1989). Therefore, in the case of non-viscous fermentation broths, where no "out of phase" operating conditions are to be expected, a shaking diameter of 3 mm is superior to higher shaking diameters. The experiments with the two shaking diameters of 3 and 25 mm were conducted up to the maximum shaking frequency at which the liquid is spilled out. Therefore, the same liquid heights of the fluids within the well were reached. There must be a fundamental difference between the flow regimes at the different shaking diameters.

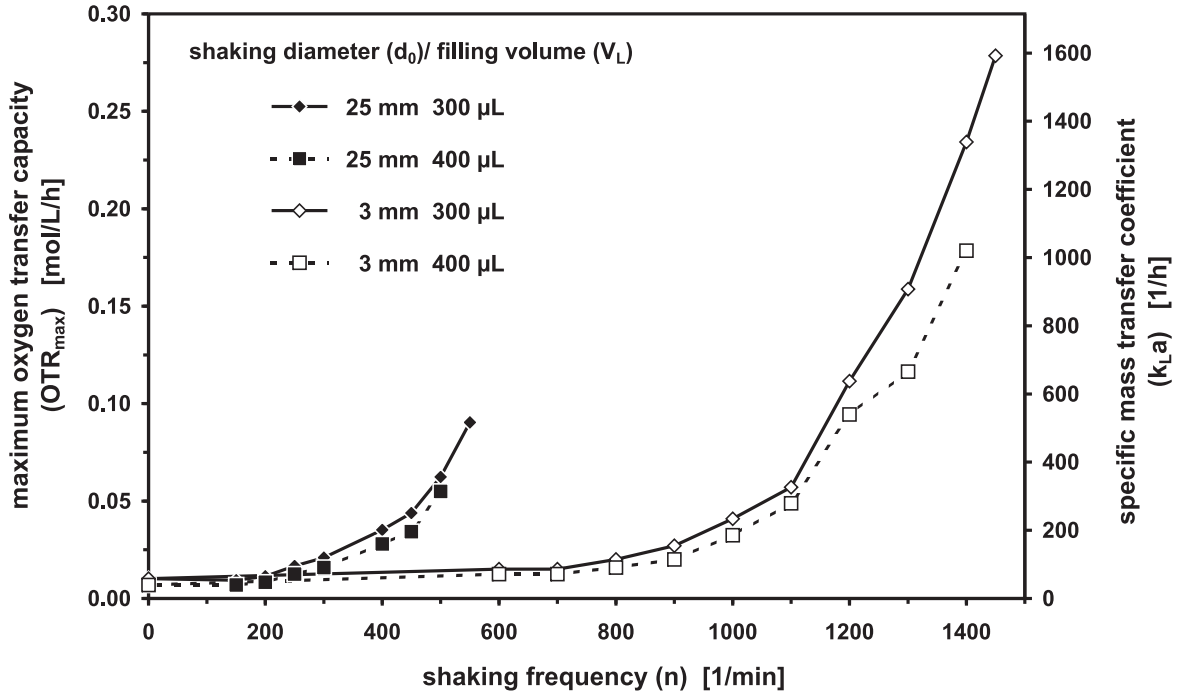


Figure 2.4: Maximum oxygen transfer capacity (OTR_{max}) and specific mass transfer coefficient (k_La) in conventional 48-well microtiter plates with small filling volumes of 300 µL and 400 µL at shaking diameters of 3 mm and 25 mm versus shaking frequency.

Experimental conditions: 25 °C, 0.5 M Na_2SO_3, 10^{-7} M $CoSO_4$, $2.4 \cdot 10^{-5}$ M Bromothymol blue, 0.012 M phosphate buffer, initial *pH* 8.

To understand the physical background of this phenomenon, the liquid flow at the two shaking diameters was photographed from the top and from the side of the

microtiter plate. In Figure 2.5 the photographs are shown. The side views clearly show that at a shaking diameter of 25 mm the fluid occupies only a part of the inner surface of the well, whereas at a shaking diameter of 3 mm the fluid is distributed over the complete inner surface of the well. The top-view confirms this. At a shaking diameter of 25 mm the fluid forms a half-moon on the well bottom, whereas at a shaking diameter of 3 mm it forms a circle. As a consequence, the thickness of the formed fluid layer on the well wall at a shaking diameter of 3 mm is significantly thinner than at a shaking diameter of 25 mm, which can contribute to a faster penetration of oxygen into the liquid. Additionally, the gas/liquid mass transfer area is larger in the case of a shaking diameter of 3 mm. This results in a higher OTR_{max}.

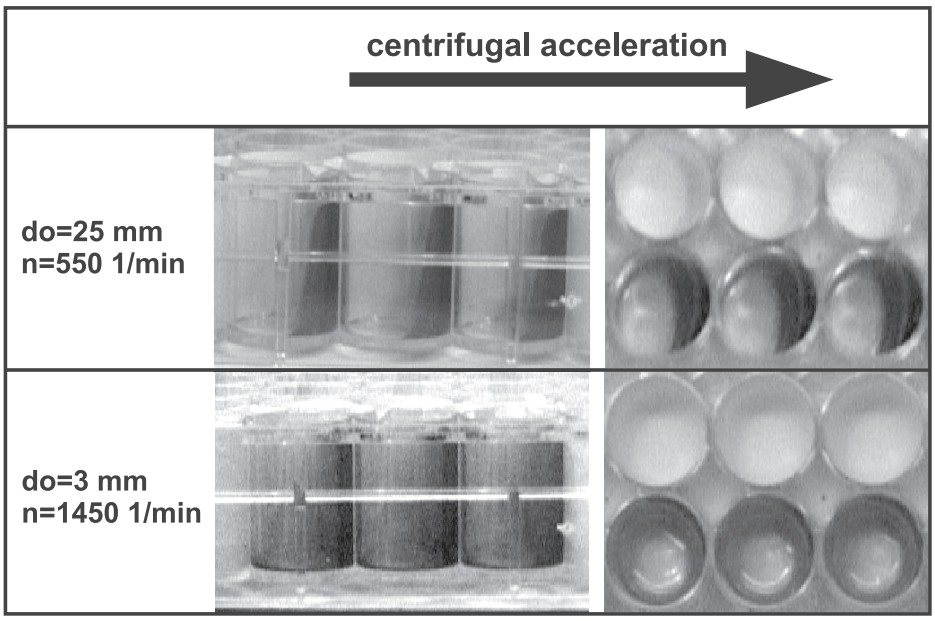


Figure 2.5: Flow regimes in 48-well microtiter plates at the maximum possible shaking frequency at 3 mm and 25 mm shaking diameter.

Experimental conditions: 300 µL filling volume, model fluid: water with Bromthymol blue.

2.4.4 Influence of shaking diameter on specific mass transfer area (*a*) and mass transfer coefficient (k_L)

Applying the enhanced reaction regime of the sulfite oxidation, the reaction takes place only at the gas-liquid interface. Thus, it became possible to determine the specific mass transfer area (*a*) (Eq. [2.4]). This investigation was performed at shaking diameters of 3 mm and 25 mm in order to study the fundamental background

of the mass transfer in 48-well MTPs on these examples. In Figure 2.6 the specific mass transfer area (*a*) and the mass transfer coefficient (k_L) for shaking diameters of 3 mm and 25 mm and for filling volumes from 200 to 600 µL are shown. The specific mass transfer area (*a*) increases exponentially with the shaking frequency (*n*) independent of the shaking diameter. For both shaking diameters a clear sequence of the specific mass transfer area (*a*) for the different filling volumes is observable. The only noticeable difference is that the specific mass transfer area (*a*) is in general slightly higher at 3 mm shaking diameter than at 25 mm. The difference Δa amounts to 350 1/m for 300 µL and 400 µL and decreases to 130 1/m and 55 1/m for 500 µL and 600 µL. The highest measured specific mass transfer area (*a)* of 2420 1/m at 3 mm and 300 µL lies well between the corresponding theoretical specific inner surface area of a cylinder of 2073 1/m and a cylinder plus bottom of 2450 1/m for a filling volume of 300 µL.

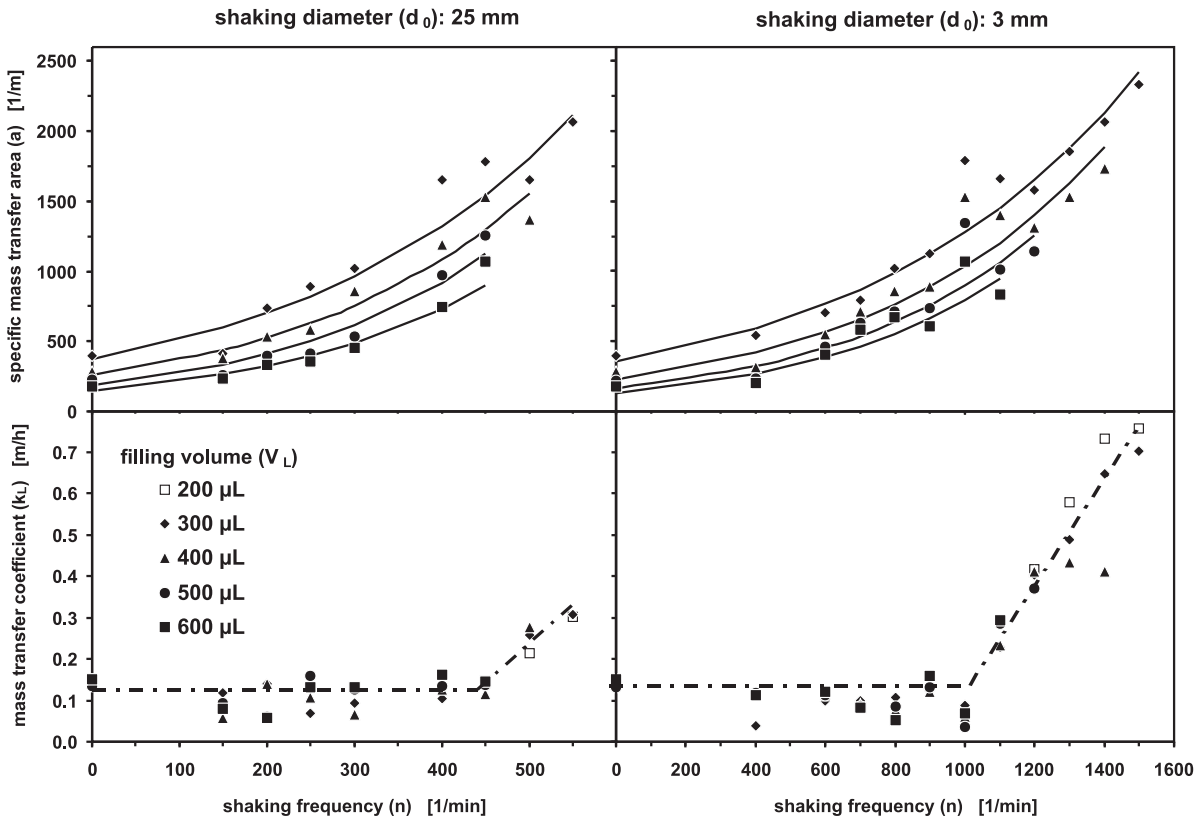


Figure 2.6: Comparison of the specific mass transfer area (*a*) and mass transfer coefficient (k_L) in conventional 48-well microtiter plates with different filling volumes at shaking diameters of 25 mm and 3 mm versus different shaking frequencies.

Experimental conditions: 25 °C, 0.5 M Na_2SO_3, 10^{-4} M $CoSO_4$, $2.4 \cdot 10^{-5}$ M Bromothymol blue, 0.012 M phosphate buffer, initial *pH* 8. Results for 200 µL filling volume are only included in the lower graphs.

The mass transfer coefficient (k_L) shown in Figure 2.6 for both shaking diameters remains at a constant level of 0.15 m/h at moderate shaking frequencies. This is similar to the measurements in conventional 96-well MTPs recently reported by Hermann et al. (2003). Starting from a critical shaking frequency of 450 1/min at a shaking diameter of 25 mm and from 1000 1/min at a shaking diameter of 3 mm, the k_L increases linearly with the shaking frequency (Figure 2.6). A sudden increase of the mass transfer coefficient (k_L) with increasing shaking frequency was also observed for square-shaped 96-deepwell MTPs above 450 1/min at a shaking diameter of 25 mm (Hermann et al., 2003). The same authers could observe no significant increase of the k_L for round-shaped 96-deepwell MTPs. In the current investigation of round-shaped 48-well MTPs, maximum k_L-values of 0.30 m/h at a shaking diameter of 25 mm and 0.76 m/h at a shaking diameter of 3 mm were reached. It should be noticed that only monotonously increasing k_L-values were encountered in shake flasks (Maier and Büchs, 2001, Maier et al., 2004).

It is noteworthy that the start of the linear increase of the mass transfer coefficient (k_L) with the shaking frequency (n) coincides with the observation that the bottom of the wells start to run dry. Thus, the main part of the liquid is distributed on the well wall and not on the well bottom. However, the detailed mechanistic reasons for the essential difference in the k_L-values between the two shaking diameters could not be elucidated in this investigation. Probably the mass transfer must be described as “two sub-reactor model” similar to shake flasks (Maier et al., 2004) to account for the different mechanisms of mass transfer in the liquid film on the well wall and the bulk of the liquid rotating within the well. In a preliminary simulation of the mass transfer coefficient (k_L) for 48-well MTPs applying the model theory of the “two sub-reactor model” developed for shake flasks (Maier et al., 2004), the experimental data lay well within the border of the Higbie´s penetration theory (describing only the liquid film) and the theory of Gnielinski (describing the rotating bulk liquid) (data not shown). For an accurate theoretical description of the mass transfer in 48-well MTPs further investigations are necessary.

2.4.5 Correlation of OTR_{max} of the chemical and biological method

In Figure 2.7, three examples of the *OTR* course over time at different shaking conditions with the same biological culture system of *Hansenula polymorpha* are given. In a biological system the OTR_{max} is reached when the aerated culture system is mass transfer limited and a constant level of the *OTR* course is obtained. Under these conditions the dissolved oxygen concentration is close to zero. Therefore, the modified RAMOS-technology for MTPs makes it possible to study whether a microbial culture is oxygen limited or not under the applied shaking conditions. The specific plateaus of the experiments at three different experimental conditions are indicated in Figure 2.7 with individual horizontal lines.

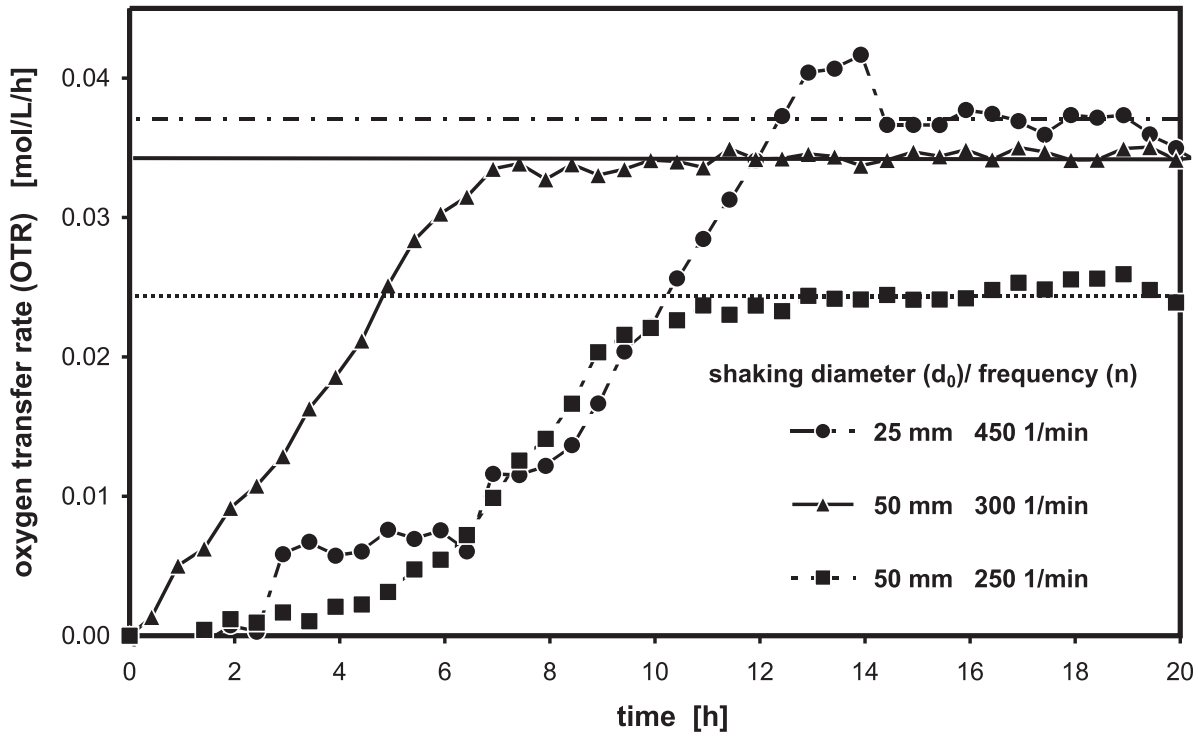


Figure 2.7: Online measurement of the oxygen transfer rate (*OTR*) during a cultivation of *Hansenula polymorpha* in 48-well microtiter plates at 25 mm and 50 mm shaking diameter at different shaking frequencies.

Experimental conditions: 500 µL filling volume, 2x YNB-Medium, *T*=37 °C. The respective maximum oxygen transfer capacities (OTR_{max}) are indicated by horizontal lines. The starting *OD* after inoculation of the culture at 50 mm and 300 1/min was *OD* 0.8, at the other shaking conditions *OD* 0.4.

Deviding these biological maximum oxygen transfer capacities (OTR_{max}) (Figure 2.7, horizontal lines) by the corresponding OTR_{max}-values of the sulfite system (Figure 2.2) results in an averaged proportionality factor (*f*) of 1.35. A proportionality factor of

larger than one means that the biological media is less concentrated than the sulfite solution and therefore, possesses a higher oxygen solubility and diffusion coefficient, which both improve the mass transfer. With the determined proportionality factor (*f)*, all the chemical OTR_{max}-values from Figure 2.2 and Figure 2.4 can be transformed to OTR_{max}-values relevant for a biological culture.

2.5 Conclusions

The choice of the adequate culture conditions for microorganisms in lab scale is still an important task. In the presented work, the gas-liquid mass transfer conditions were characterized with respect to oxygen in conventional 48-well MTPs. The influences of shaking frequency, shaking diameter, and filling volume on OTR_{max}, specific mass transfer area (*a*) and k_L were investigated. Similar to 96-well MTPs, the OTR_{max} remains constant up to a critical shaking frequency. At higher shaking frequencies the OTR_{max} increases exponentially. A phenomenon, which was formerly only described for shake flasks (Büchs et al., 2001), the “out of phase”-phenomenon, could also be verified in 48-well plates. The irregular behavior at a shaking diameter (d_0) of 1 mm could be predicted by the criteria of the Phase number (*Ph*) (Büchs et al., 2001). Thus, this shaking diameter should be abandoned for applications which need good mixing and gas-liquid mass transfer. The determination of the maximum oxygen transfer capacities (OTR_{max}) in 48-well plates using the sulfite oxidation as chemical model system revealed that very high OTR_{max} in the range of up to 0.28 mol/L/h and corresponding maximum k_La-values of 1600 1/h can be reached. The specific mass transfer area (*a*) increases exponentially with the shaking frequency, reaching values of up to 2400 1/m. The mass transfer coefficient (k_L) remains constant up to a critical shaking frequency. At higher shaking frequencies the k_L-value rises linearly. This critical shaking frequency is significantly higher than that critical shaking frequency (n_{crit}) necessary to overcome the surface tension of a liquid in small wells.

With a new device based on the RAMOS-technology (Anderlei and Büchs, 2001, Anderlei et al., 2004) it was possible to directly determine the maximum oxygen transfer capacity (OTR_{max}) of a biological culture in 48-well plates. The knowledge of

the biological OTR_{max} allowed the calculation of a proportionality factor (*f)* between the OTR_{max} of the biological system and the OTR_{max} of the chemical system, so that the obtained chemical data could be easily transformed to biological conditions. This data can serve as an ideal design tool for new experiments in the studied reactor. The 48-well MTPs proved to be an excellent alternative for microbial cultivation and expression studies combining the advantages of both the high-throughput possible with 96-well MTPs and the classical shaken Erlenmeyer flasks. The suitability of 48-well plates for most commercially available microtiter plate readers and liquid-handling systems allows fast implementation times, and finally offers the chance to automate microbial cultures under sufficient oxygen supply.

3 Characterization of Operation Conditions and Online Monitoring of Physiological Culture Parameters in Shaken 24-Well Microtiter Plates

Abstract: A new online monitoring technique to measure the physiological parameters dissolved oxygen tension *(DOT)* and *pH* of microbial cultures in continuously shaken 24-well microtiter plates (MTP) is introduced. The new technology is based on immobilised fluorophores on the bottom of standard 24-well MTPs. The sensor MTP is installed on a sensor dish reader, which can be fixed on an orbital shaker. This approach allows real online measurements of physiological parameters during continuous shaking of cultures without interrupting mixing and mass transfer like currently available technologies do. The oxygen transfer conditions at one constant shaking frequency (250 1/min) and diameter (25 mm) was examined with the chemical sulfite oxidation method. Varied filling volumes (600–1200 µL) of *Escherichia coli* cultures demonstrated the importance of sufficient oxygen transfer to the culture. Cultures with higher filling volumes were subjected to an oxygen limitation, which influenced the cell metabolism and prolongated the cultivation time. The effects could be clearly monitored by online *DOT* and *pH* measurements. A further study of different media in an *Escherichia coli* fermentation elucidated the different growth behaviour in response to the medium composition. The microtiter plate fermentations correlated very well with parallel fermentations in shake flasks. The new technique gives valuable new insights in biological processes at a very small scale, thus enabling parallel experimentation and shorter development times in bioprocessing.

3.1 Introduction

In parallel with the expansion of gene sequencing and increasing protein structure data, the knowledge to create new biomolecules is rising, and there seems to be an unlimited pool of new products. Due to the fact that many of these new molecules can only be produced by fermentation and cell culture, there exists a great interest in new processing techniques. In order to process large numbers of different clones and to handle the complexity of the biological systems, the industry turned to high-throughput screening technologies (Lye et al., 2002, Boettner et al., 2002). Coming from parallel shake flasks cultures, many companies switched to microtiter plates as cultivation and screening platform. The advantages of these miniaturized bioreactors include the possibility to operate many simultaneous cultures on a small space. Additionally, the standardized microtiter plate design is ideally suited for automated systems (Lye et al., 2002, Kumar et al., 2004, Hermann et al., 2001). If microtiter plates, as any other bioreactors, are used for the cultivation of microorganisms, care has to be taken to operate these bioreactors at suitable conditions. Basic characterization of mixing and oxygen transfer in microtiter plates were yet performed by different authors (Hermann et al., 2001 and 2003, Weiss et al., 2002, John et al., 2003). The results demonstrated that high shaking frequencies are required to provide sufficient mixing and suitable oxygen transfer conditions for microbial cultures.

For online monitoring of cultures in microtiter plates, new miniaturized sensors were implemented in these plates. Especially, optical fluorescence sensors were preferred, because of their small size, high precision, low cost and the possibility of non-invasive measurement (Kumar et al., 2004, Weiss et al., 2002, John et al., 2003b). The information from fluorescence sensors can be acquired by measuring the fluorescence intensity or the fluorescence lifetime (Bambot et al., 1994, Huber et al., 2000). General problems with measurements of fluorescence intensity include fluctuations in light intensity and detector sensitivity, irregular sensor spots, light scattering, and intrinsic fluorescence of the sample. The use of a second internal standard fluorophore is a promising strategy. Though, fluorescence lifetime is the more reliable solution, because the fluorophores can be intrinsically referenced, and,

therefore, are independent of fluctuating light intensities. The fluorescence lifetime can be detected in either the time domain or the frequency domain. The use of fluorophores with long half-life (in the order of microseconds), which are typically used in optical oxygen sensors, enables inexpensive detector systems containing light-emitting diodes (LEDs) and photodiodes (Holst et al., 1998, Hartmann et al., 1997). Additionally, the intrinsic fluorescence of complex biological matrices can be eliminated in this time regime, because the fluorescence of biological material usually decays completely within 100 ns. The fluorescence sensors (optodes) are immobilized in the middle of the MTP wells and are measured through the optically transparent bottom with an optical fibre (Weiss et al., 2002, John et al., 2003 and 2003b, Bambot et al., 1994). Commercially available fluorescence intensity based sensor microtiter plates can be read out with conventional fluorescence readers. The apparent drawback of this method is the poor shaking performance of these readers (too low shaking frequencies at small shaking diameters of 1 to 3 mm (John et al., 2003)) and the interruption of MTP shaking during the measurement. This can lead to limited oxygen transfer and mixing. As a result, the microorganisms or cells are possibly affected and change their metabolism. Additionally, the oxygen concentrations drop rapidly and measurement errors are unavoidable (John et al., 2003).

In this study a new online measurement technique for continuously shaken 24-well microtiter plates is presented. The new technique distinguishes between the currently available techniques (Weiss et al., 2002, John et al., 2003, Bambot et al., 1994) due to the direct mounting of the measurement optics and electronics on the shaker plate of a regular orbital shaker, thus, avoiding poor shaking performance and the interruption of MTP shaking of the currently available techniques. To validate the technique, growth of bacterial cultures is investigated. The oxygen transfer characteristics of the 24-well MTP is determined at one constant shaking condition for different filling volumes. The influence of different filling volumes on the culture growth is demonstrated by monitoring of the physiological parameters *DOT* and *pH* . The kinetics of *Escherichia coli* cultures in different media are compared with oxygen transfer rate results from shake flasks obtained from the Respiration Activity Monitoring System (RAMOS, see below).

3.2 Materials and Methods

3.2.1 Sensor Dish Reader for optical *DOT* and *pH* measurements in MTPs

The sensor dish reader is a commercially available 24-well MTP-reader. It was developed and provided by Precision Sensing GmbH (Regensburg, Germany). The reader consists of a controller and a compact box with 24 single optical read out units, which are localized in the center below each well of a 24-well MTP (Figure 3.1). Each read out unit consists of an excitation light source (LED) and a photon detector (photo diode). The excitation light and fluorescence light are both directed through optical filters (see Figure 3.1). The sensor dish reader was optimized by the supplier of the device that way, that no difference in the signal to noise ratio were notable between shaking and resting conditions. The *DOT*- and *pH*-measurement were conducted with specially prepared standard 24-well microtiter plates (OxoDish OD-24 and HydroDish HD-24, Precision Sensing GmbH, Regensburg, Germany). In the middle of each well of the 24-well MTP a fluorescence optode was immobilised. MTPs with only one type of optodes, for *DOT* or *pH* measurement, were available. Thus, measurements of *DOT* and *pH* could only be conducted in subsequent experiments. Both types of optodes, *DOT* and *pH*, were measured in the frequency domain detection mode. While *DOT* was calculated from simple phase-shifts of the emitted light, *pH* was analysed by the dual lifetime referencing (DLR) method (Hartmann et al., 1997).

All measurements with the sensor dish reader were performed in a dark room. The *DOT* optodes were calibrated with 10 g/L sodium sulfite solution for zero reading and air saturated water (aqua bidest.) for the 100% reading. The *pH* optodes were calibrated with a six point calibration at *pH* 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. As calibration solutions acetate, phosphate and TRIS buffers with constant ionic strength of 120 mmol/L NaCl (Fluka, Neu-Ulm, Germany) were used. All calibrations were performed before each experiment at the experimental temperature. The intrinsic fluorescence of each medium caused some slight deviations of the *DOT* values from 100% and 0%. Thus, a background correction of the measured data of the *DOT*-optodes were necessary. Therefore, the *DOT* signals were recalibrated after the cultivation experiments. The measured phase shift at the end of the culture was

taken as 100% *DOT* and the phase shift of a spontaneous baseline during exponential culture growth (e.g. Figure 3.3, 1200 µL) was taken as 0% *DOT*. The recalibration was calculated with a modified Stern-Volmer equation, which was implemented in an oxygen calibration software provided by the manufacturer of the sensor dish reader (Precision Sensing GmbH, Regensburg, Germany).

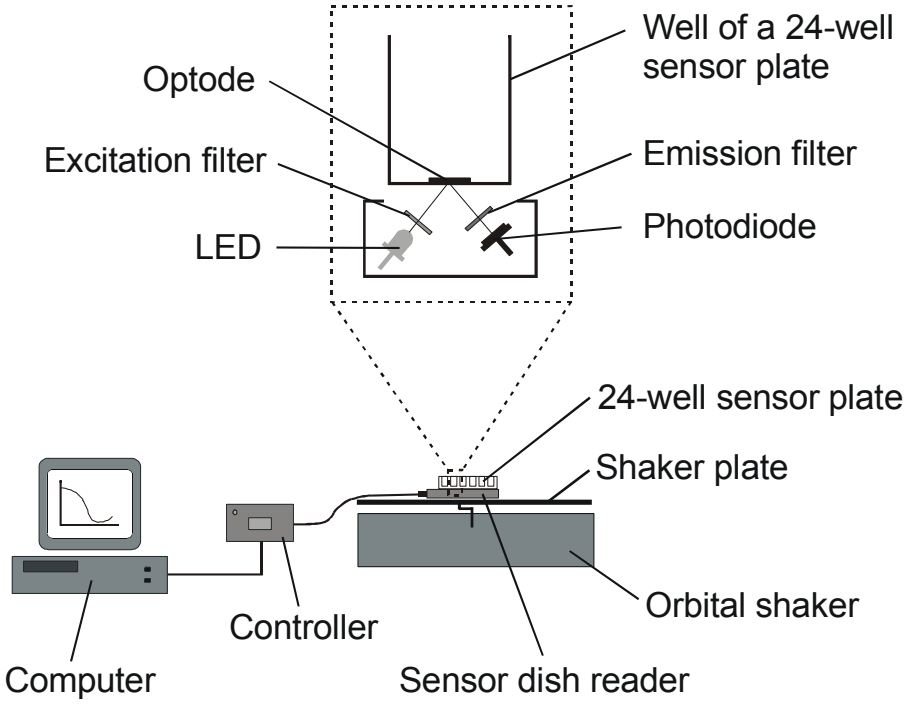


Figure 3.1: Experimental set up for the optical measurement of dissolved oxygen tension (*DOT)* and *pH* in shaken 24-well microtiter plates.

3.2.2 Characterization of oxygen mass transfer in 24-well MTPs

To characterize the oxygen mass transfer conditions in 24-well MTPs at one fixed shaking frequency (n: 250 1/min) and shaking diameter (d_0: 25 mm) with different filling volumes (V_L: 400, 500, 600, 800, 1000 and 1200 µL), the chemical sulfite oxidation method was applied (Kosch et al., 1998). Hermann et al. (2001 and 2003) modified this method for the application in small scale reactors without requiring any oxygen probe by applying a *pH* indicator, which was used to indicate the end of the complete oxidation. Thus, it was possible to determine the maximum oxygen transfer capacity (OTR_{max}) by simple stoechiometric balancing of the oxidation reaction. The maximum oxygen transfer capacity (OTR_{max}) is generally defined as:

$$OTR_{\max} = k_L a \cdot C_{O2}^{*} = k_L a \cdot L_{O2} \cdot p_G \qquad (3.1)$$

With the knowledge of the oxygen solubility in the liquid phase (L_{O2}) ($7.46 \cdot 10^{-4}$ mol/L/bar at 30°C according to Weisenberger and Schumpe (1996)) and the oxygen partial pressure in air (p_G) (0.2095 bar), the volumetric mass transfer coefficient ($k_L a$) can be calculated.

The experiments were performed by shaking an unsealed 24-well MTP with *DOT*-optodes together with the sensor dish reader (Figure 3.1) at 30°C, a shaking frequency of 250 1/min and at a shaking diameter of 25 mm (Lab-Shaker LS-W, Kühner AG, Basel, Switzerland). Different from Hermann et al. (2001 and 2003) the sulfite system (0.5 M Na_2SO_3 (98% purity, Carl Roth GmbH & Co. KG, Karlsruhe, Germany), 10^{-7} M $CoSO_4$ (Fluka, Neu-Ulm, Germany), 0.012 M Na_2HPO_4/NaH_2PO_4 phosphate buffer (Merck, Darmstadt, Germany), initial *pH* 8) were used without the *pH*-indicator Bromothymol blue because the depletion of sulfite oxidation could directly monitored by the *DOT* increase at the end of the reaction. Generally the experiments last between 7 to 20 hours at the applied conditions.

In order to transfer the oxygen transfer conditions to biological culture systems one have to take into account that the OTR_{max} is slightly different due to physico-chemical differences of biological media in comparison to the sulfite system. The differences are caused by different oxygen diffusion coefficients (D_{O2}) and oxygen solubilities in the liquid phase (L_{O2}). In case of synthetic media these parameters can be estimated by methods presented by Akita (1981) and Weisenberger and Schumpe (1996). In case of complex media these parameters can not be calculated theoretically. In former works these effects were lumped together in a proportionality factor (*f*), where the biological maximum oxygen transfer capacities (OTR_{max}) were related to the corresponding OTR_{max}-values of the sulfite system (Kensy et al., 2005, Duetz and Witholt, 2004, Maier et al., 2004). The proportionality factor (*f*) normally will be in the range of 1 to 3 due to the fact that biological media are less concentrated than the applied sulfite solutions (Kensy et al., 2005, Duetz and Witholt, 2001, Maier et al., 2004). The most convenient way to determine the proportionality factor (*f*) is to

measure the OTR_{max} (under oxygen limitation, where C_L is 0%) in the biological culture and in the sulfite system at the same operation conditions (V_L, n, d_o) with the respiration activity monitoring system (RAMOS, see below). With the knowlegde of the proportionality factor (f) and the OTR_{max} from the sulfite system, it is now possible to choose adequate operation conditions during fermentations that allow sufficient oxygen supply.

3.2.3 Cultivation of *E. coli* cultures in MTPs and RAMOS

Escherichia coli JM109 (ATCC 53323, DSMZ, Braunschweig, Germany) was used for culture studies in 24-well MTPs with different filling volumes. The strain was maintained in glycerol stocks (LB-medium with 50 g/L glycerol) at –70 °C. The experiment with the different filling volumes in 24-well MTP were directly inoculated from the glycerol stocks with 5%-v/v of the filling volume resulting in an initial OD_{600nm} of 0.3 (0.12 g/L). It should be noted that due to the direct inoculation from the glycerol stocks into the main culture also a small amount of glycerol was transfered. Therefore, the culture medium contains 2.5 g/L glycerol and is termed LB2.5G-medium in this work. The 24-well MTP was incubated on the sensor dish reader at 30°C, a shaking frequency of 250 1/min and at a shaking diameter of 25 mm (Lab-Shaker LS-W, Kühner AG, Basel, Switzerland). *Escherichia coli BL 21 (DE3) pET3-alcdh* (*E. coli ADH*) is a strain expressing an alcohol dehydrogenase (*acldh*), which was kindly delivered by the laboratory of Prof. Tadao Oikawa (Department of Biotechnology, Kansai University, Osaka, Japan). This strain was used in the RAMOS experiments and in the media studies in the 24-well MTP. The strain was maintained on LB-agar Petri dishes at 4°C. The inoculum culture was incubated over night in 20 ml LB medium in a 250 ml-Erlenmeyer flask at 30°C at a shaking frequency of 300 1/min and a shaking diameter of 50 mm. Cells were separated from the inoculum medium and resuspended in the main culture medium. After inoculation, the culture started with an *OD*600nm of 0.5. The 24-well MTP was incubated on the sensor dish reader at 30°C, a shaking frequency of 250 1/min, and a shaking diameter of 25 mm. During these experiments the 24-well microtiter plates were sealed with gas permeable adhesive seals (art.-no.: AB-0718, Abgene, Hamburg, Germany) to protect the culture from contamination.

The experiments were carried out with three different types of bacteria culture media: complex, semi-synthetic and synthetic. The media had the following composition: Luria Bertani medium (LB): 10 g/L tryptone (Difco from Becton Dickinson, Franklin Lakes, USA), 5 g/L yeast extract (Carl Roth GmbH & Co. KG, Karlsruhe , Germany) and 5 g/L NaCl. The *pH* was close to 6.7 without adjustment. All components were mixed and dissolved in deionized water and autoclaved for 20 min at 120 °C. Glucose M9Y medium (GM9Y): 5.0 g/L yeast extract (Carl Roth GmbH & Co. KG, Karlsruhe , Germany), 1.0 g/L NH_4Cl, 0.5 g/L NaCl, 6.0 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.011 g/L $CaCl_2$, 0.241 g/L $MgSO_4$, *pH*= 7.0. All components were mixed and dissolved in deionized water and autoclaved for 20 min at 120 °C. After autoclaving, 4.0 g/L glucose (Carl Roth GmbH & Co. KG, Karlsruhe , Germany) was added to the solution after separate autoclaving in a 200 g/L glucose stock solution. The WR-medium is a completely synthetic medium and is normally used for high cell density fed-batch fermentations. It was prepared according to (Wilms et al., 2001). All chemicals were delivered by Fluka (Neu-Ulm, Germany) unless specified otherwise.

Recently, a Respiration Activity Monitoring System (RAMOS, Hitec Zang, Herzogenrath, Germany) for the online determination of the oxygen transfer rate (*OTR*) and carbon dioxide transfer rate (*CTR*) in shake flasks has been introduced and described in detail (Anderlei and Büchs, 2001, Anderlei et al., 2004). In this work RAMOS was used as reference to monitor the culture growth in the described media. All experiments were performed with 10 ml filling volume in a modified 250 ml-Erlenmeyer shake flask (see Anderlei and Büchs, 2001, Anderlei et al., 2004). The cultures were incubated at 30°C at 250 1/min (d_o= 50 mm).

3.3 Results and Discussion

3.3.1 Influence of the filling volumes (V_L) on the oxygen transfer capacity (OTR_{max})

The OTR_{max} was studied at different filling volumes (400, 500, 600, 800, 1000 and 1200 µL) and constant shaking conditions, at a shaking diameter (d_0) of 25 mm and a shaking frequency (n) of 250 1/min. The data are presented in Figure 3.2 versus the reciprocal filling volume ($1/V_L$) to illustrate the dependencies of the oxygen

transfer. A straight linear increase of the OTR_{max} and k_La over the reciprocal filling volume is notable. Similar as Hermann et al. (2003) and John et al. (2003) reported for 96-well MTPs, the oxygen transfer depends only on the gas-liquid interfacial area (*A*), which is a typical property of surface aerated reactors (Hermann et al., 2003, John et al., 2003). At moderate shaking conditions the gas-liquid interfacial area (*A*) remains constant at the different filling volumes, thus with increase of the filling volume the specific mass transfer area (*a*) (*a*= *A/V*) and thereby the OTR_{max} and k_La decrease. The upper OTR_{max} and k_La-values are slightly higher than the OTR_{max} and k_La-values of 96-well MTPs measured by Hermann et al. (2003) at 22°C, whereas they are much lower as values reached in 48-well MTP at much higher shaking frequencies (Kensy et al., 2005).

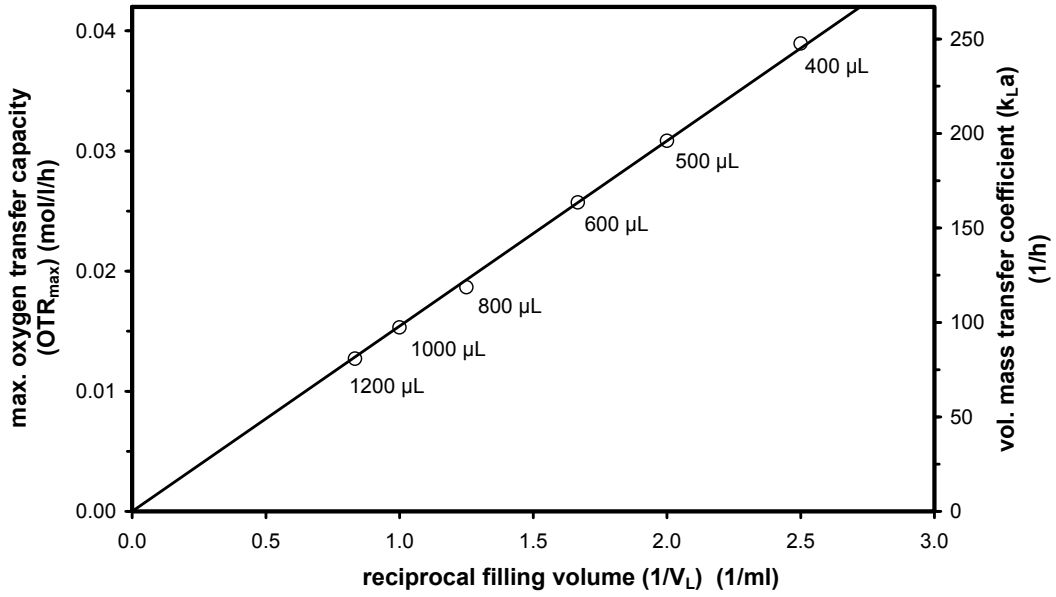


Figure 3.2: Maximum oxygen transfer capacity (OTR_{max}) and volumetric mass transfer coefficient (k_La) in conventional 24-well microtiter plates at different filling volumes.

Experimental conditions: 400-1200 µL at a shaking diameter (d_0) of 25 mm and a shaking frequency (*n*) of 250 1/min presented versus the reciprocal filling volume ($1/V_L$); 30 °C, 0.5 M Na_2SO_3, 10^{-7} M $CoSO_4$, 0.012 M phosphate buffer, initial *pH* 8.

By this experiment, it became obvious that filling volumes of 400 µL and 500 µL are not very adequate for the online measurement of physiological parameters like *DOT* or *pH*. The immobilized optodes in the middle of the wells are not covered by liquid at this filling volumes, thus, no correct detection of this parameters is possible. Depending on the evaporation rate during the cultivation this problem could occur

even with higher filling volumes. Under the studied conditions 33% of the water in the wells evaporated until the end of the experiment after 20.5 hours, because no further precautions were taken to prevent evaporation in this experiment.

3.3.2 Cultivation of *E. coli* at different filling volumes

Cultivations of *E. coli JM 109* in LB2.5G-medium were conducted at filling volumes of 600, 800, 1000, and 1200 µL. Huge differences in the *DOT* and *pH* courses of each single batch can be noticed (Figure 3.3). The *DOT* and *pH* measurements were performed in different subsequent experiments with the same inoculation (from glycerol stock) and culture conditions. The *DOT* values for the highest filling volume show the fastest drop until oxygen limited conditions are reached. The *pH* courses indicate only small deviations until 3.8 h of cultivation. When the cultures reached oxygen limitation, indicated by very small *DOT* values, the *pH* starts to decrease linearly in sequence from the highest to the lowest filling volume (V_L). It is well known that an oxygen limitation of aerobic bacterial cultures can induce the production of organic acids like acetic acid (Losen et al., 2004). Therefore, a stronger oxygen limitation will result in a more pronounced decrease of the *pH*, as illustrated in Figure 3.3. The spontaneous increase of the *DOT* signals indicate the depletion of the primary carbon sources (glycerol together with yeast extract). At the same time also the *pH* signal exhibits a turning-point. With the start of the metabolization of a second carbon source of the LB2.5G-medium, the *pH* increases. This increase continues until the end of the cultivation. During this increase of the *pH* signals, the *DOT* signals drop down to low levels again and finally increase after all carbon sources are exhausted. It can be assumed that the organic acid produced during the oxygen limitation is reutilized by the organism, which leads to the increase of the *pH* signal as depicted in Figure 3.3 (Losen et al., 2004).

During the second growth phase again a clear separation of the *DOT* and *pH* courses can be noticed between cultures performed at the different filling volumes (V_L). This results in a maximum prolongation of the cultivation of 4 hours between the smallest filling volume (600 µL) and the largest filling volume (1200 µL).

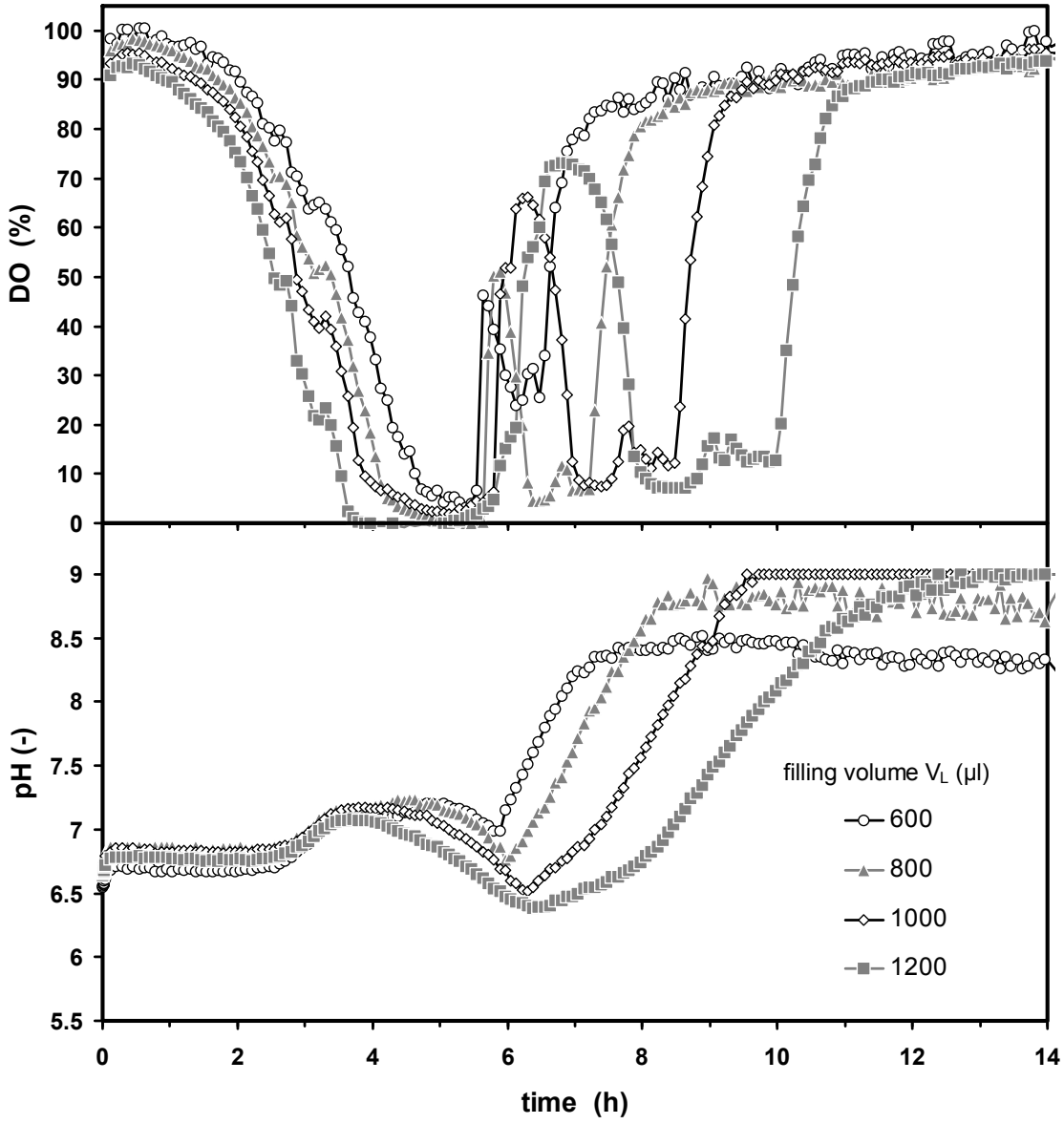


Figure 3.3: Impact of different filling volumes (600-1200 µL) on dissolved oxygen tension (*DOT*) and *pH* of a biological culture of *E. coli JM 109* in LB2.5G-medium.

Experimental conditions: measured at a shaking diameter (d_0) of 25 mm and a shaking frequency (n) of 250 1/min, 30 °C.

This very simple example of varying oxygen transfer conditions (adjusted by different filling volumes at otherwise constant shaking conditions) demonstrates the importance of suitable culture conditions. Unknown oxygen transfer conditions can lead to misinterpretations of the experimental results. Especially, in the case of functional genomic and proteomic research it might be important to avoid unexpected gene regulation and protein translation effects. The new technique can help to clarify

gene regulation and protein translation under different oxygen transfer or enviromental (e.g. *pH*) conditions in microfermentations.

3.3.3 Monitoring of *E. coli* cultures in different media

In the following experiment the sensor dish reader was used to monitor cultivations of *E. coli ADH* in different media. Again, the *DOT* and *pH* measurements were performed in different subsequent runs with the same inoculation and culture conditions. As reference the same cultivations were performed in the RAMOS system and the oxygen transfer rates (*OTR*) were monitored during the fermentations. Figure 3.4A clearly depicts the typical characteristics of bacterial growth on the different media. Different OTR_{max} were reached. The *E. coli ADH* culture on LB-medium reached an OTR_{max} of 0.022 mol/L/h similar as reported before (Losen et al., 2004). Higher OTR_{max} could be reached on the semi-synthetic medium GM9Y (0.051 mol/L/h) and on the full synthetic medium WR (0.057 mol/L/h). After an initial exponential increase of the *OTR,* all media show a second phase, where residual carbon source or by-products were metabolised. Due to the different nature of the media the cultivations ended at different times. Whereas in the media with complex ingredients (LB, GM9Y) the metabolic activity terminated at around 6 hours, *E. coli ADH* required 14 hours to complete growth on pure synthetic medium (WR).

In comparison to the RAMOS-cultivations the measured *DOT* and *pH* data of these different cultivations are presented in Figure 3.4B-D. The experiments were conducted with a filling volume (V_L) of 600 µL to guarantee a maximum oxygen transfer (among the applied filling volumes here, see comments on Figure 3.2) and sufficient volume for a steady coverage of the optode by the culture liquid (see evaporation problems above).

The *DOT* courses in the different media appear as inverse projections of the respective *OTR* courses in RAMOS. The *DOT* minimum occur at very similar times as the *OTR* peaks (LB, GM9Y: ~3-4 h and WR:~8-9 h).

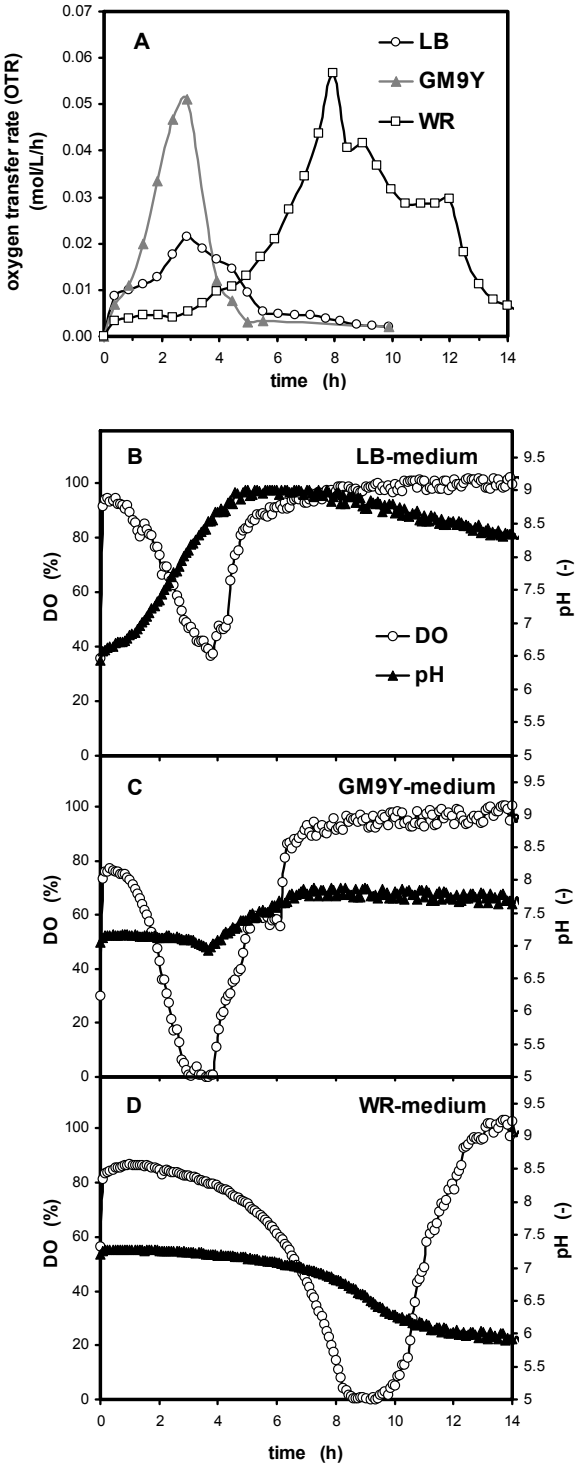


Figure 3.4: Comparison of the oxygen transfer rate (*OTR*) with *DOT* and *pH* over time of *E. coli ADH* in three different standard culture media (LB, GM9Y and WR-medium).

Experimental conditions: A: measured in a RAMOS device; 30 °C, filling volume: 10 mL, shaking diameter (d_0): 50 mm, shaking frequency (n): 350 1/min, bioreactor: modified 250 mL-Erlenmeyer flasks (Anderlei and Büchs, 2001). B-D: measured in the Sensor Dish Reader, *E. coli ADH* in the same culture media for bacteria (B. LB-, C. GM9Y-, and D. WR-medium) incubated in a 24-well microtiter plate; 30 °C, filling volume (V_L): 600 µL, shaking diameter (d_0): 25 mm and shaking frequency (n): 250 1/min.

Obviously, cultures on the GM9Y- and the WR-medium in the 24-well MTPs were subjected to a slight oxygen limitation as the *DOT* reached very low levels. The respiration activity in the 24-well MTP finally terminates at around 6 h for the LB- and GM9Y-medium and at 14 h for the WR-medium, which is very similar to the cultures in the RAMOS system (Figure 3.4A).

The *pH* course of the cultures can provide additional information. The steady increase of the *pH* value on LB-medium (Figure 3.4B) is typical for this medium (Losen et al., 2004). This occurs due to the deamination of amino acids or peptides and the release of ammonia to the medium (Losen et al., 2004, Alfoldi and Rasko, 1970, Robbins and Taylor, 1989).

In GM9Y-medium (Figure 3.4C), the turning point at 3.8 h indicates the switch of metabolism from the consumption of the first carbon source (glucose) to the second (yeast extract). The subsequent increase of *pH* is typical for growth on complex media components. Synchronously with the end of respiration activity, the *pH* became stationary. The culture in WR-medium (Figure 3.4D) displays a typical *pH* course of cultures growing on synthetic media with steadily decreasing *pH* (Wilms et al., 2001).

3.4 Conclusions

A new technique to measure *DOT* and *pH* online in microbial cultures is presented. It permits online measurements of these physiological parameters in 24-well microtiter plates during continuous shaking of the culture. It is possible to monitor the real physiological state of the microorganisms without interrupting mixing and mass transfer. Thus, especially in case of *DOT* measurements the problems associated with interrupted shaking operation, typical for read-out with common MTP readers (John et al., 2003), can be avoided. Measurements with *E. coli* cultures at different filling volumes demonstrated the importance of adequate and sufficient oxygen transfer. Neglecting these aspects may result in prolongation of experiments, formation of anaerobic by-products and misinterpretation of results. Therefore, the characterization of oxygen transfer at the specific experimental conditions should be

in any case obligatory. The presented chemical model system offers an easy way to characterize the oxygen transfer. The study of different media in 24-well MTP elucidated that the medium composition has great impact on the oxygen uptake rate of the cells. It became apparent that at the applied shaking conditions weak oxygen limitations occurred with two media (GM9Y and WR). The online measurements of *DOT* and *pH* worked reliably and gave important process information at a very small scale. The operation conditions with the described set up applied for biological culture experiments depend strongly on the oxygen requirement of the investigated microorganisms and on the prerequisite that the culture liquid is in contact with the optodes. Evaporation can cause problems, so that optodes may not be covered with culture liquid during the experiment. Humidity control in the incubator or improved gas-permeable membranes with less evaporation can solve this problem. Generally, the new technique provides an excellent tool for strain screening, media development, toxicity screening and many more automated screening applications.

4 Validation of a High-Throughput Fermentation System based on Online Monitoring of Biomass and Fluorescence in Continuously Shaken Microtiter Plates

Abstract: An advanced version of a recently reported high-throughput fermentation system with online measurement, called BioLector, and its validation is presented. The technology combines high-throughput screening and high-information content by applying online monitoring of scattered light and fluorescence intensities in continuously shaken microtiter plates. Various examples in calibration of the optical measurements, clone and media screening and promoter characterization are given. Bacterial and yeast biomass concentrations of up to 50 g/L cell dry weight could be linearly correlated to scattered light intensities. In media screening, the BioLector could clearly demonstrate its potential for detecting different biomass and product yields and deducing specific growth rates for quantitatively evaluating media and nutrients. Growth inhibition due to inappropriate buffer conditions could be detected by reduced growth rates and a temporary increase in NADH fluorescence. *GFP* served very well as reporter protein for investigating the promoter regulation under different carbon sources in yeast strains. A clone screening of 90 different *GFP*-expressing Hansenula polymorpha clones depicted the broad distribution of growth behavior and an even stronger distribution in *GFP* expression. The importance of mass transfer conditions could be demonstrated by varying filling volumes of an E.coli culture in 96-well MTP. The different filling volumes caused a deviation in the culture growth and acidification both monitored via scattered light intensities and the fluorescence of a *pH* indicator, respectively. The BioLector technology is a very useful tool to perform quantitative microfermentations under engineered reaction conditions. With this technique, specific yields and rates can be directly deduced from online biomass and product concentrations, which is superior to existing technologies such as microplate readers or optode-based cultivation systems. In particular, applications with strong demand on high-throughput such as clone and media screening and systems biology can benefit from its simple handling, the high quantitative information content and its capacity for automation.

4.1 Introduction

In order to process large numbers of different clones and to handle the enormous complexity of biological and biochemical systems in modern biotechnology, many research groups have developed new microbioreactor systems. In industry and academic research, the demand for high-throughput and high information content about time-dependent processes has driven the development of microbioreactors. A wide variety of individual solutions for microbioreactors has been suggested and prototyped. Among these, miniature stirred tank reactors (Betts and Baganz, 2006), gas-inducing impeller systems (Puskeiler et al., 2005), bubble columns including *in-situ* electrochemical oxygen generation (Doig et al., 2005, Maharbiz et al., 2004) and a combination of magnetic stirrer and membrane surface aeration (Zanzotto et al., 2004) have been developed. Whereas these research groups mainly developed individual solutions for their own laboratories, only a few systems have been commercialized and, therefore, are accessible for a broader research society.

One commercial solution is the SimCell system from Bioprocessors, Inc.. *pH*, dissolved oxygen tension (*DOT*) and optical density (*OD*) values can be recorded in a separate reading station apart from the incubation chamber. This system does not provide sufficient oxygen to microbial cells (specific mass transfer coefficient k_La= 10 1/h, SimCell, 2009) and, thus, is limited to applications with higher eukaryotic cells such as mammalian cell cultures.

Another miniature bioreactor system (MBR) provided by Microreactor Technologies, Inc. is based on a shaken and gas-sparged 24-well microplate with measurement and control of temperature, *pH* and *DOT*. Maximum k_La values of 56 1/h have been reported for shaking frequencies of 800 1/min and non-sparged conditions (Isett et al, 2007).

Whereas new designs of microbioreactors require adapted infrastructure, microtiter plates (MTPs) are already the industrial standard in biotechnology. Therefore, it would be most convenient to maintain this standard and properly adapt operation conditions, geometric design of wells and the measurement method to researcher's

needs. Betts and Baganz (2006) have reported that unlike other MBRs, MTPs have the unique advantage, that they provide intrinsic high-throughput capacity and allow automation. MTPs are well characterised in respect to mass transfer. Relatively small k_La values of 150-160 1/h are found for standard round 96-well plates (Hermann et al., 2003, Ortiz-Ochoa et al., 2005), whereas very high k_La values of up to 860 1/h and 1600 1/h are found for square 96-deep well plates and standard round 48-well plates at 200 µL and 300 µL filling volume, respectively [Hermann et al., 2003, Kensy et al., 2005). These high k_La values can only be achieved with very small filling volumes which are often not compatible for further offline analysis or for online analysis in small wells. When working on a small scale, one may likely gain more insight into the micro reactions. Sampling further reduces the filling volume and probably interferes with the reaction. Therefore, online measurements are most favorable.

The most popular and widespread measurement techniques for MTPs are microplate readers. They are equipped with different optical arrangements and generally detect absorbance or fluorescence. Lu et al. (2004) performed a study on promoter regulation in *E.coli* with green fluorescent protein (*GFP*) as reporter protein in a standard microplate reader. Common microplate readers have only very poor shaking capacities and no humidity control, simply a temperature control. Therefore, the operation conditions in such readers are also poor with respect to oxygen supply. Inhomogeneous evaporation can lead to misinterpretation of results. Because of the fact, that the measurement procedure in these microplate readers is generally uncoupled from shaking, sedimentation of cells during the measurement process can also cause artifacts. A widespread microplate reader to monitor cell growth is the BioScreen C device (BioScreen, 2009). This system features two covered honeycomb plates, each consisting of 100 wells, making it possible to run 200 samples simultaneously. Even through the transparent cover of the plates allows absorption measurements and it reduces the gas exchange with the environment. k_La values in the range of that approved for 96-well MTPs (k_La= 150-160 1/h, (Hermann et al., 2003) or even below are supposed, thus limiting its application to anaerobic or low density cultures.

Weiss and John reported about standard 96-well MTPs with immobilized fluorescent dyes on the bottom of the wells. As these so-called optodes are sensitive to *pH* or *DOT*, they can only detect one measurement parameter at a time during an experiment (Weis et al., 2002, John et al., 2003). These plates can be read with standard microplate readers, yet with the same aforementioned drawbacks. Especially, *DOT* measurements are hindered by shaking interruptions (Wittmann et al., 2003). The same sensors are currently also available in 24 MTPs (Kensy et al., 2005b). To read these optodes, the SensorDish Reader from Precision Senssing GmbH (Regensburg, Germany) can be applied (PreSens, 2009). Installed on a shaker, this system can provide real online data without interruption from the shaking process. Here, k_La values of up to 250 1/h have been reported with a filling volume of 400 µL (Kensy et al., 2005b). At higher filling volumes (e.g. 1mL), that are more suitable to optode measurements because the liquid has continuously contact to the optode, k_La values are reduced to 100 1/h (Kensy et al., 2005b). These mass transfer conditions can be oxygen-limiting for many fermentations with microbial cells (Anderlei et al., 2004, Losen et al., 2004).

Samorski et al. (2005) introduced a new measurement technique which is capable of detecting biomass concentrations via scattered light, NADH, riboflavin or fluorescent proteins through fluorescence in continuously shaken microtiter plates. For the first time, this technique provides real online data about kinetics of biomass and product formation from microbioreactors without any interference of the cultivation.

This study presents substantial improvements in the online monitoring technique in continuously shaken microtiter plates based on the technique first presented by Samorski et al. (2005) and gives several examples of feasible applications. The quantitative detection of biomass concentrations via scattered light for standard microbial expressions systems such as the bacterium *Escherichia coli* and the yeast *Hansenula polymorpha* was studied. Moreover, the influence of different media compositions was evaluated by monitoring of biomass and protein formation while using green fluorescent protein (*GFP*) as a model protein. This technique was further applied to strain and media screening, promoter characterization and evaluation of

operation conditions. The application of a soluble fluorescent *pH* indicator for online detection of *pH* during fermentation was also studied.

4.2 Materials and Methods

4.2.1 Microorganisms and Media

Standard microbial expression systems, the bacterium *Escherichia coli* and the yeast *Hansenula polymorpha*, were used to evaluate the improved measurement device. The microorganisms were provided by several cooperation partners. The strain *E.coli BL21-Pet 28A ytvAC62A*, expressing a flavin mononucleotide (*FMN*)-based fluorescent protein (*FbFP*) (Drepper et al., 2007), was kindly delivered by Thorsten Eggert from evocatal GmbH (Düsseldorf, Germany, *FbFP*s reporter proteins are commercialized under the name evoglow®). The strains *Hansenula polymorpha RB11-pC10-Mox-GFP* and *H.p. RB11-pC10-FMD-GFP* express *GFP* under the control of the *MOX* and the *FMD* promoter, respectively (Amuel et al., 2000). They were kindly provided by Carsten Amuel from the Institute of Microbiology (Heinrich-Heine-University, Düsseldorf, Germany). For the biomass calibration, wild type strains of *Escherichia coli JM109* (ATCC 53323) and *Hansenula polymorpha wt* (ATCC 34438, synonym: *Pichia angusta*) were applied.

The *E.coli* experiments were carried out with three different types of bacterial culture media: complex medium Luria Bertani (LB), Terrific Broth (TB) and the synthetic medium (WR) for *E.coli* fedbatch fermentations reported by Wilms et al. (2001). These media had the following compositions: LB medium: 10 g/L tryptone (Difco from Becton Dickinson, USA), 5 g/L yeast extract (Carl Roth GmbH & Co. KG, Karlsruhe , Germany) and 5 g/L NaCl, *pH*~6.7 without adjustment; TB medium: 5 g/L glycerol (Merck, Germany), 12 g/L tryptone, 24 g/L yeast extract, 12.54 g/L K_2HPO_4, 2.31 g/L KH_2PO_4, *pH*~7.2 without adjustment; WR medium: 2.0 g/L Na_2SO_4, 2.68 g/L $(NH_4)_2SO_4$, 0.5 g/L NH_4Cl, 14.6 g/L K_2HPO_4, 4.0 g/L Na_2HPO_4 x 2 H_2O, 1.0 g/L $(NH_4)_2$-H-citrate, 0.5 g/L $MgSO_4$ x 7 H_2O, 0.01 g/L thiamine, 3 ml/L trace element solution (TES), 20 g/L glucose or glycerol, *pH* was adjusted to 7.2 with 1 M NaOH. TES contains: 0.5 g/L $CaCl_2$, 0.18 g/L $ZnSO_4$ x 7 H_2O, 0.1 g/L $MnSO_4$ x H_2O, 10.05 g/L Na_2-EDTA, 8.35 g/L $FeCl_3$, 0.16 g/L $CuSO_4$, x 5 H_2O, and 0.18 g/L $CoCl_2$ x 6

H_2O. The *E.coli* cultures were induced with 0.5 mM isopropyl-β-D-thiogalactopyranosid (IPTG, Biomol, Germany).

To study *H. polymorpha* with different media, the following media were applied: YPG, YPD, YNB-G, YNB-D (buffered and unbuffered). YP medium contained: 20 g/L peptone (Difco from Becton Dickinson, USA), 10 g/L yeast extract (Carl Roth GmbH & Co. KG, Karlsruhe , Germany) and 10 g/L glycerol (YPG) or 20 g/L glucose (YPD), *pH*~7.6 without adjustment. YNB medium contained: 5 g/L $(NH_4)_2SO_4$ and 1.7 g/L YNB without ammonium sulfate and amino acids (Difco from Becton Dickinson, USA) and 10 g/L glycerol (YNB-G) or 20 g/L glucose (YNB-D). After all medium components were dissolved, the *pH* was adjusted to 6.0 with 1 M NaOH. The buffered YNB medium was supplemented with Na_2HPO4 / NaH_2PO_4 buffer in a concentration of 0.1 M to maintain the *pH* at about 6 (starting value pH_0= 6.0) during the batch fermentation. In the experiments with different glycerol concentrations, the glycerol concentration in the YNB medium was simply varied during media preparation, whereas the other components remained constant. All the applied chemicals were of analytical grade and were delivered by Fluka (Neu-Ulm, Germany), unless specified otherwise.

4.2.2 Measurement device

As the measurement device, the same setup like that formerly reported by Samorski et al. (2005) was applied with only few but influential changes referring to signal qualities. The major change was varying the distance between the optical light fiber and the microtiter plate bottom as well as the tilting angle. The distance of the optical light fiber to the microtiter plate bottom was reduced from 7 mm to 4 mm, and the tilting angle was increased from 23° to 35°. This adjustment mainly reduced the back scattering of light from internal reflections within the wells, thus stabilizing the measurement signals. Moreover, the flashes of the xenon flash lamp during one measurement were reduced from 200 to 50 flashes to improve the life-time of the lamp. The biomass concentrations were measured via scattered light at 620 nm excitation without an emission filter. The *GFP* concentrations were monitored through an excitation filter of 485 nm and an emission filter of 520 nm. Furthermore, NADH

was monitored by an excitation of 340 nm and an emission of 460 nm. The *FbFP* preferred an excitation of 460 nm and an emission of 520 nm. The sensitivity of the photomultiplier (Gain) was adapted to the different measurement tasks and, therefore, different signal intensities were obtained. The entire device is called “BioLector” in the following text to facilitate referencing of the measurement device. The BioLector holds a data reproducibility of smaller than 5% standard deviation, when cultivating the same clone in the same medium on a microtiter plate. Due to small standard deviation and the high information content, error bars in the figures were omitted.

The *pH* was measured by adding a sterile solution of HPTS (8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt, art.-no.: 56360, Fluka, Neu-Ulm, Germany) to TB medium before inoculation with cells. The soluble fluorescent *pH* indicator was applied in a final concentration of 20 mg/L in the fermentation media. This indicator was excited by filtered xenon light with a wavelength of 410 nm and 460 nm and the emission was detected for both excitation wavelengths at 510 nm. The *pH* value could be derived from a calibration with buffers in which the same concentration of HPTS (20 mg/L) as in the culture medium was added. Buffers ranging from *pH* 4.0 to 9.0 and having an ionic strength of 120 mM (20 mM buffer and 100 mM NaCl) were applied to calibrate the measurement device. For each buffer condition, the intensity ratio I_R was calculated as follows:

$$I_R = \frac{I_{em\ 510nm,ex\ 460nm}}{I_{em\ 510nm,ex\ 410nm}} \tag{4.1}$$

After determining I_R for the different buffers, the *pH* values were correlated with the Boltzmann equation (Schulte et al., 2006) as follows:

$$pH = pH_O + dpH \cdot \ln\left(\frac{I_{R,min} - I_R}{I_R - I_{R,max}}\right) \tag{4.2}$$

The calibration parameters pH_O, d*pH*, $I_{R,min}$ and $I_{R,max}$ were calculated with an Excel sheet by using the implied Solver function, determining the least square root of the function (4.2).

The experiments were exclusively carried out with black standard round 96-well microtiter plates with an optical bottom from Greiner Bio-One, Germany (µclear, art.-no.: 655087), that were covered with a gas permeable membrane from Abgene, UK (art.-no.: AB-0718). If not otherwise specified, the experiments were conducted with 200µL working volume of culture or medium and normally 995 1/min shaking frequency (shaking diameter of 3 mm). At this operation condition a k_La value of 150 1/h was achieved (Hermann et al., 2003).

4.2.3 Biomass calibration

High concentrations of biomass were required to correlate scattered light intensities and biomass concentrations. To reach high biomass concentrations, the wild type strains of *E.coli* and *H.polymorpha* were cultivated in the complex TB and YPG media in two 250 ml shake flasks over night (20 mL, 37 °C, 300 1/min, 50 mm shaking diameter). The cultures were harvested and concentrated ten times by centrifugation (3000 1/min, 5 min, Rotina 38R, Hettich, Germany). One sample (2 mL) of each concentrated cell suspension was taken to determine *OD* (absorption at 600 nm using a photospectrometer (UVIKON 922, Kontron Instruments, UK)) and cell dry weight (*CDW*). *CDW* was determined by washing the cells twice in physiological salt solution (9 g/L NaCl) and drying the cells at 105°C until the mass remained constant. Duplicates of dilutions of the concentrated cell suspension in the respective cultivation medium were then distributed to a fresh microtiter plate and read with the BioLector at the same operating conditions as in the culture experiments. The reference measurement of *OD* was carried out at 600 nm in a microplate absorption reader (Powerwave X, BioTek, USA) with 200 µL working volume and without any cover.

4.3 Results and Discussion

4.3.1 Biomass calibration

In biotechnology labs, it is very common to follow biomass development by monitoring the optical density. However, it is well known that the correlation between the optical density and biomass concentration is only linear in a very small range -

normally between 0.1 to 0.3 *OD*. For higher biomass concentrations, therefore, it is necessary to adequately dilute the samples and to recalculate the real *OD* of the sample. It is often mentioned in the literature, that scattered light measurements can compensate for this inconvenience and that biomass concentrations can be correlated up to high densities without dilutions (Junker et al., 1988). To prove this, cell suspensions of different biomass concentrations (*CDW*) of *Hansenula polymorpha wt* in YPG medium were compared by scattered light and optical density measurements.

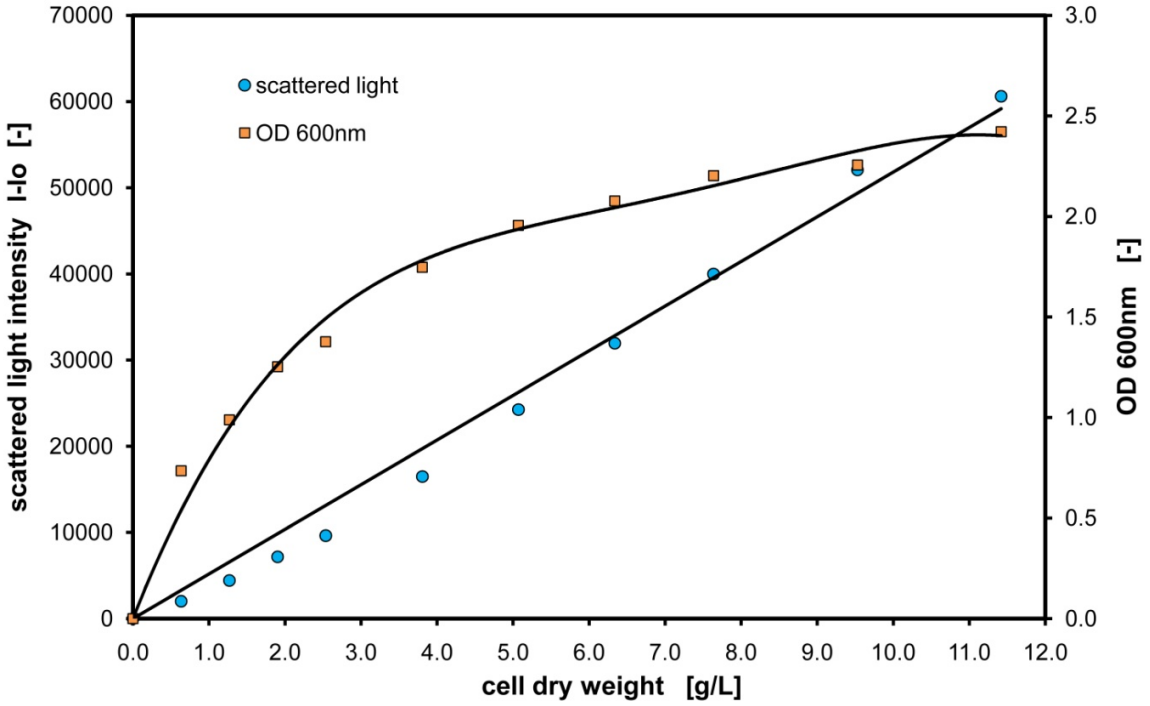


Figure 4.1: Comparison between scattered light and optical density measurements (absorption).

Experimental conditions: *Hansenula polymorpha wt* culture in YPG medium measured in 96-well MTP, with 200 µL filling volume, at 37 °C temperature, 995 1/min shaking frequency and 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, Gain: 40).

Figure 4.1 shows the correlation of measurement signals from *OD* and scattered light versus the cell dry weight concentrations of *H. polymorpha*. The *OD* signals follow a typical saturation curve and confirm that linearity is limited already at concentrations well below 1 g/L *CDW*. However, the scattered light signals show a good linear correlation with the cell dry weight concentration versus the applied concentrations of up to 11 g/L *CDW*, which is a normal concentration reached in batch fermentations (Puskeiler et al., 2005). Additionally, it is observable that the resolution of the

biomass signal with scattered light is very high. Thus, each g/L cell dry weight can be resolved with 5500 scattered light units at the applied sensitivity (Gain= 40). As the *OD* measurement loses resolution with higher biomass concentration, it is not recommendable to use this method with undiluted samples. Thus, this method is not applicable for online monitoring of cultures.

There are two further aspects which make *OD* measurements impractical for online monitoring of growing cultures in microtiter plates. First of all, gas-permeable membranes are normally used to seal the wells, to avoid contaminations and to reduce evaporation. This makes transmission measurements almost impossible. Second, the *OD* measurements are normally conducted when the shaking of the plate is interrupted. This results in measurement artifacts through the sedimentation of cells during the measurement. Furthermore, the decrease in oxygen mass transfer and effectiveness of mixing during the shaking interruption has to be taken into account with sensitive microorganisms or when studying fast metabolic reactions.

4.3.2 Limits of biomass detection with scattered light

To determine the limits of the scattered light measurements, a concentrated biomass solution of *E.coli JM109* and *H.polymorpha wt* after growth in shake flasks was diluted, distributed on a microtiter plate and measured with the BioLector. The results are presented in Figure 4.2.

In Figure 4.2, the scattered light intensities of the dilutions series is shown versus the cell dry weight of the cell suspension. It is notable that the scattered light signals show a good linear correlation with the cell dry weight concentrations for both cell types, *E. coli* and *H. polymorpha,* over a broad concentration range. Up to cell concentrations of 50 g/L cell dry weight, the scattered light intensities show a linear correlation with the cell dry weight concentrations. At cell concentrations higher than 50 g/L cell dry weight, the scattered light signal for *H. polymorpha* becomes saturated, for the described measurement setup. At this point, resolution dramatically decreases and further monitoring of undiluted samples is not recommendable. The cell suspension of *E. coli* was followed only up to cell dry weight concentrations of 50

g/L, because the biomass yield of the applied shake flask cultivations was limited to this value.

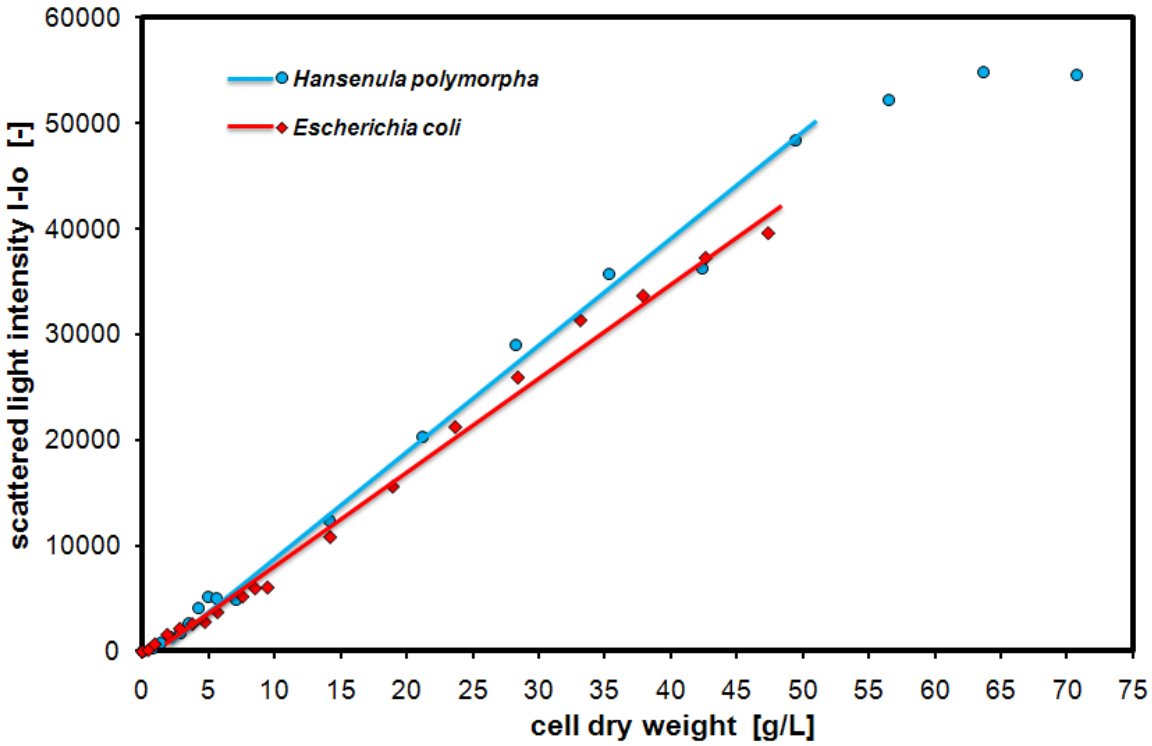


Figure 4.2: Limits of biomass monitoring with scattered light.

Experimental conditions: *Hansenula polymorpha wt* in YPG medium; *E.coli JM109* in TB medium, both were measured in 96-well MTP, with 200 µL filling volume, at 37 °C temperature, 995 1/min shaking frequency and 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, Gain: 5).

Nevertheless, it is assumed that the detection limit of *E.coli* cell suspensions lies also in the range of 50 g/L cell dry weight, because up to this concentration the signals for yeast cells behave similarly to those of bacteria. However, this conclusion cannot be generalized and extended to any other cell type, media composition or application. To ensure reliable measurement data, it is recommendable to perform a specific biomass calibration when working with new cell types and media. In general, the broad linear range and sensitivity of non-invasive scattered light measurements reflecting biomass concentrations, for the first time, enable online monitoring of cell growth in microbioreactors. Thereby, dilutions of samples, contaminations due to open vessels and interruption of shaking is avoided. If 50 g/L cell dry weight is accepted as upper detection limit for a linear range calibration of scattered light measurements, there is obviously no limitation of biomass detection for batch

fermentations. This most commonly applied operation mode in screening (Büchs, 2001) reaches a cell dry weight of usually below 15 g/L (Puskeiler et al., 2005). The minimal detection limit for biomass concentration ranges from 0.1 to 0.2 g/L *CDW* (*OD*600 ~ 0.2 to 0.6) for bacteria and yeast cells (data not shown here). When flash lamps are used as excitation light (such as in our example), the flashed light can cause oscillations of the scattered light signals at the beginning of the cultivation with normally small cell concentrations as well as in non-inoculated media. This results from interferences of the flashed excitation light and the continuously changing path length of the shaken liquid inside the wells. At small cell densities or in pure media, the light can completely penetrate the liquid and scatter back from the adhesive sealing membrane covering the microtiter plate. With higher cell densities, the light cannot completely penetrate through the liquid anymore, and the path length of the liquid has a reduced effect on signal stability.

4.3.3 Variation of the amount of carbon source

Another possibility to calibrate or reference the scattered light signals to biomass concentrations is to grow cells on different amounts of carbon source, assuming a constant biomass yield on the carbon source and no by-product formation.

To analyze this, YNB medium with four different concentrations of glycerol (5, 10, 15 and 20 g/L) was prepared and used in a *H. polymorpha* fermentation in microtiter plates. Stöckmann et al. (2003) reported that *H. polymorpha* strains do not produce by-products during cultivation on glycerol even under oxygen-limited culture conditions.

Figure 4.3 presents the results of this experiment. A parallel growth of *H. polymorpha* in the exponential growth phase on all four media is observed. Obviously, there is no substrate inhibition on growth. Remarkably, the culture attains the stationary phase at different times. The scattered light levels depend on the available amount of carbon source (glycerol). The more glycerol is present proportionally more biomass can be produced, which is well depicted by the scattered light signals.

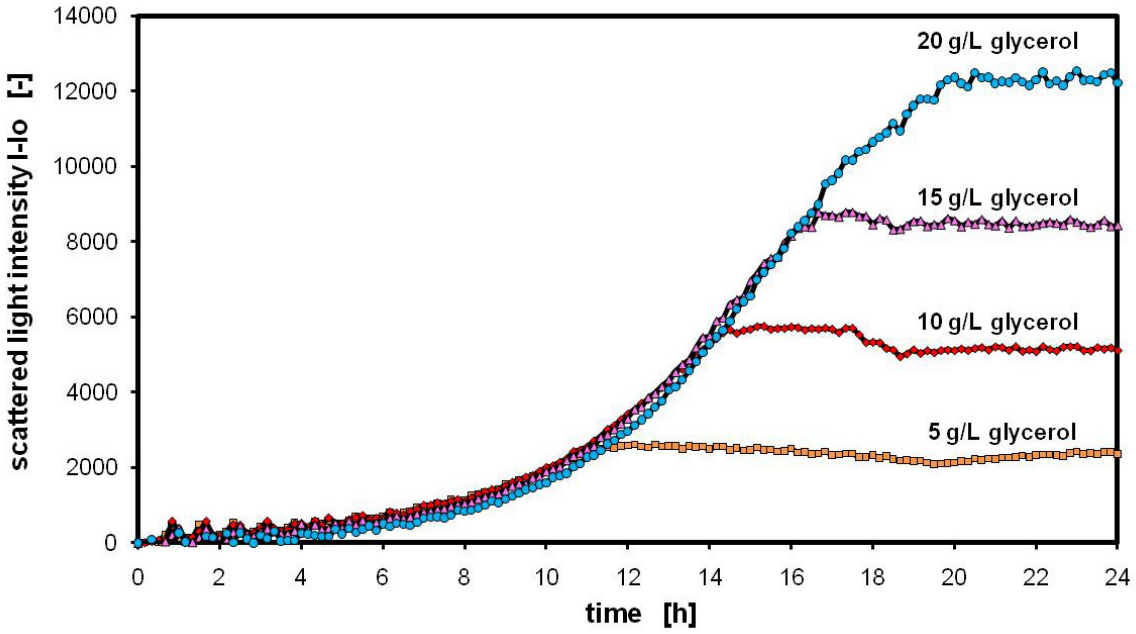


Figure 4.3: Comparison of different media - variation of carbon source concentration monitored with scattered light intensities.

Experimental conditions: *Hansenula polymorpha RB11-pC10-FMD-GFP* culture in YNB-G medium with varying glycerol concentrations (5, 10, 15 and 20 g/L); measured in 96-well MTP, with 200 µL filling volume, at 30 °C temperature, 995 1/min shaking frequency and 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, Gain: 10).

4.3.4 Influence of *pH* conditions on growth

A large number of different culture media has to be tested to optimize the environment for production strains and to ensure maximum productivity. Unfortunately, *pH* conditions are often not considered. The most simple way to handle this aspect in small scale cultures is to use buffers.

Figure 4.4 depicts the effect of buffers on growth of *H. polymorpha*. Here, the cell growth in buffered and unbuffered YNB media is presented. The biogenic NADH pool is simultaneously monitored in both media. The scattered light of the growing yeast cells on buffered medium follows the typical exponential growth curve until entering the stationary phase at 19 hours. The NADH signal also follows an exponential growth curve after a slight decay of the signal at the beginning. In the stationary phase, the NADH signals stay constant, while the scattered light signals decrease slightly and continuously due to probable morphological changes of the cells. The cells in the unbuffered medium, however, behave differently. There seems to be no

difference in the monitored signals up to 12 hours of the culturing time. But then, due to a *pH* decrease below 4.0 (data not shown here), the cells change their metabolism, thereby reflecting the higher proton gradient in the cell environment. This also leads to a decreased growth rate. This point is also reflected by a spontaneous increase in the NADH signal. Probably the respiratory chain is blocked and the NADH pool fills up.

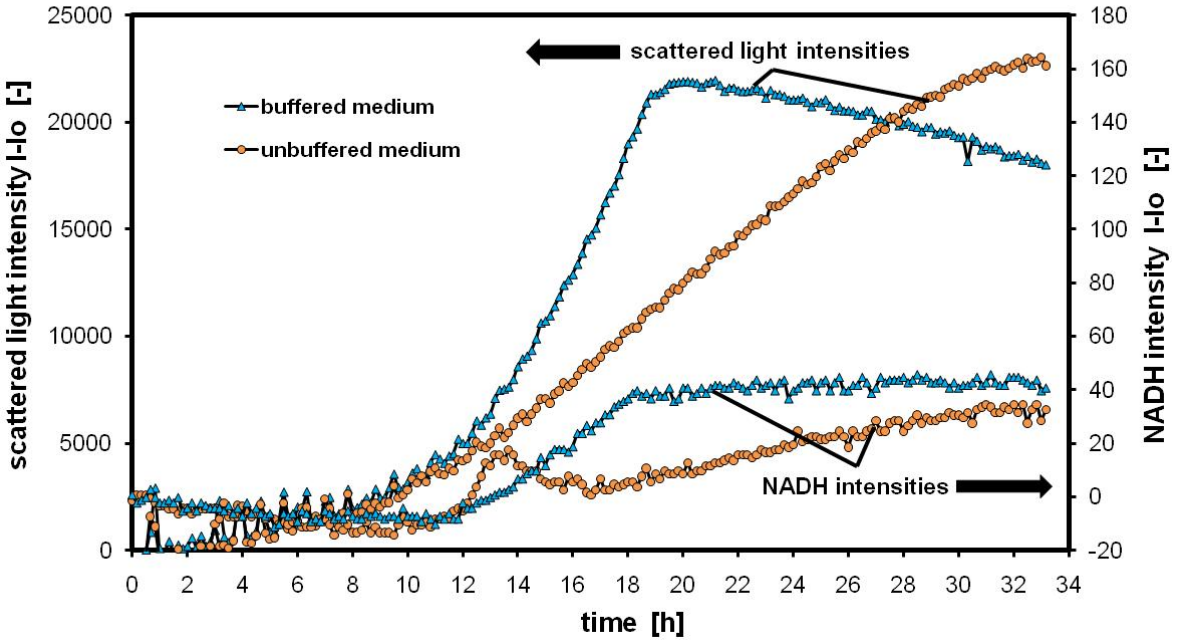


Figure 4.4: Influence of *pH* conditions on growth - buffered/unbuffered medium monitored with scattered light intensities and NADH fluorescence intensities.

Experimental conditions: *Hansenula polymorpha RB11-pC10-FMD-GFP* culture in buffered YNB medium with 10 g/L glycerol and 100 mM phosphate and unbuffered YNB medium with 10 g/L glycerol without phosphate; measured in 96-well MTP, with 200 µL filling volume, at 30 °C temperature, 995 1/min shaking frequency and 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, Gain: 20), NADH (ex: 340 nm/em: 460 nm, Gain: 20).

After a metabolic switch, the cells grow at a reduced growth rate, and the NADH pool is continuously decreased until 17 hours when it reached the normal level of intracellular NADH (see as reference NADH of the culture in the buffered medium). From this point on, the NADH signal continuously increases again in parallel to the growing cell mass. Due to the unsuitable *pH* conditions, the yeast can only grow linearly, not exponentially which leads to a much larger cultivation time (Δt= +14h). Here, there is no evidence that other by-products are formed, because the biomass still reaches the same scattered light levels. By applying other microorganisms or media, the behavior can completely differ with respect to by-product formation,

product formation and biomass development. Especially sensitive microorganisms such as e.g. *E.coli* can stop growth when passing below a critical *pH*~5.0 (Losen et al., 2004).

4.3.5 Comparison of different media with *Hansenula polymorpha*

It is also interesting to compare biomass yield, growth rate, protein expression on different mineral and complex media. Therefore, the yeast *H. polymorpha* was grown on different standard expression media for small scale cultures and the growth behavior was monitored with the BioLector.

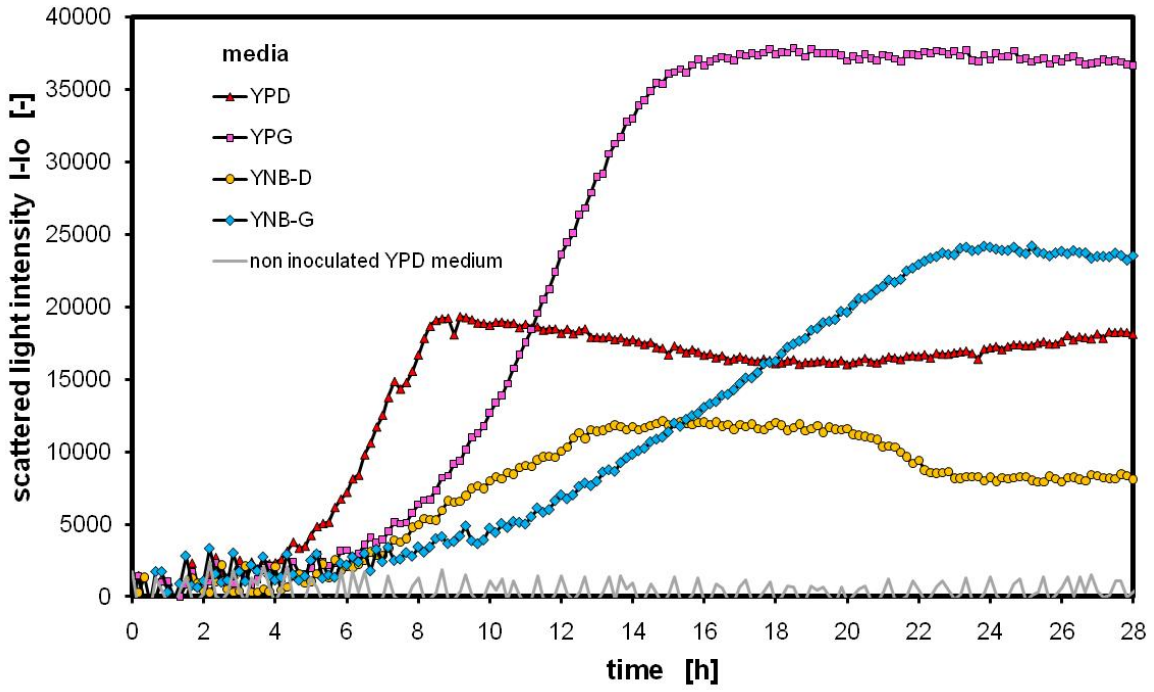


Figure 4.5: Comparison of different media – *Hansenula polymorpha* on complex and synthetic media with glucose and glycerol as carbon source monitored with scattered light intensities.

Experimental conditions: *Hansenula polymorpha RB11-pC10-Mox-GFP* culture in YPG, YPD, buffered YNB-D and YNB-G media; measured in 96-well MTP, with 200 µL filling volume, at 37°C temperature, 995 1/min shaking frequency and 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, Gain: 20).

Figure 4.5 depicts the differences in growth of the yeast *H. polymorpha* on YPD, YPG, YNB-D and YNB-G medium. The YP medium consists of complex ingredients such as yeast extract and peptones, whereas the YNB medium consists only of a pure mineral basis. The biomass development is clearly graduated with respect to the biomass yield as follows: YPG > YNB-G > YPD > YNB-D and with respect to the

specific growth rate as follows: YPD > YPG > YNB-D > YNB-G. It can be clearly stated for both media compositions that glucose (D) is consumed by the cells significantly faster than glycerol (G) and that the biomass yield on glycerol is approximately double that on glucose (at normalized molar mass, as applied here). On the same carbon source, the complex media gained higher biomass yields than the synthetic media resulting from the additional carbon sources supplied by the yeast extract and peptones. Also, this beneficially contributes to the growth rates, because many catabolites are immediately available in the medium and do not have to be synthesized by the cells as in case of the mineral medium YNB.

4.3.6 Comparison of different media with *Escherichia coli*

To prove the applicability of the BioLector in media screening, a media comparison was also performed with *E. coli*. The growth and the expression of *FbFP* (fluorescence protein) were studied on standard media for *E. coli*: LB, TB and WR, the latter being a synthetic fedbatch fermentation medium. Figure 4.6 presents the results of this experiment.

Interestingly, a clear difference in the biomass development can be seen in this experiment. *E.coli* grows to the highest biomass concentration on TB medium, whereas half the biomass concentration is attained on WR medium and 1/3 of the maximum concentration on LB. While a typical exponential growth curve is observable on the WR medium, the cultures on LB and TB medium show only a linear slope. Moreover, a shift in growth on the TB medium is displayed at 7 h. This has already been described previously by Losen et al. (2004) and can be related to a carbon source shift from glycerol to peptone. This shift is also expressed in a turnaround of *pH* from a decrease to a *pH* increase at this point (Losen et al., 2004). The cultures can grow much faster on complex media (LB and TB) than on the synthetic media due to the availability of key intermediates and building blocks for growth in the complex media. Concerning to the protein expression in this media, it is notable that the expression of the *FbFP* in TB and WR medium gained almost the same level, although the biomass level in TB medium is significantly higher. Even though the expression of the *FbFP* in LB medium is very low, this can also be

attributed to the late induction time for the LB culture. At the induction time (7.3 h), the culture already enters the stationary growth phase and no carbon source is left to produce the protein. Prior to induction in all media, there is only a small fluorescence intensity observable which is explained by biogenic fluorescence of the cells or a small constitutive expression of *FbFP*. After the induction, the promoters are activated and fluorescence of the *FbFP* increases strongly in TB and WR medium. The specific productivity of the *E.coli* culture in WR medium seems to be higher than in the TB medium, because almost the same *FbFP* fluorescence is detected at approximately half the biomass concentration in WR medium in comparison to TB medium.

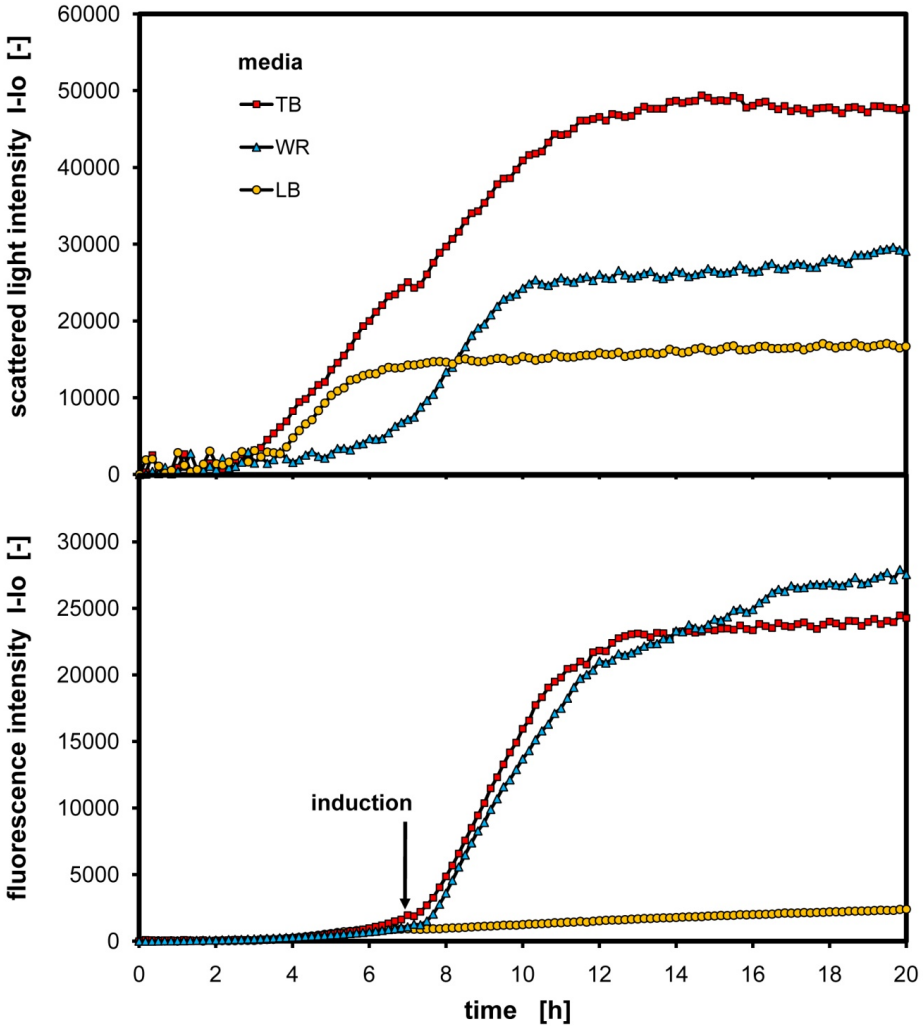


Figure 4.6: Comparison of different media – growth and protein expression of a flavin mononucleotide (*FMN*)-based fluorescent protein (*FbFP*) in *E.coli.*

Experimental conditions: *E.coli BL21-Pet 28A ytvAC62A* culture in LB, TB and WR media; measured in 96-well MTP, with 200 µL filling volume, at 30 °C temperature, 995 1/min shaking frequency and 3 mm shaking diameter, induction at 7.3 h with 0.5 mM IPTG, scattered light (ex: 620 nm/em: -, Gain: 20), *FbFP* (ex: 460 nm/em: 520 nm, Gain: 10).

Distinct differences in biomass concentrations and growth rates can be seen using varying carbon source quantities, media backgrounds and buffer conditions. With these results, the applicability of the BioLector technology is confirmed for quantitative biomass and fluorescent protein monitoring as well as for media screening.

4.3.7 Clone screening

The BioLector was then applied in clone screening with *H. polymorpha,* expressing *GFP* as a model protein for simplifying quantification. The clones were passaged 8 times for integration of the *GFP*-gen into the *Hansenula* genome and then stabilized (Stöckmann et al., 2003b). The screening was performed with glycerol as sole carbon source; that means that the applied *FMD* and *MOX* promoters were derepressed and, therefore, they actively expressed *GFP*.

Figure 4.7 illustrates the results from a screening of 90 different clones. The scattered light and the fluorescence of *GFP* were monitored during the batch fermentation in a 96-well microtiter plate. Most of the clones grow exponentially, and the majority of clones enter the stationary phase between 8 and 13 h. About seven clones show a significantly reduced growth rate and enter the stationary phase after 15 h and more. Two strains do not grow and form a baseline together with the two wells of non inoculated media. Notably, the different clones achieve different biomass yields, which is expressed in the different scattered light intensities. As the measurement device has a standard deviation of smaller than 5%, the deviation of +/- 15% around the median of the scattered light signals in the stationary phase demonstrates that the biomass yields differ significantly. Figure 4.7B illustrates the huge diversity of *GFP* formation kinetics and the *GFP* expression levels ranging from a few thousand fluorescent units (FUs) up to 30.000 FUs. It has to be assumed that this huge difference in protein level is also observed in the case of the expression of real target proteins instead of *GFP*. The *GFP* expression kinetics in Figure 4.7B show a similar exponential curve like the biomass curves in Figure 4.7A but with a much broader distribution. As formerly reported, the *GFP* protein is very stable (March et

al., 2003). That is proven here by the constant *GFP* fluorescence in the stationary phase.

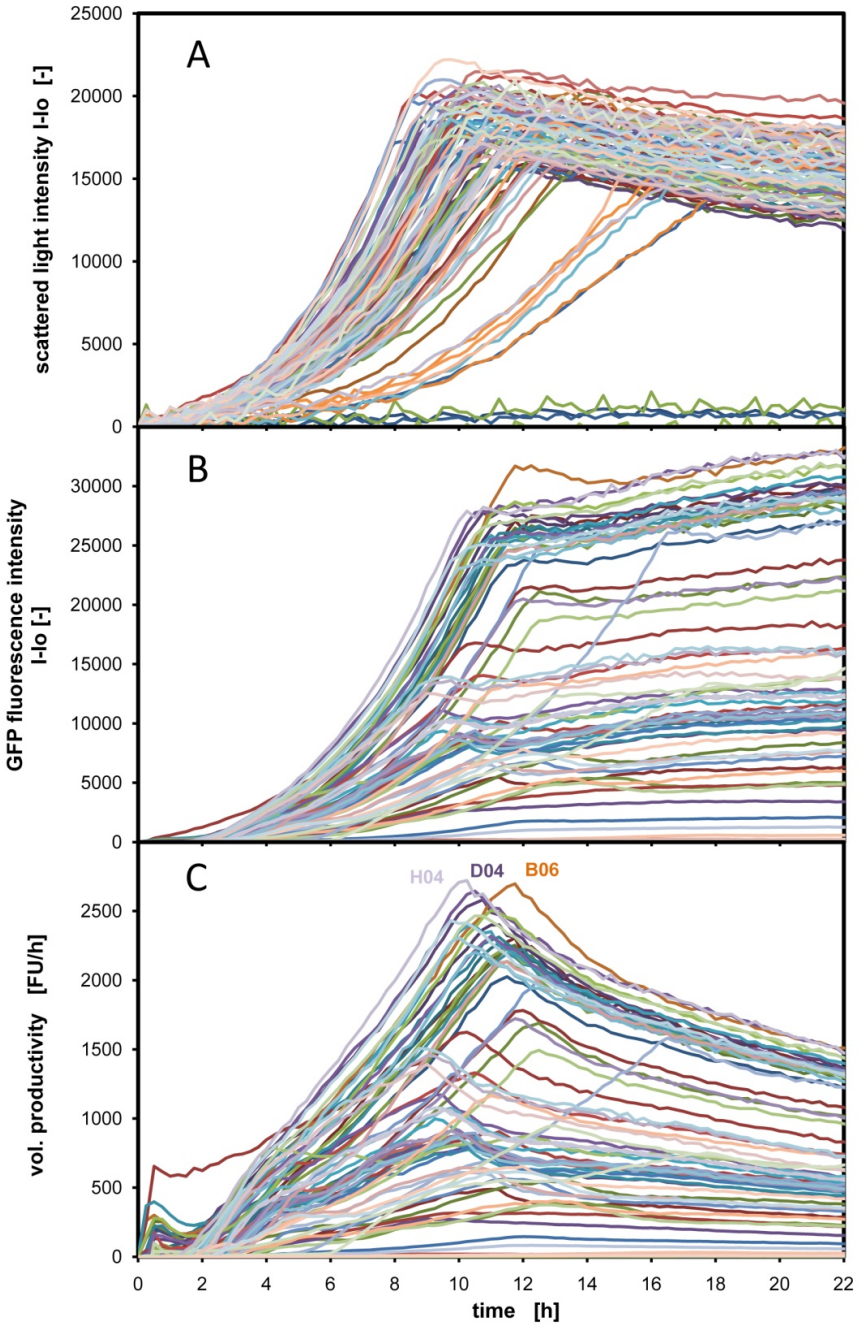


Figure 4.7: Clone Screening - comparison of growth and *GFP* protein expression of 90 different *Hansenula polymorpha* clones.

(A) growth via scattered light intensities (B) protein expression via *GFP* fluorescence intensities; (C) volumetric productivity (P_V) - calculated as *GFP* formation rate without consideration of setup time of the equipment, the best clones are depicted by given the well number in the diagram; 45 clones of *Hansenula polymorpha RB11-pC10-Mox-GFP* and 45 clones of *Hansenula polymorpha RB11-pC10-FMD-GFP* in buffered YNB-G medium; measured in 96-well MTP, with 200 µL filling volume, at 37 °C temperature, 995 1/min shaking frequency and 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, Gain: 20), *GFP* (ex: 485 nm/em: 520 nm, Gain: 10).

Other proteins probably show degradation and very often are exposed to proteolysis (Weydemann et al., 1995, Viaplana et al., 1997). In this case, online monitoring of biomass development and protein formation kinetics can provide valuable information in early bioprocess development. Generating this extensive amount of online information necessitates further data analysis. One simple example of analysis is applying typical evaluation criteria such as the specific product yield ($Y_{P/X}$) which expresses the relation of the formed product to biomass.

Figure 4.8 reveals the calculated specific product yield at 18h of all *H. polymorpha* clones described (criteria: all clones entered the stationary phase). The whole bar chart of Figure 4.8 depicts all clones, whereas the zoomed bar chart presents the ten best clones (TOP10). The bar chart discloses the huge diversity of the specific product yield and, therefore, the necessity of sophisticated screening.

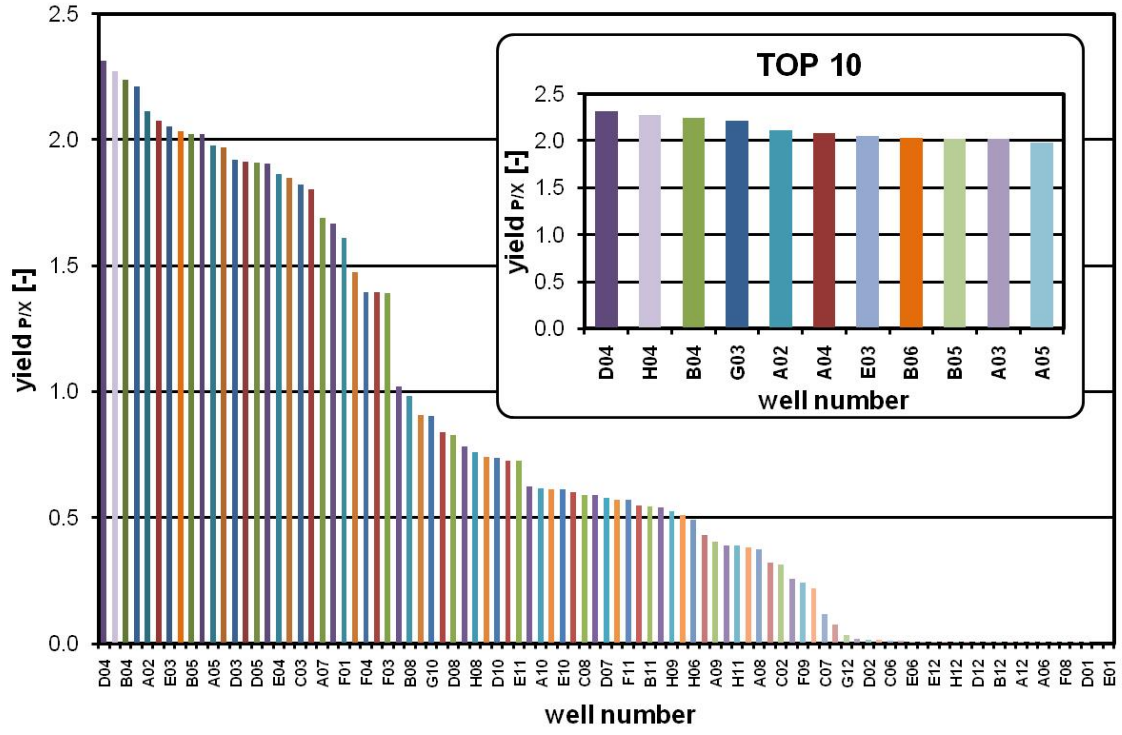


Figure 4.8: Derivation of specific product yield ($Y_{P/X}$).

Data taken from the clone screening of Fig. 7, specific product yield $Y_{P/X}$ calculated as ratio of *GFP* intensities (protein concentration) to scattered light intensities (biomass concentration); data arranged to present the best clones from left to right given the well number on the abscissa, TOP10 represents the best ten clones in respect to $Y_{P/X}$.

Another evaluation criteria of a bioprocess could be the volumetric productivity (P_V) of the process. Figure 4.7C visualizes the development of the volumetric productivity in all 96 wells of the microtiter plate. The volumetric productivity is derived from the *GFP* fluorescence units (FU equivalent to product concentrations ~ mg/L) divided by the cultivation time (net productivity, without setup time). Again, very diversified curves are observable. All volumetric productivities reach a peak where the cultures should be harvested. The well numbers of the best clones are highlighted in the diagram of Figure 4.7C. With respect to the volumetric productivity, it would be most advisable to continue the bioprocess development with these clones. The point of maximum volumetric productivity reflects the expected process performance and the optimum harvest point. Again, the online measured *GFP* fluorescence can be regarded as a convenient model in this case, but alternatives such as fusion proteins with fluorescent reporter proteins or offline protein analysis could be a more realistic case in respect to the industrial praxis. Thus, a sophisticated evaluation of clones and fermentation conditions can already be performed with the hereby presented tools in a very early stage in bioprocess development.

4.3.8 Characterization of promoters

Promoter regulation is another very important aspect when an expression system or a bioprocess is evaluated. It is still difficult to generate meaningful data on promoter regulation. Currently many laborious techniques are applied, e.g. fluorescence microscopy or fluorescence activated cell sorting (FACS). It is possible to obtain kinetics of the promoter activity or the protein expression through painstaking sampling from culture vessels. Very often promoter activity is controlled by addition of specific inductors or by growth on specific substrates.

Figure 4.9 presents one example of promoter regulation with *H. polymorpha*. Two different promoters, *MOX* and *FMD*, were studied together with the wild type strain as negative control. Both promoters were inserted in front of the *GFP* gene in the transformation plasmid to follow promoter activity by the fluorescence of *GFP* (Amuel et al., 2000). The yeast strains were cultivated on two different carbon sources to study the promoter regulation on glucose and glycerol.

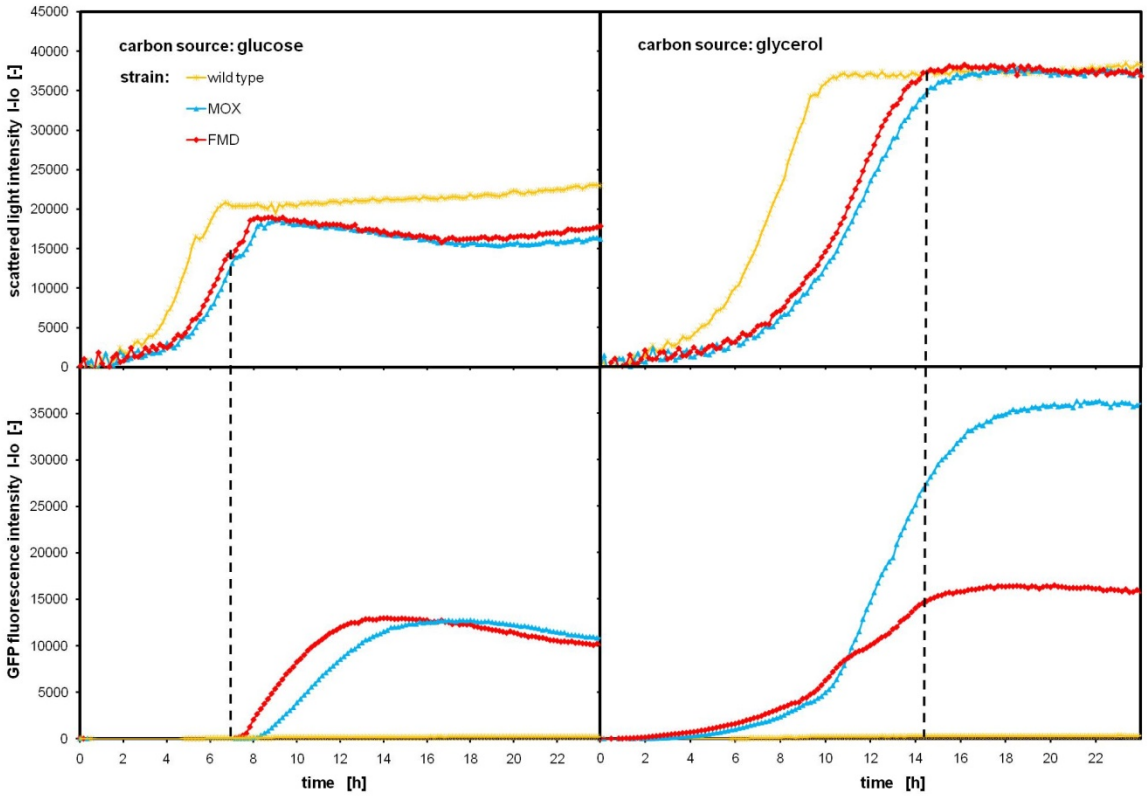


Figure 4.9: Characterization of promoters - *MOX* and *FMD* promoter regulation in *Hansenula polymorpha* on glucose and glycerol growth medium monitored via *GFP* fluorescence intensities and parallel measurement of scattered light intensities.

Experimental conditions: *Hansenula polymorpha wt, RB11-pC10-Mox-GFP* and *RB11-pC10-FMD-GFP* in YPD (10 g/L glucose) and YPG (20 g/L glycerol) medium; measured in 96-well MTP, with 200 µL filling volume, at 37 °C temperature, 995 1/min shaking frequency and 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, Gain: 20), *GFP* (ex: 485 nm/em: 520 nm, Gain: 10).

Figure 4.9 clearly depicts the differences in growth and promoter activity of the strains on the different media. On both carbon sources, the sequence of growth is equal, whereas the wild type strain grows faster than the recombinant strains. In addition, these two recombinant strains grow at a similar growth rate, with the *FMD* strain growing slightly faster. Proving the findings in Figure 4.5, the growth rates of all strains are higher on glucose than on glycerol. The biomass yield on glycerol seems to be twice as high as on glucose (on the same molar mass). On glucose, there seems to be a second growth phase following a slight decrease in the scattered light signals at 5 h for the wild type strain and at ca. 7 h for the *MOX* and *FMD* strains. In this latter phase, the cells metabolize the overflow metabolites, ethanol and acetic acid, formed during the growth on glucose (Stöckmann et al., 2003, Jeude et al., 2006). The lower diagram of Figure 4.9 shows the *GFP* formation of the strains. On

glucose, there seems to be no promoter activity at all in the presence of glucose (first growth phase). Just after the switch to the second growth phase (on ethanol and acetic acid), the promoters are activated and *GFP* is formed. The *FMD* and *MOX* strains form *GFP* at similar concentrations and in the same growth sequence. Probably also complex components of the medium (YP basis), such as peptone and yeast extract, are utilized to form *GFP*, as no other substrate is available and *GFP* is still formed in the stationary phase. As expected, the wild type does not form any *GFP*. On glycerol however, the promoters behave completely different. Already during the growth phase and in the presence of elevated glycerol concentrations, the promoters are activated and *GFP* is produced. The activity of both promoters is very similar up to 10-11 hours. After that point, the *MOX* promoter activity is boosted while the *FMD* promoter declines. After entering the stationary phase, both promoter activities slow down and the *GFP* fluorescences remain constant. At the end of the fermentation, the *GFP* fluorescence intensity of the *MOX* promoter is about 2.5 times higher than that of the *FMD* promoter, demonstrating the superiority of the *MOX* promoter under the applied experimental conditions. Again, the wild type does not form any *GFP*. This scheme of promoter regulation for *H. polymorpha* is also reported in the literature, where on glucose, *MOX* and *FMD* promoter are repressed by the substrate, whereas on glycerol, these promoters are derepressed at relatively high substrate concentrations (Stöckmann et al., 2003, Jeude et al., 2006, Hartner and Glieder, 2006, Oh et al., 2004).

With the help of the BioLector, it is possible to quantitatively characterize promoter activities under different regulatory conditions. At the same time, these promoter activities can be assigned to different growth phases and substrate uptakes.

4.3.9 Influence of filling volume

The operation conditions in small-scale cultivations should be characterized and generally be transferable to larger scales. Upon working with microbial expression systems, one of the most relevant engineering parameters is the oxygen transfer rate. By simply varying the culture volume, the oxygen transfer rate is changed. A

good example of the influence of filling volume and, therefore, the oxygen transfer rate in small scale cultures is given by Figure 4.10.

In Figure 4.10, growth and *pH* of an *E.coli* culture are monitored in the complex TB medium which is known to be a strong oxygen-demanding medium (OTR~ 0.08 mol/L/h, Losen et al., 2004). Different culture volumes of 200 µl, 230 µL, 260 µL and 290 µL are applied in a standard 96-well MTP. With higher culture volume, the growth rate is reduced and the fermentation time is prolonged due to a reduced oxygen transfer rate. Additionally, the *pH* decay is more pronounced in the cultures with higher volumes, because under limited oxygen transfer conditions, the bacteria are forced to produce organic acids from glycerol. More acids are produced under higher oxygen limitation. The diauxic shift of the culture from glycerol to peptones, reflected by the slight decay of the scattered light signals in the middle of the growth curves, also correlates very well with the *pH* shift from a descending to ascending trend. At first glance, the different mass transfer conditions seem to have no influence on the final biomass yield and the final *pH*; but, of course, different growth rates and acidifications are observed.

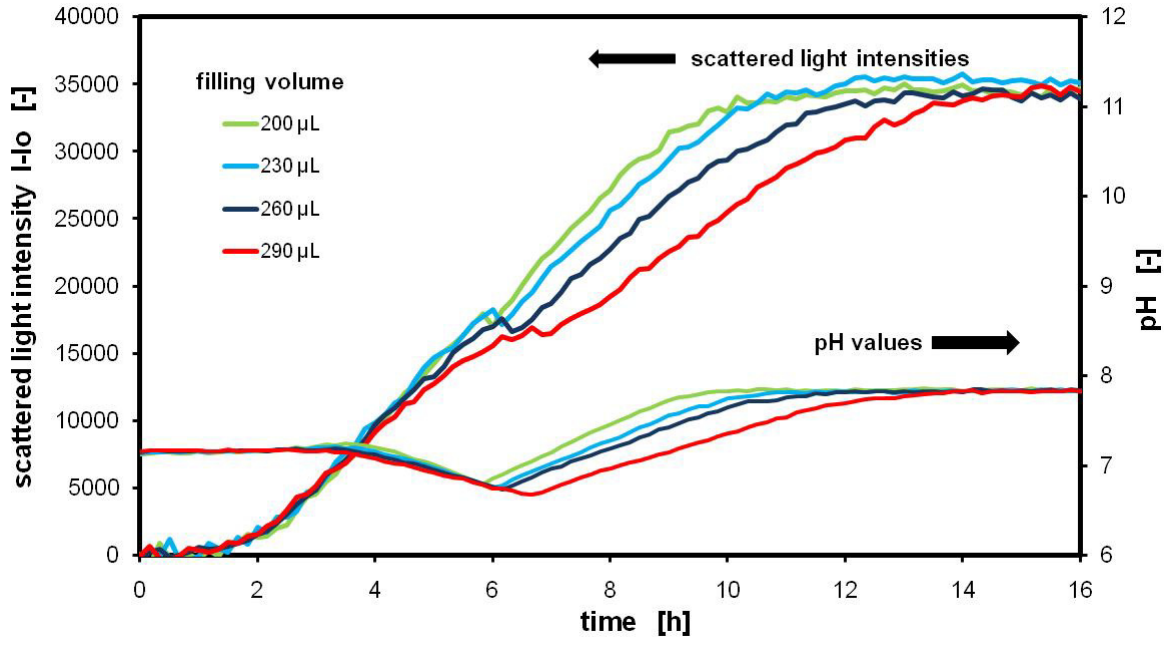


Figure 4.10: Influence of filling volume on microbial growth and *pH*.

Growth and *pH* monitoring of *E.coli* cultures with filling volumes from 200 µl to 290 µL; *E.coli BL21* culture in TB medium, measured in 96-well MTP, with 200, 230, 260 and 290 µL filling volume, at 30 °C temperature, 995 1/min shaking frequency and 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, Gain: 20), HPTS (ex: 410 nm and 460 nm/em: 510 nm, Gain: 10), calibration parameters for the Boltzmann equation: I_R,min= 0.00, I_R,max= 3.00, d*pH*= 0.50, pH_0= 7.20.

It has to be taken into account that even inferior mass transfer conditions and media with lower buffer capacity, than those applied here, could also lead to adverse effects such as termination of biomass growth or instability of the produced protein of interest. In conclusion, undefined culture conditions can result in a unattended selection pressure, the wrong design of a process and finally can cause unwanted problems during scale up.

The soluble fluorescent *pH* dye (HPTS) applied here could be a good alternative to normally expensive *pH* optodes. It is easy to apply in any kind of microtiter plate and can be read out with normal fluorescence plate readers. The main drawback of this method is the intensive calibration which has to be performed by the researcher itself and on any new reader. In contrast, *pH* optodes are normally delivered with calibration parameters.

4.4 Conclusions

The presented microtiter plate based fermentation system can be used to quantify microbial biomass concentrations of up to cell dry weights of 50 g/L without any dilution and in a linear correlation with offline biomass values. This allows the reliable online monitoring of biomass development during fermentation without the need of sampling and dilutions. The parallel monitoring of fluorescent protein concentrations (e.g. *GFP* or derivatives) or other fluorescent analytes in the culture broth allows, for the first time, the online calculation of typical evaluation criteria of a bioprocess such as yield, specific growth and product formation rates as well as volumetric productivity. Of course, online product data are not available in every product screening, however, continuous development in molecular biology and biomarker research will eventually create broader applications of fluorescent reporter proteins in the future (Su, 2005). A currently applied solution is fusing *GFP* or any other fluorescent protein to the target protein (Li et al., 2001). This provides a fluorescent signal of the fusion protein and the possibility for online detection. After expression of the fusion protein, the fluorescent protein partner can be cleaved from the target protein during the downstream processing by specific proteases (Zhang et al., 2001). Another more extensive method to evaluate the product yield is to combine offline

analyzed data with online data. Therefore, manual or automated sampling out of the microtiter plate would be helpful. To reduce the sampling frequency and, therefore, the volume removed during sampling, the online biomass signal can be used for triggering the sampling. A good sampling point could be the middle of the exponential growth phase, the entry into the stationary phase and the late stationary phase. Combining these methods of online monitoring and offline protein and nutrient analysis can deliver more sophisticated information and evaluation criteria for bioprocesses. Another interesting metabolite, NADH, can also be monitored with the BioLector technology. Under aerobic and unlimited growth conditions, the NADH fluorescence can be correlated with the biomass concentration, whereas under oxygen, *pH* or nutrient limitations, the signal can be a valuable indicator of limitations or metabolic changes (Marose et al., 1998). Furthermore, the technique can be utilized to evaluate different media by comparing such parameters as biomass concentration, specific growth rate, yield and product formation rate. Even diauxic growth shifts can be detected upon using media with different carbon sources. Thus, this quantitative method can positively reverse media development from merely being empirical experimentation to quantitative and knowledge-based media design. The combination of the BioLector technology with modern genetic engineering enables the real-time characterization of genetic elements such as promoters combined with fluorescent proteins directly during the cultivation. A huge variety of different substrates, chemical compounds and fermentation parameters can be easily studied via the fluorescence of the reporter protein. One aspect which is very often underrated in small-scale fermentations is the effect of engineering parameters. Upon performing high-throughput experimentation on a small scale, it would be most attractive, if the experimental results can be directly transferred to larger reactor scales. Therefore, small-scale reactors such as microtiter plates should be well characterized regarding relevant engineering parameters. OTR_{max} and k_La values for common microtiter plates of the 48- and 96-well type have already been characterized (Hermann et al., 2003, Kensy et al., 2005). In respect of validation of the data from micro-scale fermentations with lab fermenter data, further work is necessary. In conclusion, the BioLector technology is a powerful tool to generate more insight in bioprocesses in a high-throughput and quantitative manner. It is very suitable for clone screening, media optimization, systems biology and early

bioprocess development. Future research in this area should advance this technology towards the integration of optodes for monitoring *pH*, *DOT* and other fermentation parameters. The proof of scalability to stirred tank fermenters and the extension to other cell types such as plant, mammalian and human cell lines are highly desirable.

5 Scale-up from Microtiter Plate to Laboratory Fermenter: Evaluation by Online Monitoring Techniques of Growth and Protein Expression in *Escherichia coli* and *Hansenula polymorpha* Fermentations

Abstract: In the past decade, an enormous number of new bioprocesses have evolved in the biotechnology industry. These bioprocesses have to be developed fast and at a maximum productivity. Up to now, only few microbioreactors were developed to fulfill these demands and to facilitate sample processing. One predominant reaction platform is the shaken microtiter plate (MTP), which provides high-throughput at minimal expenses in time, money and work effort. By taking advantage of this simple and efficient microbioreactor array, a new online monitoring technique for biomass and fluorescence, called BioLector, has been recently developed. Nevertheless, the scalabilty of results from the micro-scale to laboratory or even larger scales is very important for short development times. Therefore, engineering parameters regarding the reactor design and its operation conditions play an important role even on a micro-scale. In order to evaluate the scale-up from a microtiter plate scale (200 µL) to a stirred tank fermenter scale (1.4 L), two standard microbial expression systems, *Escherichia coli* and *Hansenula polymorpha*, were fermented in parallel at both scales and compared with regard to the biomass and protein formation. Volumetric mass transfer coefficients (k_La) ranging from 100 to 350 1/h were obtained in 96-well microtiter plates. Even with a suboptimal mass transfer condition in the microtiter plate compared to the stirred tank fermenter (k_La= 370-600 1/h), identical growth and protein expression kinetics were attained in bacteria and yeast fermentations. The bioprocess kinetics were evaluated by optical online measurements of biomass and protein concentrations exhibiting the same fermentation times and maximum signal deviations below 10% between the scales. In the experiments, the widely applied green fluorescent protein (*GFP*) served as an online reporter of protein expression for both strains. The successful 7000-fold scale-up from a shaken microtiter plate to a stirred tank fermenter was demonstrated in parallel fermentations for standard microbial expression systems. This confirms that the very economical and time efficient platform of microtiter plates can be very easily scaled up to larger stirred tank fermenters under defined engineering conditions. New online monitoring techniques for microtiter plates, such as the BioLector, provide even more real-time kinetic data from fermentations than ever before and at an affordable price. This paves the way for a better understanding of the bioprocess and a more rational process design.

5.1 Introduction

Industrial bioprocesses mainly involve microbial or mammalian fermentations. To develop a productive bioprocess, it is necessary to screen a vast number of different clones and media. Moreover, for industry, it is crucial to develop the bioprocess as fast as possible to reduce time to market. Meanwhile, companies are pressured by regulatory authorities (e.g. FDA, EMEA) to substantiate the actual knowledge of the process applied (Gnoth et al., 2007, Mandenius and Brundin, 2008). The Process Analytical Technology Initiative (PAT) by the FDA is one of the major programs to improve process understanding, and, therefore, the quality of pharmaceutical products. Consequently, in recent years high-throughput technologies have become well-established in research laboratories to perform high-throughput experimentation and to gain more insights into bioprocesses.

Microtiter plates (MTPs) play the predominant role as the microbioreactor of choice for high-throughput screening applications. They are applied for drug discovery, cell cultivation, enzymatic assays and immunoassays (Houston and Banks, 1997, Duetz et al., 2000, Minas et al., 2000, Girard et al., 2001, Ferreira et al., 2005). This microbioreactor platform is mainly used as a batch reactor, and the experimental results are very often simply evaluated by endpoint analysis. The application of MTPs under shaken conditions can further improve mixing and mass transfer conditions in cultivations. Recognizing the great potential of MTPs as a serious reaction platform, some groups have started to characterize engineering parameters in these formats. Besides the mixing time, the oxygen transfer capacity (OTR_{max}) and the volumetric mass transfer coefficient (k_La) are generally characterized (Duetz et al., 2000, Weiss et al., 2002, Hermann et al., 2001 and 2003, Kensy et al., 2005). In order to better exploit the results of MTP experiments, it is important to precisely ascertain the scalability of MTPs to standard laboratory fermenters. Only if the microbioreactor performs identically to laboratory stirred tank fermenters (STF), it is possible to scale-up the data generated in high-throughput screenings and, thus, speed up the development timeline.

Micheletti et al. (2006) and Islam et al. (2008) compared microbial and mammalian fermentations as well as biotransformations in MTPs with those in a STF. The k_La value hereby emerged as one of the key scale-up factors. Using matched k_La values, they obtained comparable results at both the MTP and STF scale. Their research could be considered as the starting point of the scale-up from MTP to STF fermentations. This research, however, was based on laborious and error-prone sampling methods from the different reactor scales. In general, MTPs are recognized as being inexpensive, highly standardized and easy to handle. Nonetheless, MTPs would be an ideal platform for all tasks in fermentation science and bioprocess development if sampling and process data acquisition were easier to automate. Thus, Samorski et al. (2005) introduced a new online monitoring technique for continuously shaken microtiter plates which was further advanced and validated by Kensy et al. (2009). This technique, called BioLector, was able to resolve this lack of online information from MTPs and facilitated the processing of high-throughput fermentations; it now provides all relevant fermentation parameters online.

The aim of this study is to elaborate a scale-up methodology from microtiter plate to stirred tank fermenter. The validation of the performed microbial fermentations was based on defined mass transfer conditions and online monitoring signals of biomass and protein concentrations at both reactor scales. Parallel fermentations of standard microbial expression systems such as the bacteria *Escherichia coli* and the yeast *Hansenula polymorpha* were performed in MTP with 200 µL and STF with 1.4 L scale and compared with each other.

5.2 Materials and Methods

5.2.1 Microorganisms and Media

Standard microbial expressions systems, the bacteria *Escherichia coli* and the yeast *Hansenula polymorpha,* were used for the scale-up experiments. Moreover, the widely used green fluorescent protein (*GFP*) was chosen as a model protein for protein expression, because it can be monitored online (Su, 2005). The strain *E. coli BL21(DE3) pRSET B GFP-S65t* which expresses *GFP* was kindly delivered by Markus Sack from Fraunhofer IME, Germany. The *E. coli GFP* expression was

controlled by the strong, inducible *T7* promoter (inducer: isopropyl-β-D-thiogalactopyranosid - IPTG). The *GFP*-S65T mutant processes a point mutation at position 65 from Serine to Threonine, which results in a single red shifted excitation peak, a more intensive fluorescence, and in an approximately four times faster fluorescence response than wild type *GFP* (Heim et al., 1995). The strain *Hansenula polymorpha RB11-pC10-FMD-GFP* expresses *GFP* under the control of the *FMD* promoter. The *FMD* promoter is repressed under the presence of glucose and derepressed under the presence of glycerol in the culture medium (Amuel et al., 2000). It was kindly provided by Carsten Amuel from the Institute of Microbiology (Heinrich-Heine-University, Düsseldorf, Germany).

The *E. coli* experiments were carried out with the synthetic medium Wilms-Reuss (WR) (Wilms et al., 2001). The medium had the following composition: WR medium: 20 g/L glycerol, 2.0 g/L Na_2SO_4, 2.68 g/L $(NH_4)_2SO_4$, 0.5 g/L NH_4Cl, 14.6 g/L K_2HPO_4, 4.0 g/L Na_2HPO_4 x 2 H_2O, 1.0 g/L $(NH_4)_2$-H-citrate, 0.5 g/L $MgSO_4$ x 7 H_2O, 0.01 g/L thiamine, 3 ml/L trace element solution (TES), *pH*-value was adjusted to 7.2 with 1M NaOH. TES contains: 0.5 g/L $CaCl_2$, 0.18 g/L $ZnSO_4$ x 7 H_2O, 0.1 g/L $MnSO_4$ x H_2O, 10.05 g/L Na_2-EDTA, 8.35 g/L $FeCl_3$, 0.16 g/L $CuSO_4$, x 5 H_2O, and 0.18 g/L $CoCl_2$ x 6 H_2O. The *E. coli* cultures were induced with 0.5 mM isopropyl-β-D-thiogalactopyranosid (IPTG, Biomol GmbH, Germany).

The *Hansenula polymorpha* experiments were carried out with the synthetic medium SYN6-MES, which had the following composition (Hellwig et al., 2005): 20 g/L glycerol, 1.0 g/L KH_2PO_4, 7.66 g/L $(NH_4)_2SO_4$, 3.3 g/L KCl, 3.0 g/L $MgSO_4$ x 7 H_2O, 0.3 g/L NaCl, 27.3 g/L MES Pufferan (Carl Roth GmbH & Co. KG, Karlsruhe, Germany). After dissolution of all medium components, the *pH*-value was adjusted to 6.4 with 1M NaOH or 1M H_2SO_4. Then, the medium was autoclaved. After autoclaving of the basic medium, 6.67 mL/L micro element stock solution, 6.67 mL/L vitamin stock solution, 3.33 mL/L trace element stock solution (that have all been filter sterilized) and 6.67 mL/L calcium stock solution (that has been autoclaved) were added to the medium. The stock solutions had the following compositions: micro element stock solution: 10 g/L $(NH_4)_2Fe(SO_4)_2$ x 6 H_2O, 0.8 g/L $CuSO_4$ x 5 H_2O, 3.0 g/L $ZnSO_4$ x 7 H_2O, 4.0 g/L $MnSO_4$ x H_2O, 10 g/L EDTA (Titriplex III, Merck,

Darmstadt, Germany); vitamin stock solution: 60 mg/L D-Biotin, 20 g/L thiamine chloride hydrochloride; trace elements stock solution: 100 mg/L $NiSO_4$ x 6 H_2O, 100 mg/L $CoC1_2$ x 6 H_2O, 100 mg/L H_3BO_3, 100 mg/L KJ, 100 mg/L Na_2MoO_4 x 2 H_2O; calcium stock solution: 150 g/L $CaCl_2$ x 2 H_2O.

The preculture for the fementations of both bacterial and yeast strains were prepared by using 5 cryo vials which were inoculated into 100 mL of the respective fermentation medium (WR for *E. coli* or Syn6-MES for *H. polymorpha*). Each inoculated culture volume (100 mL) was then distributed equally among five 250 mL Erlenmeyer shake flasks and was respectively incubated at a shaking frequency of 300 1/min, a shaking diameter of 50 mm, and a temperature of 30 °C on an orbital shaker (LS-X, Kühner AG, Birsfelden, Switzerland). The starting optical density (OD_0) of the *E. coli* preculture was 0.5, and that of the *H. polymorpha* preculture was 1.0. The precultivation of *H. polymorpha* took 18 hours and that of *E. coli* took 16 hours. After the preculturing, the five shake flasks were again pooled and 90 mL of the pool were inoculated into the fermenter resulting in a starting fermenter volume of 1.41 L after inoculation.

The precultures for the Respiration Activity Monitoring System (RAMOS) experiments were prepared by using only one cryo vial for inoculating 20 mL of the respective fermentation medium (WR for *E. coli* and Syn6-MES for *H. polymorpha*). The incubation conditions were the same as mentioned above.

All chemicals were of analytical grade and were delivered by Fluka/Sigma-Aldrich Chemie GmbH GmbH (Buchs, Switzerland) unless specified otherwise.

5.2.2 Microtiter plate fermentation (BioLector)

The microtiter plate fermentations were conducted in the BioLector which was originally introduced by Samorski et al. (2005) and recently improved and validated by Kensy et al. (2009). The general set-up of the BioLector is depicted in Figure 5.1A. The BioLector mainly consisted of an optical measurement unit (Fluostar, BMG Lab Technologies, Offenburg, Germany), an optical Y-fiber bundle (Prinz Optics

GmbH, Stromberg, Germany), a X-Y mover (BMG Lab Technologies, Offenburg, Germany) and an orbital shaker (LS-X, Kühner AG, Basel, Switzerland). In the presented experiments the above described BioLector prototype was exclusively applied. The complete device has now been commercialized by m2p-labs GmbH (Aachen, Germany). The biomass concentrations were measured via scattered light at 620 nm excitation without an emission filter. The *GFP* concentrations were monitored using an excitation filter of 485 nm and an emission filter of 520 nm. The sensitivity of the photomultiplier (gain) was adapted to the different measurement tasks and the detailed data is mentioned in the respective figures. The BioLector possessed a data reproducibility of below 5% standard deviation, upon cultivating the same clone in the same medium on a microtiter plate. Due to small standard deviation and the high information content, error bars in the figures were omitted. The experiments were exclusively carried out with black, standard round 96-well microtiter plates with an optical bottom from Greiner Bio-One GmbH (µClear, art.-no.: 655087, Frickenhausen, Germany) that were covered with a gas permeable membrane from ABgene Ltd. (art.-no.: AB-0718, Epsom, UK). If not otherwise specified, the experiments were conducted with 200 µL working volume of culture or medium, a shaking frequency of 995 1/min and a shaking diameter of 3 mm.

5.2.3 Stirred tank fermenter

As standard stirred tank fermenter, a Visual-Safety-Fermenter (VSF, Bioengineering AG, Wald, Switzerland) with a nominal volume of 2 L was used. The fermenter possessed a standard *in-situ* dissolved oxygen tension (*DOT*) and *pH* electrode. The system was sterilized by autoclaving with an *in-situ* rod heater. The fermenter had a height-to-diameter ratio of 3 and two six-bladed Rushton turbines. The fermenter was aerated at a constant gas flow of 1 vvm controlled by a mass flow controller (5850TR, Brooks Instruments, Hatfield, PA, USA). The offgas from the fermenter was additionally analyzed by an online offgas analyzer (O_2 by Magnos 106, ABB AG, Mannheim, Germany and CO_2 by Unor 6N, Maihak AG, Hamburg, Germany). For the online measurement of *OD* and *GFP*, a liquid bypass was connected to the fermenter (Figure 5.1B). The fermentation broth was pumped from a bottom sample port of the fermenter by using a peristaltic pump (Fixo, Ismatec Laboratoriumstechnik GmbH,

Wertheim-Mondfeld, Germany) through the bypass. This bypass contained a bubble trap (self-made) to avoid gas bubbles entering the optical detection line. For the *OD* detection, a flow cuvette with 0.5 mm path length (170-QS, Hellma GmbH& Co. KG, Müllheim, Germany) and a photometer (Photometer 6000, Skalar Analytical B.V., Breda, The Netherlands) were applied. The *OD* was measured with a 630 nm interference filter. Moreover, a flow cuvette (176.051-QS Hellma GmbH & Co. KG, Müllheim, Germany) and a fluorimeter (Fluorimeter 6300, Skalar Analytical B.V., Breda, The Netherlands) were applied for the *GFP* fluorescence measurements. The *GFP* was detected using an excitation filter of 480 nm and an emission filter of 530 nm. After passing through the flow cuvettes, the fermentation broth was recycled into the fermenter (Figure 5.1B). All online detected data were collected via a data acquisition module ADAM-4520 (Advantech Europe GmbH, Düsseldorf, Germany) and controlled by a self-developed bioprocess control program under LabVIEW (National Instruments Germany GmbH, München, Germany) on a PC.

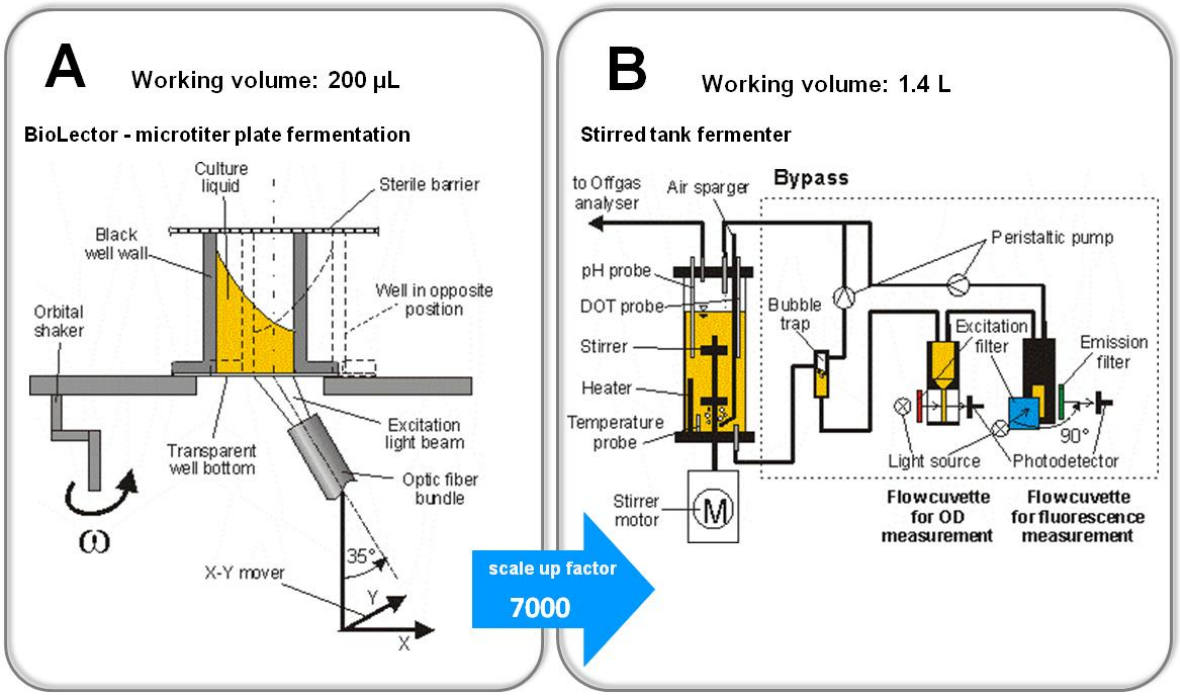


Figure 5.1: Comparison of the experimental set up.

(A) Measurement principle of the microtiter plate fermentation in the BioLector via back scattering of light from cells and fluorescence emission of molecules; (B) Measurement principle of the stirred tank fermentation with online measurement of *OD* and fluorescence in the bypass.

The oxygen transfer rate (*OTR*) and carbon dioxide transfer rate (*CTR*) of the fermentation was calculated corresponding to the following equations [5.1] and [5.2], respectively (Knoll et al., 2005, Knoll et al., 2007):

$$OTR = \frac{\dot{V_G}}{V_m \cdot V_L} \cdot \left[y_{O2}^{IN} - \left(\frac{1 - y_{O2}^{IN} - y_{CO2}^{IN}}{1 - y_{O2}^{OUT} - y_{CO2}^{OUT}} \right) \cdot y_{O2}^{OUT} \right] \quad (5.1)$$

$$CTR = \frac{\dot{V_G}}{V_m \cdot V_L} \cdot \left[y_{CO2}^{OUT} \cdot \left(\frac{1 - y_{O2}^{IN} - y_{CO2}^{IN}}{1 - y_{O2}^{OUT} - y_{CO2}^{OUT}} \right) - y_{CO2}^{IN} \right] \quad (5.2)$$

where *y* is the mole fraction of oxygen or carbon dioxide in the gas phase of the in or out flowing gas stream, $\dot{V}_G$ the norm gas flow rate and V_m is the molar gas volume. From the online derived *OTR* measurement, the $k_L a$ value of the fermenter could be determined as follows, assuming a completely mixed gas phase (Knoll et al., 2005):

$$k_L a = \frac{OTR \cdot He_{O2}}{\left(y_{O2}^{OUT} - \frac{DOT}{100} \cdot y_{O2,cal} \right) \cdot p_R} \quad (5.3)$$

where He_{O2} stands for the Henry constant for oxygen, p_R for the reactor pressure, *DOT* for the dissolved oxygen tension and $y_{O2,cal}$ for the mole fraction of oxygen from the calibration gas of the *DOT* electrode. A Henry constant for oxygen of 1012 bar L/mol for the *H. polymorpha* fermentation and 993 bar L/mol for the *E. coli* fermentation was applied in the calculations (estimated from Weisenberger and Schumpe, 1996).

The *E. coli* fermentation was conducted at a working volume of 1.4 L, a specific aeration rate of 1 vvm, a stirring speed of 1000 1/min and a temperature of 32 °C. Unlike this fermentation, the *H. polymorpha* fermentation was operated at a working volume of 1.4 L, a specific aeration rate of 1 vvm, a stirring speed of 800 1/min and a temperature of 30 °C. After the initial inoculation of the fermenter and a 5 min delay time for mixing of the cell suspension, a 10 mL sample was taken from the fermenter and was then distributed on the MTP to guarantee the same inoculation conditions. The same procedure was repeated with the *E. coli* fermentation after inducing the culture to have a second point of reference between the fermenter and the MTP.

5.2.4 Respiration Activity Monitoring System

In the presented study, the Respiration Activity Monitoring System (RAMOS) was applied to determine the correlation between oxygen transfer rates (*OTR*s) obtained from the chemical sulfite system and biological cultures. RAMOS was first introduced and described in detail by Anderlei and Büchs (2001). Basically, the system consists of particular shake flasks, that in the lower part resemble equal to a standard Erlenmeyer shake flask and, in the headspace, contains an oxygen sensor in the gas phase to measure the respiration activity of cells. With this technique, the *OTR*s of the sulfite system, the *E. coli* fermentation in WR medium and the *H. polymorpha* fermentation in SYN6-MES medium (the same media later applied in the fermenter) were measured at different filling volumes of 10 mL, 15 mL and 20 mL at a shaking frequency of 250 1/min and a shaking diameter of 50 mm. The sulfite oxidation was performed at 25°C and the fermentations at 30°C. Under oxygen limiting conditions, the *OTR* curve of the sulfite oxidation and the fermentations forms a plateau. This plateau level of the different filling volumes can be correlated to each other, thereby giving a proportionality factor (*f*) between the *OTR*s of the biological media and the sulfite system as follows in equation [5.4] (Kensy et al., 2005, Maier et al., 2004):

$$proportionality\ factor\ (f) = \frac{OTR_{Bio}}{OTR_{Sulfite}} \tag{5.4}$$

The proportionality factor (*f*) represents the ratio between the different oxygen solubilities (C^*) and diffusion coefficients (D_{O2}) of the applied media.

5.2.5 Determination of oxygen transfer rates (*OTR*) in surface-aerated bioreactors

For years, the sulfite oxidation served very well as a model system for the characterization of small-scale, surface-aerated bioreactors. Hermann et al. (2001, 2003) introduced this technique together with a simple color shift of a colorimetric *pH*-indicator making it possible to read out the reaction kinetics with a simple camera. In the presented work, this sulfite oxidation method from Hermann was again applied to characterize the oxygen mass transfer conditions in 96-well microtiter plates with various filling volumes ranging vom 100 µL up to 260 µL. The 96-well microtiter plate

was operated with a gas-permeable cover membrane (ABgene Ltd., art.-no.: AB-0718, Epsom, UK) at a temperature of 25 °C, a shaking frequency of 995 1/min and a shaking diameter of 3 mm.

5.2.6 Calibration alignment between different optical measurement methods

Due to different optical measurement methods applied in this study, the obtained measurement signals had to be calibrated with a standard analytical method. The scattered lights intensities from the BioLector and *OD* from the fermenter bypass were correlated to cell dry weight (*CDW*) as the biomass calibration unit. The cell dry weight was determined gravimetrically after washing the cells twice in physiological salt solution (9 g/L NaCl) and drying the cells at 105 °C until the mass remained constant. The calibration of the measurement signals, i.e. scattered light and *OD*, with cell dry weight was conducted at the end of the fermentations. The contents of the fermenter analogous to the normal fermenter operation were recycled through the fermenter bypass. Simultaneously, the measurements, *OD* and *GFP* fluorescence, were analyzed. During the calibration procedure, the fermenter contents were continuously diluted with new medium, directed through the bypass and again measured. After each dilution step, a 5 mL sample was taken from the well mixed fermenter in order to collect scattered light intensities in the BioLector and to analyze cell dry weights from the same samples. This procedure was repeated for the fermentations of *E. coli* and *H. polymorpha*. Finally, these measurement signals could be correlated to each other (Figure 5.2A and B). Since no offline protein analysis was available in our laboratory for the detection of *GFP* concentrations, the *GFP* fluorescence signals of both measurement systems, the fermenter and the MTP, were directly correlated with each other (Figure 5.2C). From the literature, it is known that the *GFP* fluorescence signals can be well correlated with real *GFP* protein concentrations in well aerated systems such as fermenters (Li et al., 2001).

5.3 Results and Discussion

5.3.1 Calibration of the measurement apparatus

In order to compare the growth and protein expression of both studied cultivation systems, i.e. the shaken microtiter plate and the stirred tank fermenter, it is very important to align the online measured parameters. Therefore, the biomass measurements of scattered light intensities in the MTP and those of *OD* in the STF bypass were all correlated to the cell dry weight. In the case of the protein expression parameter, *GFP* was applied as a model protein for simplifying the detection by fluorescence. *GFP* was not correlated to any offline laboratory method such as ELISA or Western Blot. Both the BioLector and the STF measurement systems were directly correlated to each other. Figure 5.2 presents the calibration of the measurement signals for the *E. coli* and *H. polymorpha* fermentations.

Figure 5.2 demonstrates that all correlations are highly precise. This is confirmed by the coefficient of determination value (R^2) of approximately 0.99 which was calculated for all linear equations. Each R^2 is presented below the respective linear equations in Figure 5.2. For the measurement of scattered light in the MTP and of *OD* in the bypass, it was necessary to perform separate calibrations for the distinct microorganisms, *E. coli* and *H. polymorpha*, because the optical biomass measurement depends strongly on the morphology of the cells. For the *GFP* fluorescence measurements, however, it was only necessary to perform one calibration, because, in this case, it served only to align the different measurement systems.

The calibrations performed here were subsequently used to calculate the cell dry weight and the *GFP* fluorescence (based on the MTP fluorescence unit) for each fermentation. Thus, the kinetic data from both fermentation systems, MTP and STF, were actually comparable.

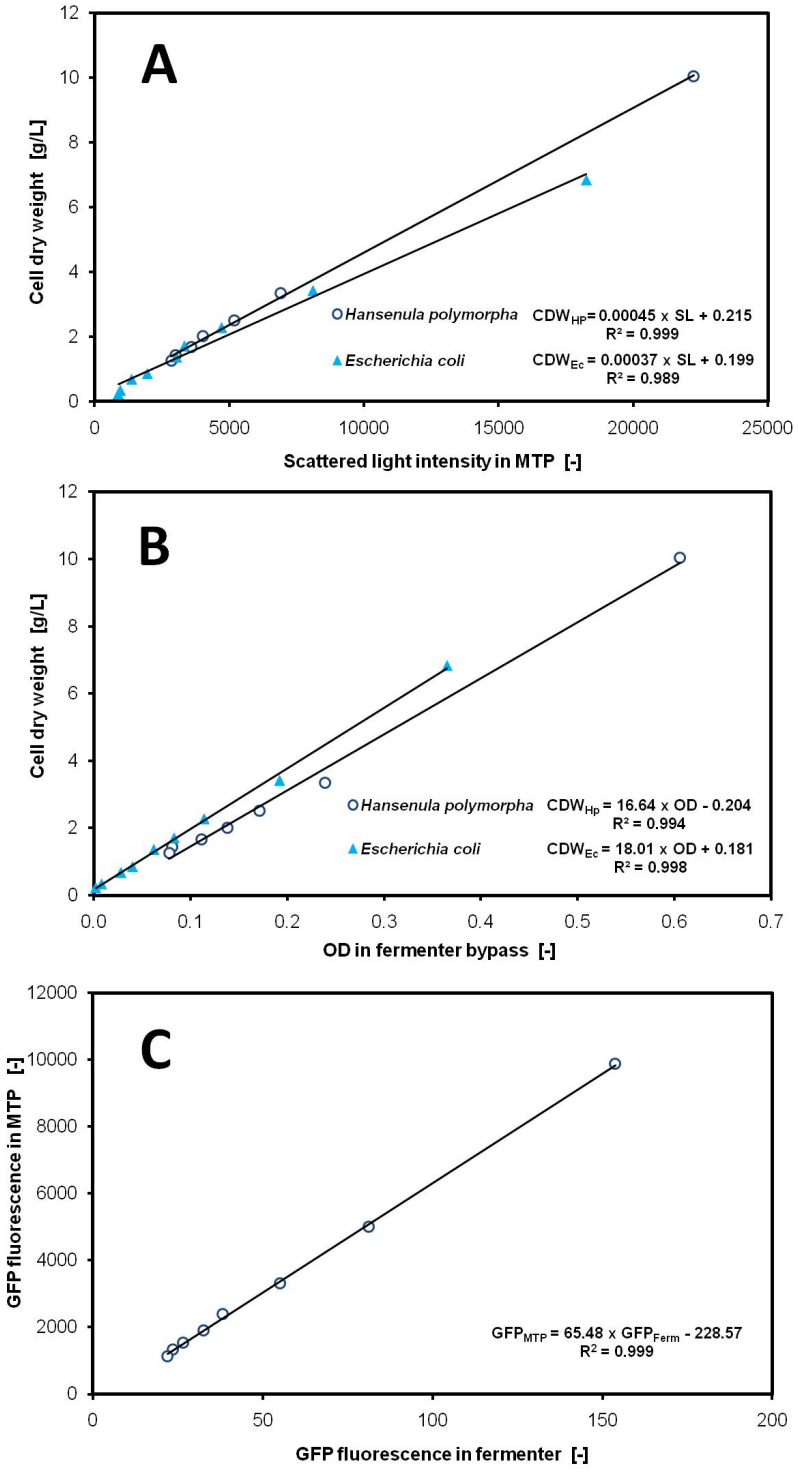


Figure 5.2: Calibration of measurement signals.

(A) Cell dry weight versus scattered light intensities measured in microtiter plates; (B) Cell dry weight versus *OD* measured in the fermenter bypass; (C) *GFP* fluorescence in the microtiter plate versus *GFP* fluorescence in the fermenter of the *H. polymorpha* fermentation; Operation conditions: MTP (*H. polymorpha/E. coli*): 200 µL filling volume, at 30 °C/32 °C temperature, 995 1/min shaking frequency and 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, gain: 20) and *GFP* (ex: 485 nm/em: 520 nm, gain: 10); fermenter (*H. polymorpha/E. coli*): 1.4 L filling volume, at 30 °C/32 °C temperature, 800/1000 1/min shaking frequency, 1 vvm specific aeration rate, *OD* (Absorption: 630 nm) and *GFP* (ex: 480 nm/em: 530 nm)

5.3.2 Transformation of chemical OTR_{max} to biological OTR_{max}

The sulfite oxidation method is a very good and simple method to characterize the maximum oxygen transfer capacity (OTR_{max}) in small-scale, surface-aerated fermentation systems as was reported by Hermann et al. (2003). Unfortunately, this chemical model system does not truly reflect the composition of biological media. In general, the actual medium composition interferes with the oxygen diffusion coefficient (D_{O2}) and the oxygen concentration at the gas-liquid interface (C^*). To determine the influence of the hereby applied fermentation media, additional RAMOS experiments with the sulfite system, the *E. coli* medium and *H. polymorpha* medium were necessary. Since no dissolved oxygen can be measured in the RAMOS device, it is important to run the experiments under oxygen-limiting conditions, where the oxygen concentration in the bulk liquid (C_L) becomes very small. Consequently, the sulfite oxidation and the fermentation were run with different filling volumes (10 mL, 15 mL and 20 mL) in the RAMOS shake flasks. The results of this experiment are presented in Figure 5.3.

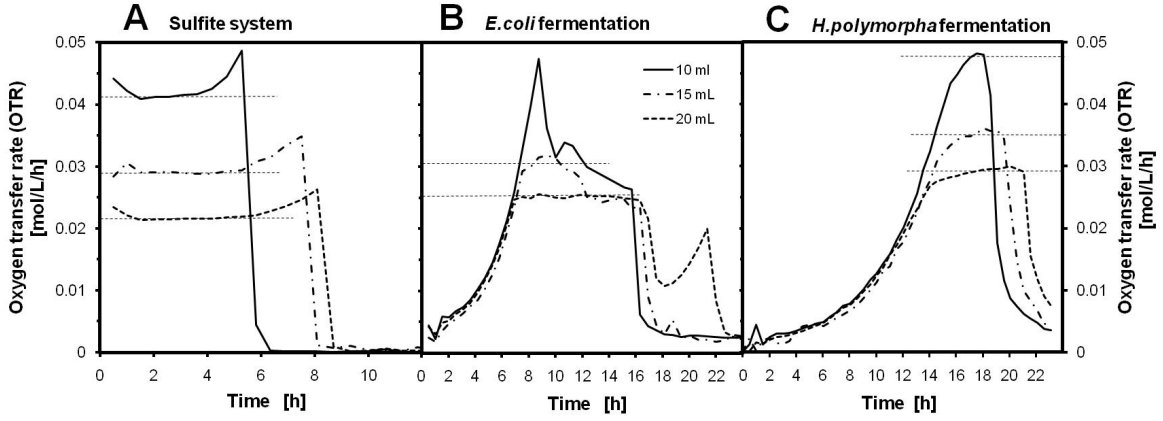


Figure 5.3: Determination of the different oxygen transfer rates (*OTR*) in RAMOS (A) for the chemical 0.5 mM sulfite system; (B) for the *E. coli* fermentation in WR medium; (C) for the *H. polymorpha* fermentation in SYN6-MES medium.

Experimental conditions: 10 mL, 15 mL and 20 mL filling volume, 250 mL Erlenmeyer shake flask for RAMOS, 250 1/min shaking frequency, 50 mm shaking diameter, at 25 °C temperature with the sulfite system and 30 °C temperature with the microorganisms. The E. coli cultures were induced at 7.17 h with 0.5 mM IPTG.

Figure 5.3 clearly depicts that most of the *OTR* curves possess a plateau, which implies the oxygen limitation (Anderlei and Büchs, 2001, Anderlei et al., 2004). Only

the *E. coli* fermentation with 10 mL filling volume does not become limited and thus, forms a sharp peak. The graphs also depict that the sulfite system reached an *OTR* plateau directly after the start of the experiment, because the oxidation is driven by a constant concentration of the cobalt catalyst under almost constant reaction conditions (Hermann et al., 2001). Therefore, the reaction runs at an almost constant rate until the end of the experiment. In contrast, the fermentation cultures pass through the typical exponential growth phase until they reach the oxygen limitation and the mentioned *OTR* plateau.

In general, it is obvious that lower filling volumes attain higher OTR_{max} values than larger filling volumes due to the higher specific surface area assessable for oxygen diffusion (Maier and Büchs, 2001, Maier et al., 2004). For both microorganisms, i.e. *E. coli* and *H. polymorpha*, the maximum *OTR* necessary to supply sufficient oxygen to the culture ranges from 0.045 – 0.050 mol/L/h as is observed with the 10 mL filling volume. From these graphs the *OTR* plateau values for the different media were correlated to each other and inserted into Equation [5.4]. The following table presents the evaluated proportionality factor (*f*):

Table 5.1: Evaluated proportionality factor (*f*) between fermentation and the sulfite system

proportionality factor (f)	$\frac{OTR_{Bio}}{OTR_{Sulfite}}$
E. coli in WR medium	**1.13**
H. polymorpha in SYN6-MES medium	**1.22**

These proportionality factors (f) were subsequently applied for calculating the maximum oxygen transfer capacities in MTPs.

5.3.3 Characterization of k_La in MTPs

Upon cultivating microorganisms in small-scale bioreactors, it is very important to conduct the experiments under well-characterized operating conditions. To meet this requirement, the operation conditions in MTPs were first characterized using the

sulfite oxidation method introduced by Hermann et al. (2001). Whereas, the shaking frequency and diameter were kept constant at 995 1/min and 3 mm, respectively, the filling volumes of the wells of the MTP were varied between 100 µL and 260 µL. The results of this characterization are presented in Figure 5.4.

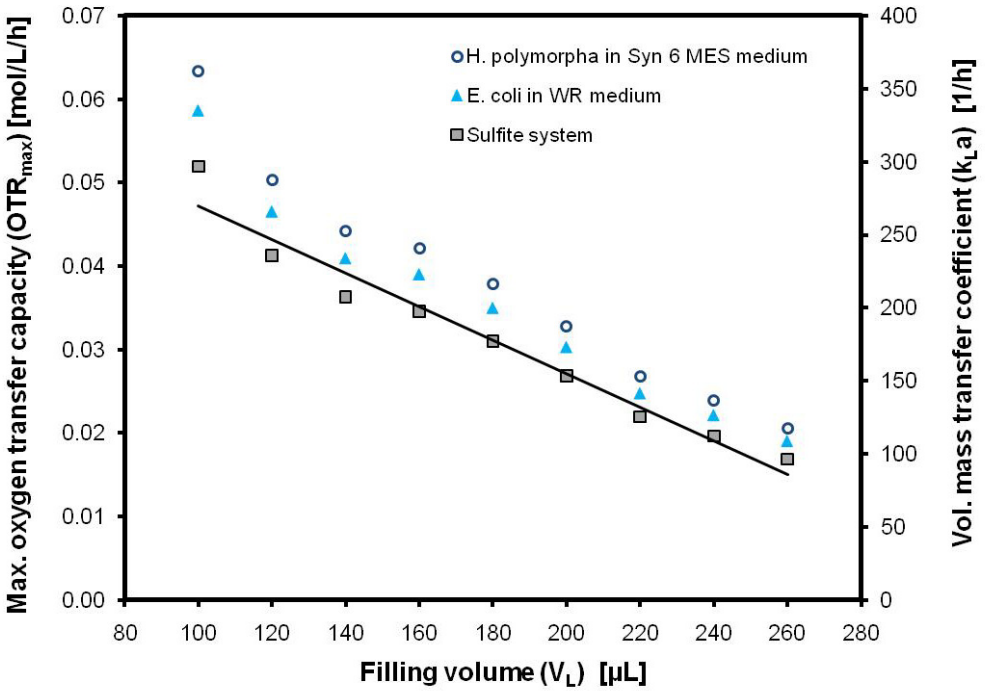


Figure 5.4: Maximum oxygen transfer capacity (OTR_{max}) and k_La values versus the filling volume for the chemical 0.5 mM sulfite system, the *E. coli* fermentation in WR medium and the *Hansenula polymorpha* fermentation in SYN6-MES medium.

The method from Hermann et al. (2001) was applied for the determination and calculation of OTR_{max} and k_La values for the sulfite system. The OTR_{max} and k_La values for the fermentations were calculated by applying the proportionality factor (*f*) from Table 5.1 on the OTR_{max} values of the sulfite system. Experimental conditions (Sulfite system/*E. coli*/ *H. polymorpha*): various filling volumes between 100-260 µL, at 25 °C/30 °C/30 °C temperature, 995 1/min shaking frequency and 3 mm shaking diameter.

It is notable that a linear correlation between the maximum oxygen transfer capacity (OTR_{max}) and the filling volume was obtained, analogously as was reported previously (Hermann et al., 2003, John et al., 2003, Kensy et al., 2005). The OTR_{max} ranges from 0.017 mol/L/h (k_La= 97 1/h) at 260 µL up to 0.052 (k_La= 297 1/h) at 100 µL for the sulfite system. These values correlate very well to those reported from Hermann et al. (2003) and John et al. (2003) for standard round 96-well MTPs and also correlate well in magnitude with other formats such as square 24-deepwell plates (Islam et al., 2008). Applying both aforementioned proportionality factors (*f*) for the fermentation media (Figure 5.4), the OTR_{max} and k_La values achievable in *E. coli*

and *H. polymorpha* fermentations lie above the line of the sulfite system, whereby *H. polymorpha* always shows the highest values.

Even though, it would be better to apply filling volumes of 100-120 µL with regard to optimize the oxygen transfer (0.045-0.050 moL/L/h from Figure 5.3), an actual filling volume of 200 µL was applied in order to ensure that sufficient liquid was available for further offline analysis. Moreover, for a precise optical detection it was important to have enough liquid on the well bottom during the continuous shaking of the MTP (Kensy et al., 2005, Kensy et al., 2005b). This implies operating the MTP at a suboptimal k_La value of 180-190 1/h compared to that of the stirred tank fermenter (k_La= 370-600 1/h). Nevertheless, the RAMOS experiments (Figure 5.3B and C) depicted that even oxygen-limiting *OTR*s of greater than 0.030 mol/L/h (k_La= 175 1/h) simply result in a prolongation of the fermentation by 1 h for *E. coli* and *H. polymorpha*, respectively. This is regarded as being non significant.

5.3.4 Comparison of parallel *E. coli* fermentations in MTP and fermenter

Figure 5.5 presents the direct comparison of parallel *E. coli* fermentations in the MTP and STF. Four different curves are plotted in Figure 5.5A and B:

- MTP, non induced (I) – the same *E. coli* fermentation without induction as a negative control
- MTP, induced (II) – the inoculated *E. coli* culture was taken from the fermenter and were distributed on the MTP, induction was performed at 7.3 h in the MTP
- MTP, induced in fermenter (III) - the *E. coli* culture was sampled from the fermenter after induction and then distributed on the MTP
- Fermenter, induced (IV) – the fermentation was completely running in the fermenter with an induction at 7.3 h.

Figure 5.5C presents the online process parameters from the fermenter and the offgas analyzer.

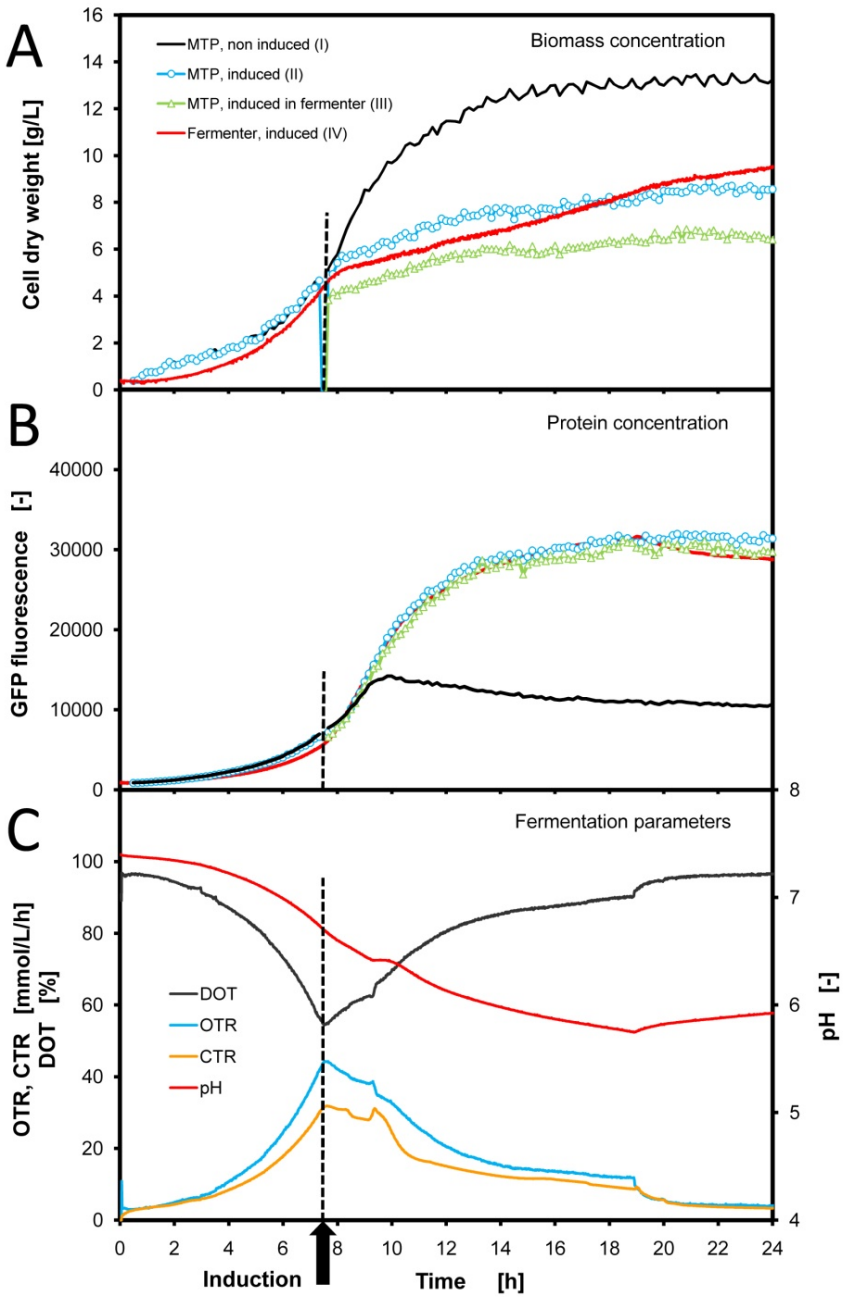


Figure 5.5: Comparison of parallel *E. coli* fermentations in microtiter plate and stirred tank fermenter.

Experimental conditions: MTP: 200 µL filling volume, at 32 °C temperature, 995 1/min shaking frequency and 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, gain: 20) and *GFP* (ex: 485 nm/em: 520 nm, gain: 10); fermenter: 1.4 L filling volume, at 32 °C temperature, 1000 1/min shaking frequency, 1 vvm specific aeration rate, induction with 0.5 mM IPTG (end concentration in the fermenter) at 7.3 h, *OD* (Absorption: 630 nm) and *GFP* (ex: 480 nm/em: 530 nm)

The development of the biomass concentrations (Figure 5.5A) depicts that, in the pre-induction phase, all three cultures (I, II and IV) have the same slope and reach a

biomass concentration of 4.5 g/L at the induction point of 7.3 h (please note: the monitoring of the culture (III) - MTP, induced in fermenter - started after the induction, in the pre-induction phase this culture is displayed as the fermenter curve). In the post-induction phase, a clear separation of the negative control (I), which was not induced, is notable. All other cultures (II, III and IV), which were induced, follow the same trend. Obviously, the *E. coli* strain seems to be growth-inhibited after induction. Only at the very end of the fermentation, the fermenter curve (IV) deviates slightly from the MTP curves (II and III). This both induced cultures running in the MTP (II and III) show exactly the same growth behavior at a slightly deviating biomass concentration.

Analyzing the protein expression (Figure 5.5B) results in a very similar behavior. In the pre-induction phase, all three cultures (I, II and IV) follow the same slope resulting in a *GFP* fluorescence of 6500 at the induction point. It seems, that *E. coli* constitutively produces the product *GFP*. Then, after the induction, the curves of the different cultures deviate. The negative control (I) continues to produce *GFP* up to 10 h. Then, the *GFP* formation stagnates. In contrast, the induced cultures (II, III and IV) continue producing the *GFP* up to 19 h. All induced cultures (II, III and IV) follow exactly the same slope of *GFP* formation, which means that the cultivation conditions are indeed comparable for the MTP and the fermenter system. It cannot be excluded that *GFP* inclusion bodies have been formed in this expression experiment, but due to the steady slope of the *GFP* fluorescence signals it is expected that *GFP* is produced in soluble form.

Figure 5.5C exhibits the corresponding process parameters measured in the fermenter. All these parameters correspond very well to the different culture phases. Due to the missing availability of *pH*-control in the MTP, the fermenter was also operated without *pH*-control. This results in a *pH* decrease from 7.2 to 5.9 over the course of fermentation, which did not limit the culture growth. The *OTR* curve points out that in this fermentation, a maximum *OTR* of 0.043 mol/L/h was reached which is comparable to those values detected in shake flasks with RAMOS. k_La-values of 600 1/h were calculated with equation [5.3] for the STF which were almost three times higher than that applied in the MTP. The *DOT* curve in Figure 5.5C demonstrates

that at this high k_La-value in the fermenter the *E. coli* culture reached a minimum *DOT* of 55%. This signifies that the culture is far away from an oxygen limitation. Even at the reduced k_La-value of the MTP (180 1/h), the culture experienced only a slight oxygen limitation which did not sustainably effect the culture growth nor protein expression as mentioned before. This minor influence of a slight oxygen limitation was also observed in the RAMOS experiments comparing the 10 mL and the 15 mL filling volumes (Figure 5.3B). In this case, a short prolongation of the fermentation with 15 mL filling volume in comparison to the 10 mL fermentation was noticeable.

Overall, the results of the parallel *E. coli* fermentations in MTP and STF demonstrate that the cultivation conditions are very comparable. All induced cultures behave very similarly and show the same growth and protein expression kinetics. The negative control of a non induced culture depicts which differences in kinetics could normally appear.

5.3.5 Comparison of parallel yeast fermentations in MTP and fermenter

The same experiment as for *E. coli* was also performed with the yeast *H. polymorpha.* Here, no induction of the promoter was necessary due to the different promoter regulation of *H. polymorpha*. The applied *FMD* promoter was simply derepressed by the substrate glycerol (Amuel et al., 2000).

Very comparable growth and protein expression kinetics were also found in these parallel fermentations (Figure 5.6). In Figure 5.6A, for both cultivation systems, MTP and STF, almost identical exponential growth curves could be observed. Exactly at about 16 h, both fermentations enter the stationary phase at a biomass concentration of 11.4 g/L *CDW* (MTP) and 10.7 g/L *CDW* (STF) resulting in a relative error of 6.3%. Both systems also showed consistent protein expression of *GFP.* Even small changes in the expression rate appeared synchronously. In general, the protein expression mirrored the exponential growth curves. The protein formation was subjected to a derepression as known from the literature (Amuel et al., 2000). At the synchronous entry into the stationary phase at 16 h, both fermentations differ only by 9.5% with regard to the *GFP* fluorescence.

The process parameters of the fermenter (Figure 5.6C) again correspond very well to the biomass and protein formation kinetics. The *OTR* and *CTR* indicate an exponential development similar to the growth curve. All dynamics terminate at 16 h, when the substrate is exhausted and the metabolic activity decreases immediately.

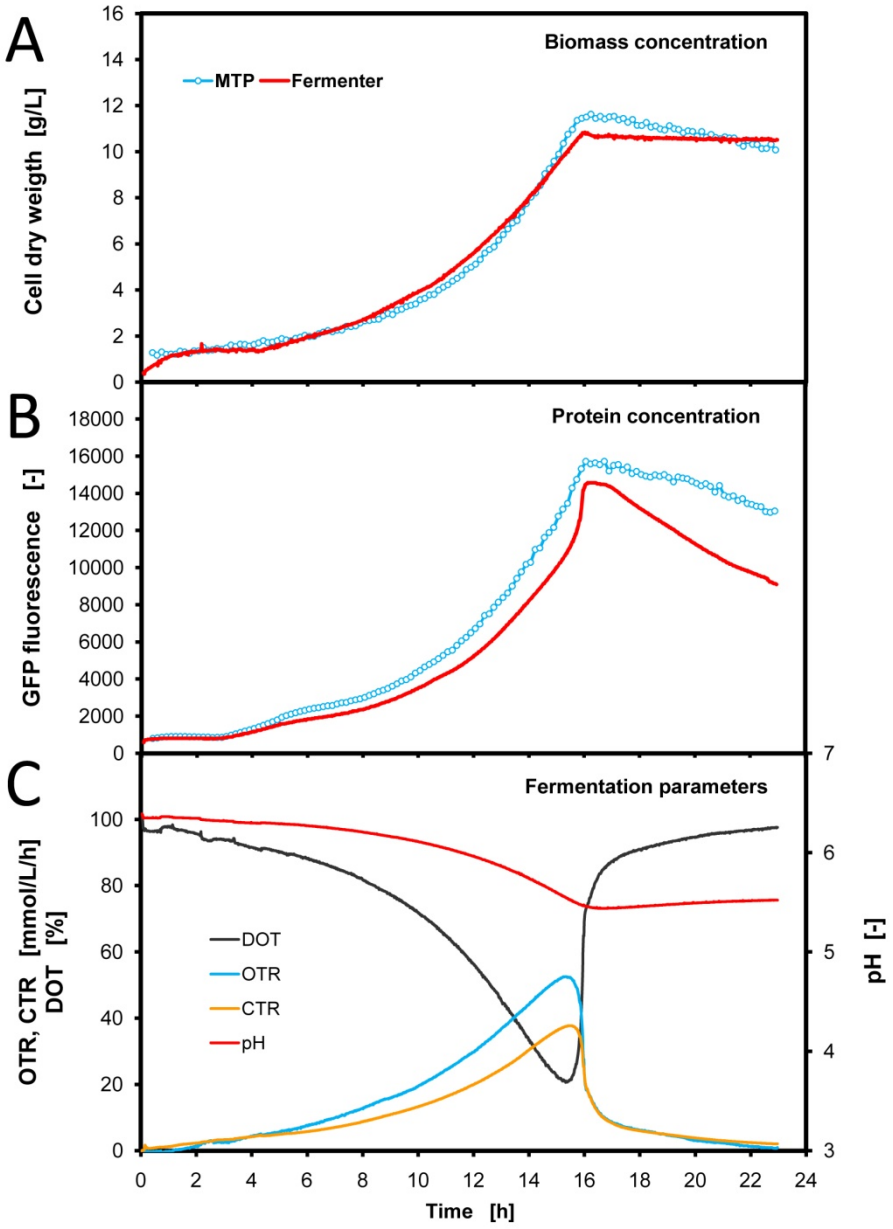


Figure 5.6: Comparison of parallel *H. polymorpha* fermentations in microtiter plate and stirred tank fermenter.

Experimental conditions: MTP: 200 µL filling volume, at 30 °C temperature, 995 1/min shaking frequency and 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, gain: 20) and *GFP* (ex: 485 nm/em: 520 nm, gain: 10); fermenter: 1.4 L filling volume, at 30 °C temperature, 800 1/min shaking frequency, 1 vvm specific aeration rate, *OD* (Absorption: 630 nm) and *GFP* (ex: 480 nm/em: 530 nm)

The uncontrolled *pH*-value decreases from 6.4 to 5.5, which does not limit growth of *H. polymorpha* (Stöckmann et al., 2003). A maximum *OTR* of 0.052 mol/L/h was reached, which is slightly higher than the values attained in the shake flasks in RAMOS (Figure 5.3C). The calculated k_La-value applied in the fermenter were 370 1/h which is double of that applied in the MTP (k_La= 180-190 1/h).

In the fermentation the *DOT* dropped down to a minimum of 21% (Figure 5.6C). This might mean that the yeast culture in the MTP fermentation could run into a slight oxygen limitation. Again here, no significant influence is noticeable concerning culture growth and protein expression in comparison to the STF. Similar ratios of *OTR* between the 10 mL and 15 mL filling volumes in the RAMOS experiments (Figure 5.3C) as that applied in the STF and MTP only led to a minor prolongation of fermentation in RAMOS of 1 h. This could not be observed in the comparison of the STF and the MTP.

In summary, the MTP can mimic the fermenter very well. Besides the *E. coli* fermentations, the parallel *H. polymorpha* fermentations in the MTP and STF also produced very comparable growth and protein expression kinetics. During metabolic activity, all online-measured data show deviations of below 10% between the two cultivation systems.

5.3.6 Characterization of specific product formation

As it was demonstrated in the previous figures, fermentations in MTPs can mimic growth and protein expression kinetics very well. Therefore, it could be very interesting to apply the online measurement data of the MTP-based BioLector to characterize clones and fermentation conditions with respect to their specific product formation capacities. Figure 5.7 gives an example of a different presentation of the online data. With the BioLector, it is now possible to present the product formation data as a function of biomass concentration. This is rarely possible with fermenters, because laboratories often lack online biomass and fluorescence sensors. This online biomass measurement would be possible by applying an online *OD* measurement (like in our case in the bypass, Figure 5.1) or an online impedance

sensor (e.g. from Aber Instruments, UK or Fogale, France). For the detection of fluorescent proteins an online fluorescence sensor could be installed (e.g. BioView from Delta, Denmark). These sensors, however, would require a much higher investment, because two additional sensors for each fermenter will be needed beside the common *pH* and *DOT* electrodes and the temperature probe.

Figure 5.7 presents the fluorescence development of *GFP* versus the cell dry weight of the *E. coli* and the *H. polymorpha* fermentation in both reactor scales, STF and MTP, which has been described earlier in this study. Whereas, the *H. polymorpha* strain shows only a linear correlation between biomass and *GFP* protein, the *E. coli* strain experiences a boost in protein production after the induction (as indicated). The graphs in Figure 5.7 clearly depict the derepressed, almost constitutive, expression for the *H. polymorpha* strain and the induced expression for the *E. coli* strain.

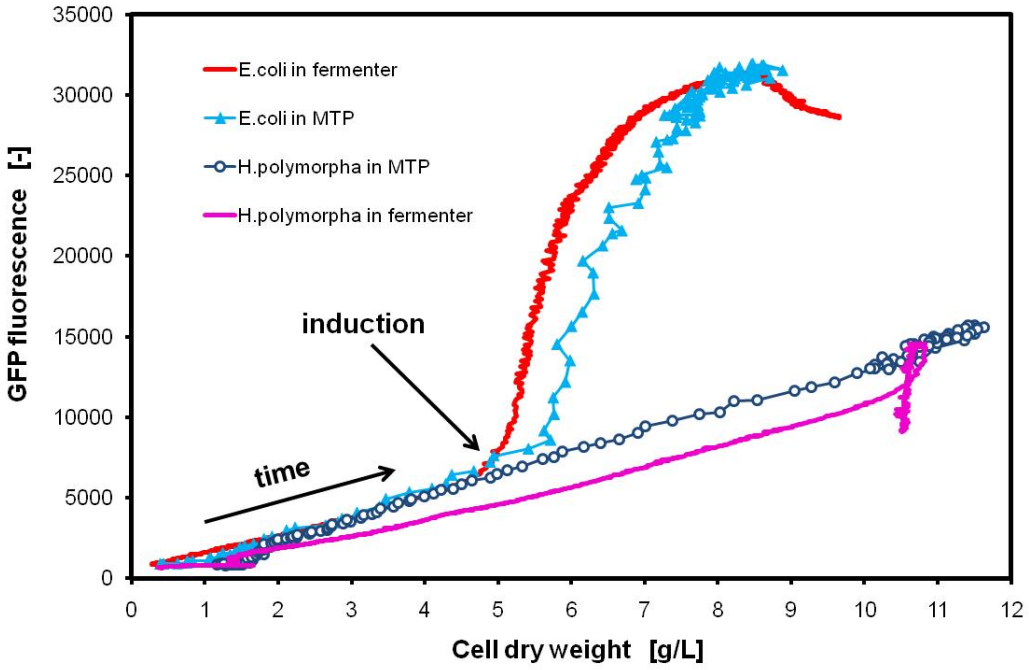


Figure 5.7: Protein expression as function of cell dry weight – characteristics of different expression systems.

Direct correlation of the protein expression via *GFP* fluorescence to the biomass concentration of the fermentations of Figure 5.5 and Figure 5.6; calibrated data from measurements in the BioLector: scattered light (ex: 620 nm/em: -, gain: 20), *GFP* (ex: 485 nm/em: 520 nm, gain: 10) and in the fermenter bypass: *OD* (Absorption: 630 nm) and *GFP* (ex: 480 nm/em: 530 nm).

Additionally, the graphs in Figure 5.7 provide the specific product yield ($Y_{P/X}$) of the strains by deriving the slope of the graphs as $Y_{P/X}$. This evaluation criteria is very often used to consider the productivity of a fermentation process (Mandenius and Brundin, 2008, Schmidt, 2005). This novel way of data presentation, i.e. protein concentration as a function of biomass concentration, has first been made possible even on a micro-scale by using new online measurement techniques such as the BioLector. This can help to facilitate the selection of a potent clone or a very productive fermentation medium or process.

5.4 Conclusions

After the validation of the online monitoring capacity of the new high-throughput fermentation system (Kensy et al., 2009), called BioLector, it was challenging to investigate the scalability from a microtiter plate to a stirred tank fermenter. Therefore, the present study was focused on the scale-up of common microbial expression systems, such as the bacteria *E. coli* and the yeast *H. polymorpha*, from microtiter plate to stirred tank fermenter. The prerequisite to perform such a study was the comprehensive characterization of mass transfer conditions in microtiter plates. In recent years, many of the common microtiter plates were already characterized by several groups, whereby a few new methods for the characterization of engineering parameters were developed (Hermann et al., 2001 and 2003, Kensy et al., 2005, Maier et al., 2004, Doig et al., 2005). In this work, the maximum oxygen transfer capacity (OTR_{max}) and the volumetric mass transfer coefficient (k_La) were determined with the sulfite oxidation method from Hermann et al. (2001). Specifically, the OTR_{max} and k_La were measured for various filling volumes in a standard 96-well microtiter plate at a constant shaking frequency of 995 1/min and a shaking diameter of 3 mm, because only little information was available at these conditions. Finally, it was necessary to convert the chemical OTR_{max} values into biological OTR_{max} values, which could be achieved with the help of a RAMOS device. Even though more practical but suboptimal filling volumes of 200 µL were applied instead of superior 100-120 µL with regard to the *OTR* demand of the cultures, excellent scalability results could be attained. The comparison of the online measurement signals, biomass concentration and the *GFP* fluorescence as a model

protein, in parallel fermentations using microtiter plate and stirred tank fermenter proved that the kinetics of growth and protein expression are well comparable between both reactor scales. This was confirmed not only by the bacteria fermentation of *E. coli*, but also by the yeast fermentation of *H. polymorpha*. Both expression systems showed nearly identical kinetics in the microtiter plate and the stirred tank fermenter, showing a maximum of 10% deviation between the measurement signals. Taking into account that the scale-up factor applied here was 7000, this was a surprisingly good result. Ultimately, the presented study pointed out that the scale-up from microtiter plates and stirred tank fermenters is possible for standard microbial expression systems with general low viscosity. As previously mentioned, one of the key factors for successful scale-up was the k_La value (Islam et al., 2008). This proven scalability of MTPs to STFs could make them ideally suited as a microbioreactor and a scale-down reactor unit. This scalability combined with high-throughput and online monitoring of important process parameters, i.e. biomass and protein concentrations when using fluorescent proteins or fusions thereof, creates a very powerful tool in screening and bioprocess development. Even though k_La values of up to 350 1/h have been reported for round 96-well MTPs in this study and for square 24-well MTPs in other publications (Islam et al., 2008), these k_La values are still not sufficient to mimic a full industrial fermenter in a scale-down model. Higher k_La values of up to 1000 1/h would be desirable to attain higher cell concentrations and to operate a fedbatch process. New microtiter plate formats with new geometrical well designs such as the recently presented Flowerplate could probably solve these limitations in the future (Funke et al., 2009).

6 Full-Parameter Monitoring in Shaken Microbioreactors at Elevated Oxygen Transfer Rates

Abstract: Microbioreactors are a promising new solution to accelerate process development in biotechnology. Besides high-throughput, there is a great demand for more information content from the biological reactions. Microtiter plates, widely applied in biotechnology, are the platform of choice for high-throughput experimentation. This platform is slowly evolving towards high-information content screening mainly applied in drug discovery. One technique for high-throughput fermentations has been recently presented. This new technique, i.e. the BioLector, combines the high-throughput of microtiter plates with non-invasive online monitoring of biomass and fluorescence. This study presents the advancement of this technology to also include the simultaneous online monitoring of *pH* and dissolved oxygen tension (*DOT*) by integrating optodes immobilized on the bottom of all 48 wells of a Flowerplate, whose baffled flower-shaped wells lead to high oxygen transfer rates. Maximum oxygen transfer capacities (OTR_{max}) between 0.04 mol/L/h and 0.14 mol/L/h (k_La range: 200-800 1/h) were achieved at two shaking frequencies (1000 1/min and 1400 1/min) for a broad range of filling volumes (500 µl-1700 µL). By using exemplary *E.coli* fermentations in both synthetic and complex fermentation media at a shaking frequency of 1000 1/min, it could be shown that the oxygen supply was limited, thereby reducing the cell growth and increasing acid production. Once the shaking frequency was increased to 1400 1/min, however, the oxygen supply became unlimited and cell growth increased. Moreover, in yeast fermentation experiments using *Hansenula polymorpha,* it was demonstrated that *pH*-variations indeed affected the yeast growth: the yeast grew slower at *pH*-values approaching neutral values of *pH* 7.4. Furthermore, with regard to protein stability investigations using *Hansenula polymorpha*, it was found that different media supplementations of plant peptones increased recombinant protein production and improved protein stability. The advancement of the BioLector technology by incorporating *pH* and *DOT* measurements, along with biomass and fluorescence measurements, transforms the BioLector into a full-parameter monitoring system for microfermentations. The combination of this technology with the Flowerplate which offers high-oxygen transfer now offers a very powerful tool to mimic laboratory fermenters. It allows researchers to transfer more R&D tasks in fermentation science to a microscale with less effort.

6.1 Introduction

Bioprocesses are increasingly replacing chemical production processes in the pharmaceutical, fine chemical, and in fuel industries. Regarding their high environmental sustainability and high reaction selectivity, bioprocesses already compete with chemical processes and are often more cost-effective (Hatti-Kaul et al., 2007). Current research efforts in systems and synthetic biology are evolving towards better understanding of metabolic pathways and bottom-up design of new pathways to further improve production of bio-molecules (Leonhard et al., 2008). All these processes have in common that they use microbial or mammalian expression hosts as cellular factories. Therefore, a huge number of individual clones has to be processed, selected and process environment has to be optimized. The current techniques to elaborate these research tasks are still simple shakes flasks and microtiter plates (MTPs). Where the former provides only moderate throughput, offers very little process information and is difficult to automate, the later, i.e. microtiter plates, provide an intrinsic capacity for high-throughput. Moreover, they are generally applied like black box systems, and the reactions are only analyzed at the end of the experiment (Büchs, 2001). MTPs can be at least integrated into robotic systems and therefore, are well suited for automation. This lack of adequate bioprocessing techniques which integrate high-throughput and more process information has in recent years led to the development of several microbioreactors, processing samples in the volume range of 10 µl to 10 mL, in which, only few have been marketed (Betts and Baganz, 2006).

All these microbioreactor concepts can be mainly classified as bubble columns, stirred tank reactors and shaken microtiter plates. A good overview of all studied technologies is given in several excellent reviews (Betts and Baganz, 2006, Weuster-Botz et al., 2005, Marques et al., 2009). The first bubble column concept was reported by Doig et al. (2005). This group characterized the volumetric mass transfer coefficient (k_La) of bubble columns in a 48-well microtiter plate format filled with 2 mL. The achieved k_La values range from 30 1/h to 324 1/h, which mimic a shake flask and a stirred tank reactor operated at moderate stirring rates (Gupta and Rao, 2003, Maier et al., 2004, Garcia-Ochoa et al., 2009). However, it was difficult to integrate

online sensors into these bubble columns. Another bubble column concept using additional orbital shaking was commercialized under the name of µ24 by MicroReactor Technologies Inc. (Mountain View, CA, USA) and marketed by Applikon Inc. (Foster City, CA, USA). For this shaken 24-well bubble column, uneven aeration rates over the plate were reported (Chen et al., 2009) and, thus, only k_La values for non-sparged operation conditions were reported in literature (Isett et al., 2007). The achieved k_La values lay at 33-56 1/h for the simple shaking operation with volumes of 4-6 mL at a shaking frequency of 500-800 1/min. These conditions could easily mimic a shaken flask operated at moderate shaking frequencies (Gupta and Rao, 2003, Maier et al., 2004). The authors also cited maximum k_La values of 624 1/h, when the columns were sparged with pure oxygen. These k_La values could well mimic those of a stirred tank bioreactor (Garcia-Ochoa et al., 2009), but safety precautions must be taken due to the use of pure oxygen in a laboratory environment. Since the µ24 technology also possesses integrated *pH*- and *DOT*-optodes for process parameter monitoring, the *pH* and *DOT* sensors were successfully applied in *Escherichia coli, Saccharomyces cerevisiae* and *Pichia pastoris* batch fermentations (Isett et al., 2007).

Mini-stirred tank reactors for high-throughput bioprocessing have been invented by the research groups of Gary Lye (University College London, GB, (Lamping et al., 2003)), Dirk Weuster-Botz (TU München, Germany, (Weuster-Botz, 2005)) and Klavs Jensen (MIT, USA (Szita et al., 2005)). Their reactor concepts are all very complex and intended for operation in fedbatch mode. Therefore, they need complex microfluidics (Szita et al., 2005) or even liquid-handling systems (Puskeiler et al., 2005) to perform the feeding and *pH* control. All concepts apply *pH*- and *DOT*-optode sensors whose data are read out at each reactor using specific LEDs and photodiodes. The concept from Jensen also includes optical density (*OD*) and fluorescence measurements (Szita et al., 2005). The basic disadvantage that all concepts share is that the higher parallelization of reactors dramatically increases complexity and investment costs. None of these concepts have yet entered the market. For more details on the mini-stirred tank reactor concepts please see the following publications (Lamping et al., 2003, Weuster-Botz, 2005, Szita et al., 2005).

Shaken microtiter plates are probably the simplest microbioreactors applied in biotechnology. Microtiter plates are already widely distributed in biotechnology laboratories and can be considered as a standard for high-throughput experimentation. Intensive research has been conducted on the characterization of engineering parameters in shaken microtiter plates in recent years (Duetz et al., 2000, Hermann et al., 2003, John et al., 2003, Doig et al., 2005, Kensy et al., 2005). Moderate k_La values of maximum 175 1/h and 250 1/h for standard round 96-well MTPs and 24-well MTPs were found, respectively (Hermann et al., 2003, Kensy et al., 2005b) and these investigations disclosed the limited application range of these plate types for microbial fermentations.

Consequently, new well geometries have been studied by Funke et al. (2009). After testing 30 different well geometries, the authors came up with a flower-shaped well geometry which provides the best performance with respect to oxygen transfer, applicable filling volume, coverage of sensors at the bottom and reduced spillage of liquid. This so-called Flowerplate® yields k_La values above 600 1/h at filling volumes of 200-600 µL and 1000 1/min (at a shaking diameter of 3 mm) under conventional aeration with air. These k_La values indicate that sufficient oxygen is available to most microbial fermentations. Thus, the Flowerplate can mimic operation conditions in a stirred tank reactors very well (Garchia-Ochoa et al., 2009).

In general, process information can be gained in microtiter plates by applying the aforementioned optodes. These optodes are currently available for *pH*, dissolved oxygen tension (*DOT*), dissolved carbon dioxide tension (DCT) and temperature (*T*). Commercially, microtiter plates with integrated *pH*- or *DOT*-optodes are available for standard 6-well, 24-well and 96-well MTPs from Precision Sensing GmbH (Regensburg, Germany). The SensorDishReader (SDR) from the same company allows only the detection of either *pH* or *DOT* in separate MTPs. This system can be installed on orbital shakers directly in incubators and provide real online kinetics of *pH* or *DOT* from each well of a 6-well or 24-well MTP (Kensy et al., 2005b).

Another possibility to better understand bioprocesses in microwells is applying common microplate readers or the BioScreen C reader (BioScreen, 2009). Normally,

optical densities can be detected via transmission measurements, but this approach requires very thin culture densities. Furthermore, the gas-permeable membrane covering the MTP, which is necessary for monoseptic operation, interferes with the measurements. The linear range of *OD* measurements lies in the range between 0.1 and 0.3 and, therefore, *OD* measurements, in general, are limited to diluted samples and are not very useful in bioprocess development, where higher biomass concentrations (> 5 g/L cell dry weight (*CDW*) ~ *OD*= 10-20) are normally desired.

Fluorescence measurements in MTPs on microplate readers are very common and widely applied in biotechnology. These measurements are normally applied in back scattering of the emitted fluorescence of a sample and, hence, do not strongly depend on path lengths and covering membranes as transmission measurements do. One important drawback of this method and as well as of the *OD* measurement is the interrupted shaking during the measurement and, therefore, interruption of mass transfer into the culture broth and probable sedimentation of cells during the measurement period.

One technique which overcomes these problems with normal microplate readers is the BioLector technology. First introduced by Samorski et al. (2005) and later improved by Kensy et al. (2009), this technology applies back scattering light measurement under a MTP to detect scattered light intensities to measure biomass concentration and subsequently to detect fluorescence either of fluorescent proteins, fusions thereof or of fluorescent biogenic metabolites such as NADH or riboflavins. This technique is a real online monitoring method, because here the non-invasive optical measurement is performed during continuous shaking. In contrary to the detection mode in the SDR and the µ24 systems which consist of a 24 and a 48 LEDs and photodiodes array, respectively, the BioLector uses one moveable glass–fiber bundle as light detector with a 6-filter changer which sequentially scans each well of a MTP. This technology enables the use of different types (24- to 96-wells) of black MTPs with a transparent bottom. The scalability of this technology to stirred tank bioreactors and the capacity for automation has already been demonstrated (Kensy et al., 2009b, Huber et al., 2009).

Based on the BioLector technology, it was challenging to further increase the online detected information content to also include *pH* and *DOT* measurements together. Therefore, the integration of the optode technology into the BioLector measurement concept was elaborated in this study. Because space at the bottom of a 96-well MTP was limited, the *pH*- and *DOT*-optodes were immobilized on the bottom of a 48-well Flowerplate. Moreover, a broader operation range of the Flowerplate with respect to oxygen transfer rates and k_La values was characterized with the sulfite oxidation method introduced by Hermann et al. (2003). The new multi-parameter measurement capability of the BioLector was validated and applied with *E.coli* fermentations at different oxygen transfer rates. In addition, the online fermentation parameters were interpreted to explain cell growth of *E. coli* cells. Moreover, in this study, the effect of different *pH* values ranging from 5.8 to 7.4 was investigated in yeast *Hansenula polymorpha* fermentations. Finally, the effect of supplementing different plant peptones to synthetic medium in yeast fermentations was studied to improve protein production and stability.

6.2 Materials and Methods

6.2.1 Microorganisms and Media

Standard microbial expressions systems - the bacteria *Escherichia coli* and the yeast *Hansenula polymorph*a - were used for the evaluation of the full-parameter monitoring experiments. The strain *E. coli BL21(DE3) pGLOW-T*^{XN} which expresses a flavin mononucleotide (FMN)-based fluorescent protein (*FbFP)*, commercialized under the name evoglow®, was kindly delivered by Michael Puls from evocatal GmbH (Düsseldorf, Germany, Drepper et al., 2007). The *E. coli FbFP* expression was controlled by the strong, inducible *T7* promoter (inducer: isopropyl-β-D-thiogalactopyranosid - IPTG). For the *pH* variation studies, a *Hansenula polymorpha* wildtype (wt) strain was used (ATCC 34438, synonym: *Pichia angusta*). In the protein production and stability testing, the *Hansenula polymorpha RB11/pFPMT MF*α*+ SAK-A* strain, which expresses the protein Staphylokinase under the control of the formate dehydrogenase (*FMD*) promoter, was applied. Staphylokinase activates plasminogen to form plasmin, which digest fibrin clots in blood (ThromboGenics NV, The Netherlands). The *FMD* promoter is repressed under the presence of glucose

and derepressed under the presence of glycerol in the culture medium (Amuel et al., 2000). The strain was kindly provided by Frank Müller from the Rhein-Minapharm Biogenetics (Cairo, Egypt).

The *E. coli* experiments were carried out with a modified synthetic medium (WR-D-MOPS) based on Wilms et al. (2001) and Terrific Broth medium (TB). The WR-D-MOPS medium had the following composition: WR-D-MOPS medium: 20 g/L glucose, 2.0 g/L Na_2SO_4, 5.0 g/L $(NH_4)_2SO_4$, 0.5 g/L NH_4Cl, 3.0 g/L K_2HPO_4, 20.93 g/L 3-(N-morpholino)-propanesulfonic acid (MOPS), 0.5 g/L $MgSO_4$ x 7 H_2O, 0.01 g/L thiamine, 3 ml/L traceelement solution (TES), *pH*-value was adjusted to 7.4 with 1M NaOH or 1M H_2SO_4. TES contains: 0.5 g/L $CaCl_2$, 0.18 g/L $ZnSO_4$ x 7 H_2O, 0.1 g/L $MnSO_4$ x H_2O, 10.05 g/L Na_2-EDTA, 8.35 g/L $FeCl_3$, 0.16 g/L $CuSO_4$, x 5 H_2O, and 0.18 g/L $CoCl_2$ x 6 H_2O. The TB medium was prepared as follows: 5 g/L glycerol (Merck, Darmstadt, Germany), 12 g/L tryptone, 24 g/L yeast extract, 12.54 g/L K_2HPO_4, 2.31 g/L KH_2PO_4, *pH*~7.2 without adjustment.

The *Hansenula polymorpha* experiments were carried out with the modified synthetic medium SYN6-red, which had the following composition: 10 g/L glycerol, 7.66 g/L $(NH_4)_2SO_4$, 3.3 g/L KCl, 3.0 g/L $MgSO_4$ x 7 H_2O, 0.33 g/L NaCl, 14.71 g/L tri-sodium citrate x 2 H_2O, 4.73 g/L NaH_2PO_4, 2.24 g/L $(Na)_2HPO_4$. After dissolution of all medium components, the *pH*-value was adjusted to 6.5 with 1M NaOH or 1M H_2SO_4. Then, the medium was autoclaved. After autoclaving, 6.67 mL/L microelement stock solution, 6.67 mL/L vitamin stock solution, 3.33 mL/L traceelement stock solution (that have all been filter sterilized) and 0.67 mL/L calcium stock solution (that has been autoclaved) were added to the medium. The stock solutions had the following compositions: microelement stock solution: 10 g/L $(NH_4)_2Fe(SO_4)_2$ x 6 H_2O, 0.8 g/L $CuSO_4$ x 5 H_2O, 3.0 g/L $ZnSO_4$ x 7 H_2O, 4.0 g/L $MnSO_4$ x H_2O, 10 g/L EDTA (Titriplex III, Merck, Darmstadt, Germany); vitamine stock solution: 60 mg/L D-Biotin, 20 g/L thiamine chloride hydrochloride; traceelements stock solution: 100 mg/L $NiSO_4$ x 6 H_2O, 100 mg/L $CoC1_2$ x 6 H_2O, 100 mg/L H_3BO_3, 100 mg/L KJ, 100 mg/L Na_2MoO_4 x 2 H_2O; calcium stock solution: 150 g/L $CaCl_2$ x 2 H_2O.

For the *pH*-variation testing, sodium phosphate was separated from the main salt mixture of the Syn6-red medium. Individual salt mixture were prepared at the required *pH*-values of 5.8, 6.5, 7.0, and 7.4, respectively. The concentrated sodium phosphate buffers (0.5 M) were analogously prepared at the same *pH* values. After autoclaving of the individual salt mixtures of the Syn 6-red medium at different *pH* values, the sodium phosphate buffer at each respective *pH* values was added to the medium to a final concentration of 0.1 M. The other supplements of the Syn 6-red medium were also added as described before.

The supplementation of peptones to the Syn 6-red medium was realized by addition of 10 g/L of each individual plant peptone to the main salt mixture from a 10x stock solution after autoclaving of the salt mixture. Peptones were taken from a testkit with plant peptones (art.-no.: 11577, Fluka, Buchs, Switzerland). This test kit contained 20 peptones, seven peptones of which were selected in a primary screening. In the secondary screening which is presented here, the following seven peptones were applied (the same numbers as they appeared in the test kit were used, (Fluka, Buchs, Switzerland)): 1. peptone (vegetable), art.-no.: 18332; 5. peptone (vegetable, no.: 2), art.-no.: 92976; 9. vegetable extract, art.-no.: 05138; 12. vegetable hydrolysate no.: 2, art.-no.: 07436; 16. peptone from broadbean, art.-no.: 93491; 17. peptone from wheat, art.-no.: 93492; 20. peptone from soybean, acid digest, art.-no.: 07022.

The precultures for the fementations of both the bacterial and yeast strains were prepared by respectively using one cryo vial of 1 mL which was inoculated into 20 mL of the respective fermentation medium (WR-D-MOPS and TB for *E. coli* or Syn6-red for *H. polymorpha*). Each inoculated culture volume (21 mL) was then filled into a 250 mL Erlenmeyer shake flask and was incubated at a shaking frequency of 300 1/min, a shaking diameter of 50 mm, and a temperature of 37°C on an orbital shaker (LS-X, Kühner AG, Birsfelden, Switzerland). The precultivation for both microorganisms *E. coli* and *H. polymorpha* was conducted over night for approximately 16 h. For the BioLector fermentations, the media were filled into the Flowerplate and were inoculated from the precultures with a starting *OD* of 1.0.

All chemicals were of analytical grade and were delivered by Fluka/Sigma-Aldrich Chemie GmbH (Buchs, Switzerland) unless specified otherwise.

6.2.2 Fermentations in the BioLector

The microtiter plate fermentations were conducted in the BioLector which was originally introduced by Samorski et al. (2005) and recently improved and validated by Kensy et al. (2009). In contrast to the former prototypes (Samorski et al., 2005, Kensy et al., 2009) of the BioLector, in this work a commercialized version of the BioLector (art.-no.: G-BL-100, m2p-labs GmbH, Aachen, Germany) was applied. This new version now combined intensity measurements for scattered light and fluorescence with the frequency-modulated fluorescence measurements for optodes in one device. The frequency-modulated fluorescence measurement of optodes was formerly described in Schroder et al. (2005) for *pH*-optodes and in Gupta et al. (2003) for *DOT*-optodes. The measurement principle and the collocation of the sensors are given in Figure 6.1. As illustrated in Figure 6.1A, the light detector scans different positions of each well of a microtiter plate, thereby allowing the detection of scattered light and fluorescence in the cell suspension in position 1), and the detection of *DOT* at position 2) as well as that of *pH* at position 3). All wells of the MTP are first scanned for one parameter before the filters are changed and then the mechanical scanning for the next parameter begins. The complete reading of a 48-well MTP for one parameter lasts 1 min.

The biomass concentrations were measured via scattered light at 620 nm excitation without an emission filter. The riboflavin fluorescence was monitored using an excitation filter of 485 nm and an emission filter of 510 nm. However, the *pH* and *DOT* were measured in the frequency-modulation mode at 45 kHz and 10 kHz, respectively. The *pH* signal was measured at an excitation wavelength of 485 nm and an emission wavelength of >530 nm, whereas the *DOT* signal was detected at an excitation wavelength of 505 nm and an emission wavelength of >590 nm. The sensitivity of the photomultiplier (gain) was adapted to the different measurement tasks, and the detailed data was mentioned in the respective figures. The BioLector possessed a data reproducibility of below 5% standard deviation, upon cultivating the

same clone in the same medium on a microtiter plate. Due to a small standard deviation and the high information content, error bars in the figures were omitted. The experiments were exclusively carried out with 48-well Flowerplates - each well containing *pH*- and *DOT*-optodes (Figure 6.1C, art.-no.: MTP-48-BOH, m2p-labs GmbH, Aachen, Germany) - that were covered with a gas permeable membrane (art.-no.: AB-0718, Abgene Ltd., Epsom, UK). The experiments were conducted at different shaking frequencies and filling volumes, that are mentioned in the respective paragraphs or below each figure. The shaking diameter of the BioLector was 3 mm.

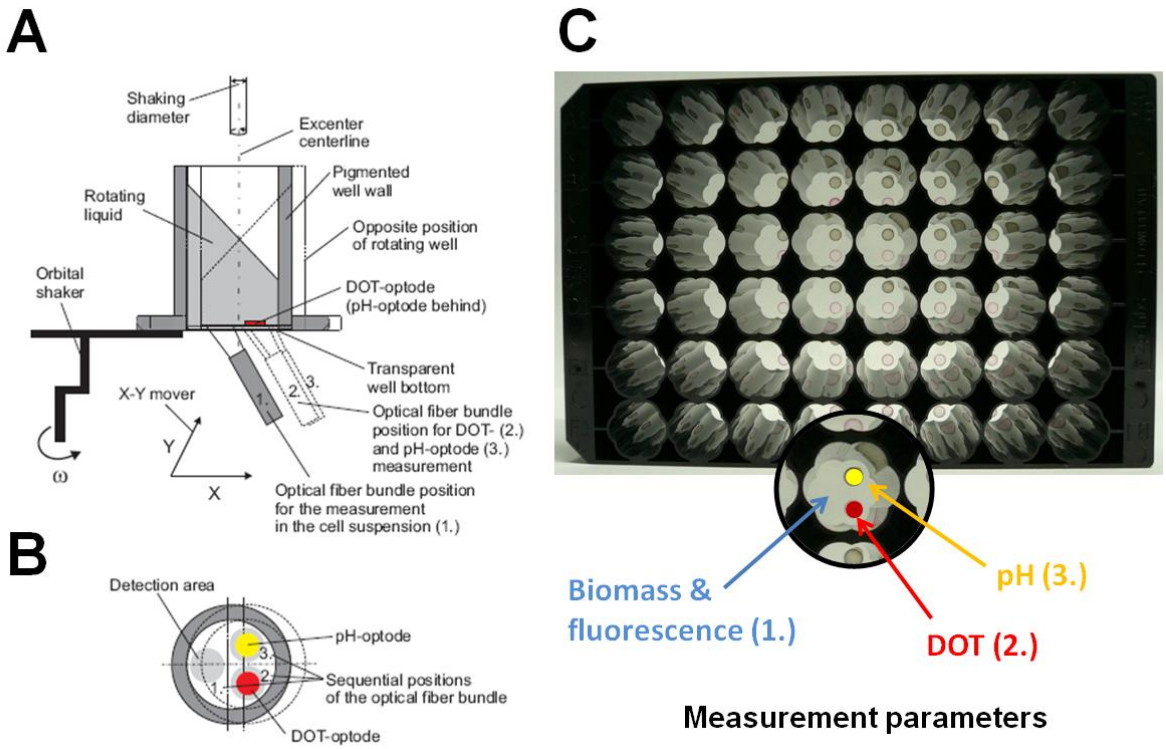


Figure 6.1: Measurement principle of the multi-parameter detection.

(A) Schematic side view of the sequential measurement of different positions under a microtiter plate well during continuous shaking. (B) Top view on a microtiter plate well with the different reading positions and sensor allocations. (C) Implementation of the multi-parameter measurement principle in the Flowerplate.

6.2.3 Determination of oxygen transfer rates (*OTR*) in surface-aerated bioreactors

The sulfite oxidation is a good and easy model system for characterizing small-scale, surface-aerated bioreactors. Hermann et al. introduced this technique together with a simple color shift of a colorimetric *pH* indicator making it possible to read out the

reaction kinetics with a simple camera (Hermann et al., 2003, Kensy et al., 2005). In the presented work, this sulfite oxidation method from Hermann was applied to amplify the characterization range of the oxygen mass transfer conditions in 48-well Flowerplates which has been initiated by Funke et al. (2009). Here, the OTR_{max} and k_La values were determined with various filling volumes ranging from 500 µL up to 1700 µL at shaking frequencies of 1000 1/min and 1400 1/min and a shaking diameter of 3 mm in the BioLector. In the current work, the method from Hermann et al., (2003) was slightly modified by substituting the *pH* indicator from bromothymol blue to 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (HPTS, art.-no.: 56360, Fluka, Buchs, Switzerland) at a concentration of 20 mg/L. The fluorescence of this fluorescent *pH* indicator was detected at an excitation of 485 nm and an emission of 510 nm and a gain of 30. The 48-well Flowerplate without optodes (art.-no.: MTP-48-B, m2p-labs GmbH, Aachen, Germany) was operated with a gas permeable cover membrane (art-no.: AB-0718, Abgene Ltd., Epsom, UK) at a temperature of 25°C. Due to confirmation measurements with a 48-well Flowerplate with DOT optodes (art.-no.: MTP-48-BO, m2p-labs GmbH, Aachen, Germany) and measured DOT values of zero for all operation conditions during the sulfite oxidation, C_L values were not corrected by mass balancing as Hermann et al. (2003) have suggested.

6.2.4 Western Blot analysis

After sampling, all fermentation samples were centrifuged immediately in 2 mL Eppi-tubes for 10 min at room temperature and were directly frozen at -80°C before continuing the Western Blot analysis. The samples were analyzed referring to the manual of the Western Breeze Immuno Detection Kit (art.-no.: WB7103, Invitrogen, USA) using Nupage Gels (12% Bis-Tris Gel, 1 mm, 10 wells, art.-no.: NP0341BOX) and the blotting membrane: nitrocellulose membrane, 0.2 µm (art.-no.: LC2000, Invitrogen, USA). The following marker, standard and antibodies were applied: molecular marker for the protein detection: SeeBlue Plus 2 (art.-no.: LC5925, Invitrogen, USA); protein standard: *E. coli* derived *SAK*; 1 mg/ml (prod.-no.: THR-174; Thrombogenics, NL); antibodies: primary: *aSAK* mouse; 2,73 mg/ml (prod.-no.: SAK20D11, Thrombogenics, NL) and secondary: aMouse *IgG* (art.-no.: A3562-5ML, SIGMA-Aldrich, USA).

6.3 Results and Discussion

6.3.1 Oxygen Transfer Rates in the Flowerplate

In order to obtain scalable results from microscale experiments inevitably one has to characterize mass transfer conditions in the applied microbioreactors. The OTR_{max} of the new Flowerplate, recently introduced by Funke et al. (2009), has already been characterized by the authors for filling volumes from 0.2 mL to 0.6 mL at different shaking frequencies (500-1000 1/min). Due to this small application range, the characterization of OTR_{max} and k_La of the Flowerplate was enlarged in this work for a volume range of 0.5 mL to 1.7 mL and shaking frequencies of 1000 1/min and 1400 1/min. For the OTR_{max} characterization, the sulfite oxidation method from Hermann et al. (2003) was applied. Figure 6.2 presents the results of this characterization study.

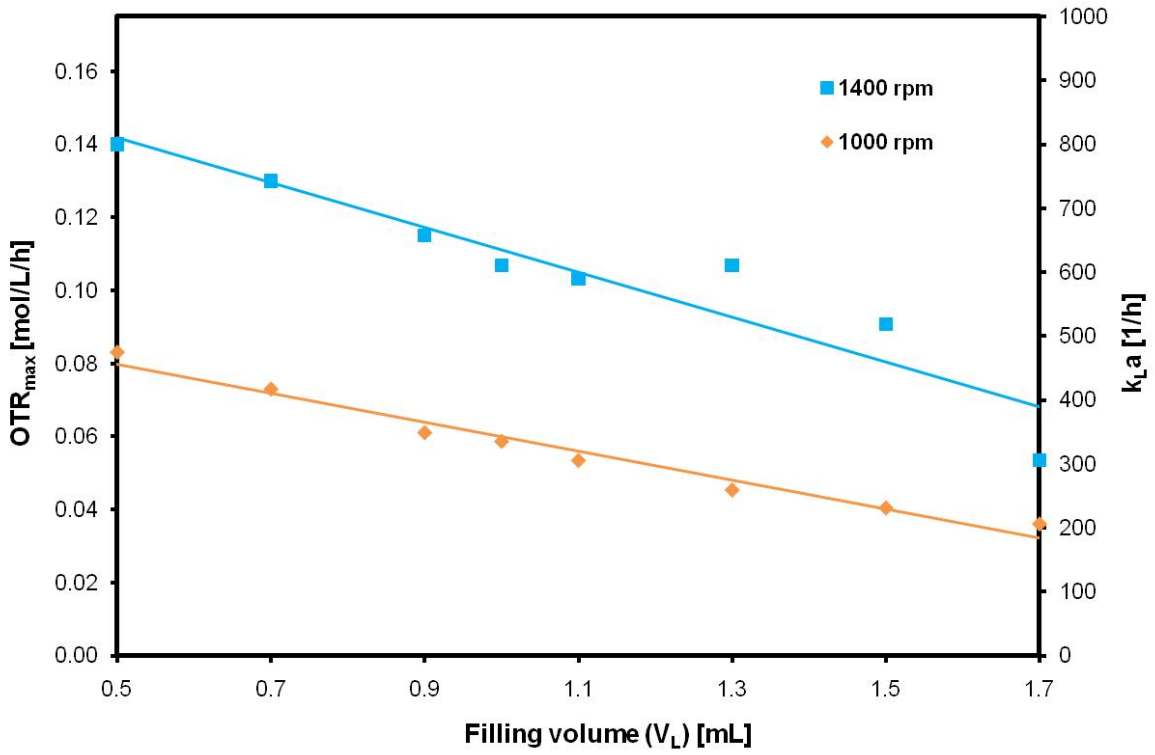


Figure 6.2: Oxygen transfer data from the Flowerplate.

Characterization of the oxygen transfer capacity (OTR_{max}) and k_La value over different filling volumes from 0.5 to 1.7 mL at 1000 1/min and 1400 1/min. Determined by the sulfite oxygenation method by Hermann et al. (2003); the correction of C_L was not applied here due to measured C_L values of zero; Experimental conditions: 0.5 M Sulfite, 25 °C, 3 mm shaking diameter, 48-well Flowerplate covered with a gaspermeable membrane from Abgene (AB-0715).

For both shaking frequencies, 1000 1/min and 1400 1/min, there was a clear linear correlation between OTR_{max} (and k_La) and the filling volume. At 1000 1/min, OTR_{max} values ranging from 0.083 mol/L/h down to 0.040 mol/L/h (k_La ranging from 470 to 200 1/h) were found for the applied filling volumes. At 1400 1/min, the lowest OTR_{max} value at approximately 0.060 mol/L/h corresponded to a median OTR_{max} value at 1000 1/min. The highest OTR_{max} values achieved at 1400 1/min lay at 0.140 mol/L/h for 500 µL filling volume, which corresponded to k_La values of 800 1/h.

The results of the oxygen transfer characterization demonstrated that with only two different shaking frequencies and a simple variation of filling volumes, the full operation range of common stirred tank fermenters with respect to k_La values (200 – 800 1/h) could be covered (Garchia-Ochoa et al., 2009). It should be noted here that for the first time k_La values of above 600 1/h are reported for microwell fermentations. This broad mass transfer range offers new applications for scale-down models of industrial fermentation processes.

6.3.2 Comparison of *E. coli* fermentations at 1000 1/min and 1400 1/min in synthetic medium

Beside the basic characterization of mass transfer conditions in microbioreactors, online fermentation monitoring can bring new insights into fermentation processes. The BioLector technology was tested with *E. coli* batch fermentations in synthetic medium (WR-C-MOPS). The fermentations were performed in a Flowerplate with three different filling volumes (500 µL, 1000 µL and 1500 µL) and conducted at the two characterized shaking frequencies (1000 1/min and 1400 1/min). Figure 6.3 depicts the results of this experiment: the scattered light signals for biomass concentrations, the dissolved oxygen tensions (*DOT*) and the *pH* values for both shaking frequencies, each as function of time.

According to Figure 6.3A, the growth of E. coli is first exponential followed by a temporary stationary phase before it again increases until saturation. With increasing filling volume and therefore reduced *OTR*, the growth is limited earlier. Regarding Figure 6.3B, the *DOT* curves decrease exponentially with increasing cell

concentration. Due to the lower *OTR* in the wells with the higher filling volume of 1500 µL, these *DOT* values decrease faster. For all filling volumes, all the *DOT* values reach the baseline of 0% between 3 and 4 hours and rise again at about 5 hours when the primary carbon source is depleted. For all volumes, the *pH* values as a function of time behave similarly at least in the detection range of 5.5–8.5 of the *pH*-optodes.

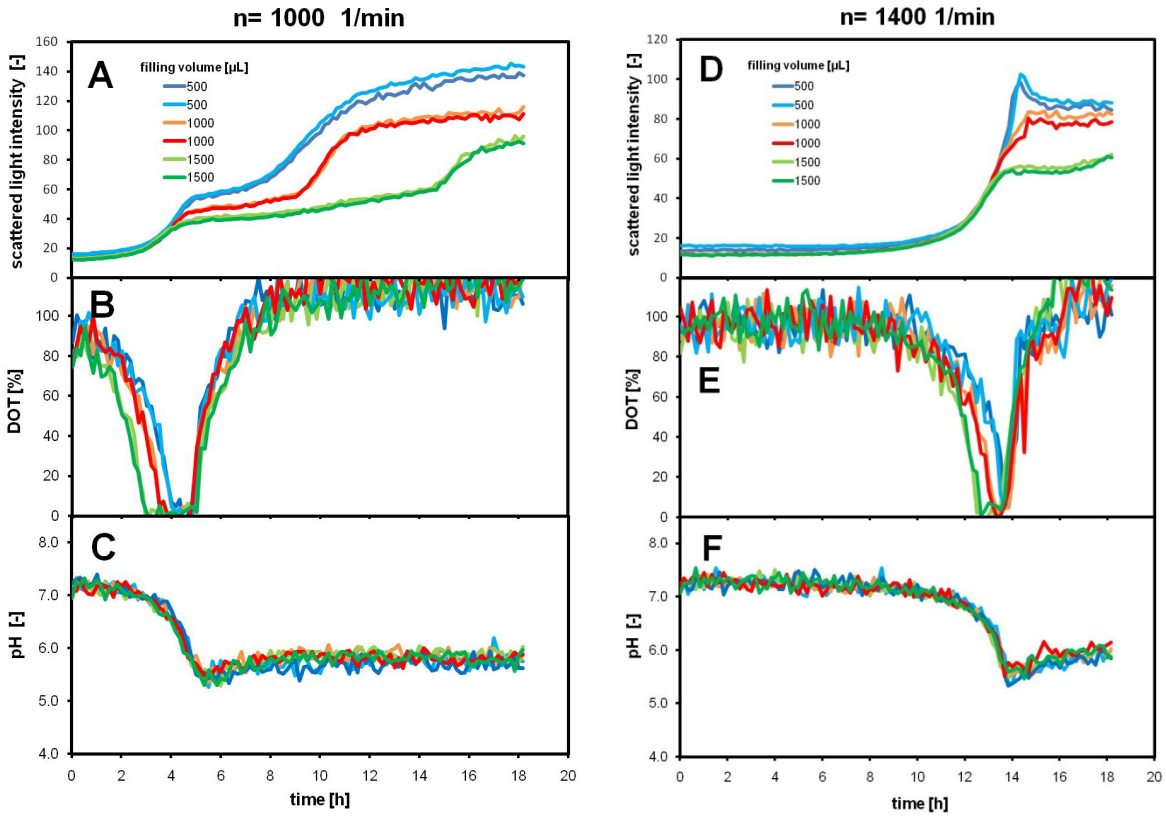


Figure 6.3: *E. coli* fermentation in synthetic medium at 1000 and 1400 1/min at three filling volumes.

(A-C) Online-Monitoring of biomass (scattered light), *DOT* and *pH* of *E. coli* fermentations in WR-D-MOPS medium with 500, 1000 and 1500 µL at 1000 1/min; (D-F) Online-Monitoring of biomass (scattered light), *DOT* and *pH* of *E. coli* fermentations in WR-D-MOPS medium with 500, 1000 and 1500 µL at 1400 1/min. Experimental conditions: 48-well Flowerplate (MTP-48-BOH) covered with a gaspermeable membrane from Abgene (AB-0715), 37°C, strain *E. coli BL21(DE3) pGLOW-T^{XN}*, 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, Gain: 20), *pH* (Gain: 50) and *DOT* (Gain: 50).

Here, there seems to be no difference between the various filling volumes, but the *pH* values of the wells detected offline decreased even down to a value of 4.5, which inhibits *E. coli* growth (Table 6.1).

Table 6.1: Offline *OD* and *pH* analysis at the end of the fermentation (with WR-D-MOPS)

	1000 1/min		1400 1/min	
Filling volume [µL]	OD_{600nm} [-]	pH_{end} [-]	OD_{600nm} [-]	pH_{end} [-]
500	33.66	4.62	40.00	5.36
1000	26.00	4.52	37.00	4.67
1500	21.50	4.57	30.83	4.38

Since all the cultures at the different filling volumes are oxygen-limited in this experiment at 1000 1/min, all the cultures eventually reach this limiting *pH* value. Indicating biomass, the curves of scattered light versus time (Figure 3A) depict that the different oxygen transfer rates, set by the different filling volumes, have a prominent impact on the growth of *E. coli*. Here, the exponential growth is interrupted by the oxygen limitation as indicated in Figure 3B. The higher the oxygen limitation is, the earlier the growth is interrupted, and the longer the transition phase up to the second growth phase lasts. Even the final biomass concentrations of the cultures with the different filling volumes are different. The wells with a filling volume of only 500 µL reach the highest biomass concentrations as is shown by the online scattered light signals and is confirmed by the offline *OD* values (see Table 6.1).

However, the same experiment conducted at 1400 1/min revealed a different growth behavior of the *E. coli* cells (Figure 6.3D). First of all, the *E. coli* cultures showed a much longer lag-phase (until 8-10 hours) than in the aforementioned experiment at 1000 1/min. The longer lag-phase can be traced back to a probable less active preculture and this should not have any effect on the general outcome of this experiment. At the elevated oxygen transfer conditions for all three applied filling volumes (conducted in duplicate), a slight oxygen limitation was observed in the *DOT* signals only for the higher volumes of 1000 µL and 1500 µL. The cultivations at a filling volume of 500 µL did not suffer an oxygen limitation and therefore, the exponential growth in these wells continued until the depletion of the carbon source. All other wells entered the transition phase where the cell probably switched to another metabolic pathway. The *pH* slopes appeared very similar to the experiments at 1000 1/min. Again here, the *pH* values dropped with the ongoing cell growth. For

all filling volumes they followed the same slope until the detection limit of the *pH* optodes at 5.5 was reached. The offline analysis at the end of the experiment depicted that the *pH* values of the wells with the 1000 µL and 1500 µL filling volumes were much lower. Due to the slight oxygen limitation, again here the *pH* decreased to values around 4.5 (Table 6.1). It is notable that the biomass concentrations at the end of the experiment are arranged in the same order as the cells experience higher oxygen transfer rates, with cultivations at 500 µL yielding to the highest biomass concentration of *OD* 40 (Table 6.1). Consequently, the oxygenation of the fermentation broth for *E coli* cells is greatly important to growth. Especially in small scale cultures where the *pH* is not actively controlled, poor oxygen transfer conditions can rapidly limit growth of cell cultures. Even in actively controlled fermentation systems, poor oxygen transfer conditions can be misleading due to oxygen limited growth of the cells and the increased consumption of base and, thus, increased ionic strengths of the fermentation broth.

6.3.3 Comparison of *E.coli* fermentations at 1000 1/min and 1400 1/min in complex TB medium

Another fermentation example was conducted with *E. coli* cells in the very widely used TB medium: the TB medium is one that promotes intense microbial growth thus leading to high oxygen consumption of the microbes (OUR ~ 0.080 mol/L/h (Losen et al., 2004)). As a result of its complex composition, it is auto-buffered by the peptones and leads to diauxic growth. The same approach, as previously used for the synthetic medium WR-D-MOPS, was applied in this *E. coli* fermentation on TB medium: duplicate cultivations of three different filling volumes (500, 1000 and 1500 µL) at shaking frequencies of 1000 1/min and 1400 1/min were tested in the Flowerplate.

Figure 6.4 presents the diagrams of all online measurement signals for 1000 1/min and 1400 1/min. In Figure 6.4A, the scattered light signals of all three filling volumes follow an exponential growth phase in the beginning. The scattered light curves split corresponding to the decrease in *DOT* signal amplitudes and the resulting entry in the oxygen limitation at around 2 hours fermentation time. The *E. coli* cultures in the wells with higher filling volumes experienced a higher oxygen limitation

(demonstrated by the longer baseline of the *DOT* signals), and therefore, the microbial growth rates are reduced (reduced slope of the scattered light signals). The fastest growing cultures cultivated in 500 µL reach the stationary phase at 4 hours, whereas the slowest cultures reach the stationary phase at around 6 hours. The entry into the stationary phase is also indicated by the spontaneous rise of the *DOT* signals. The *pH* curves of these cultures progress with the same slope until the cultures become oxygen-limited. Then, the cultures grown in the higher filling volumes experience a slightly higher acidification. Between 2.5 and 3.0 hours, the *pH* signals increase sequentially. The same behavior has been formerly observed by Kensy et al. (2009).

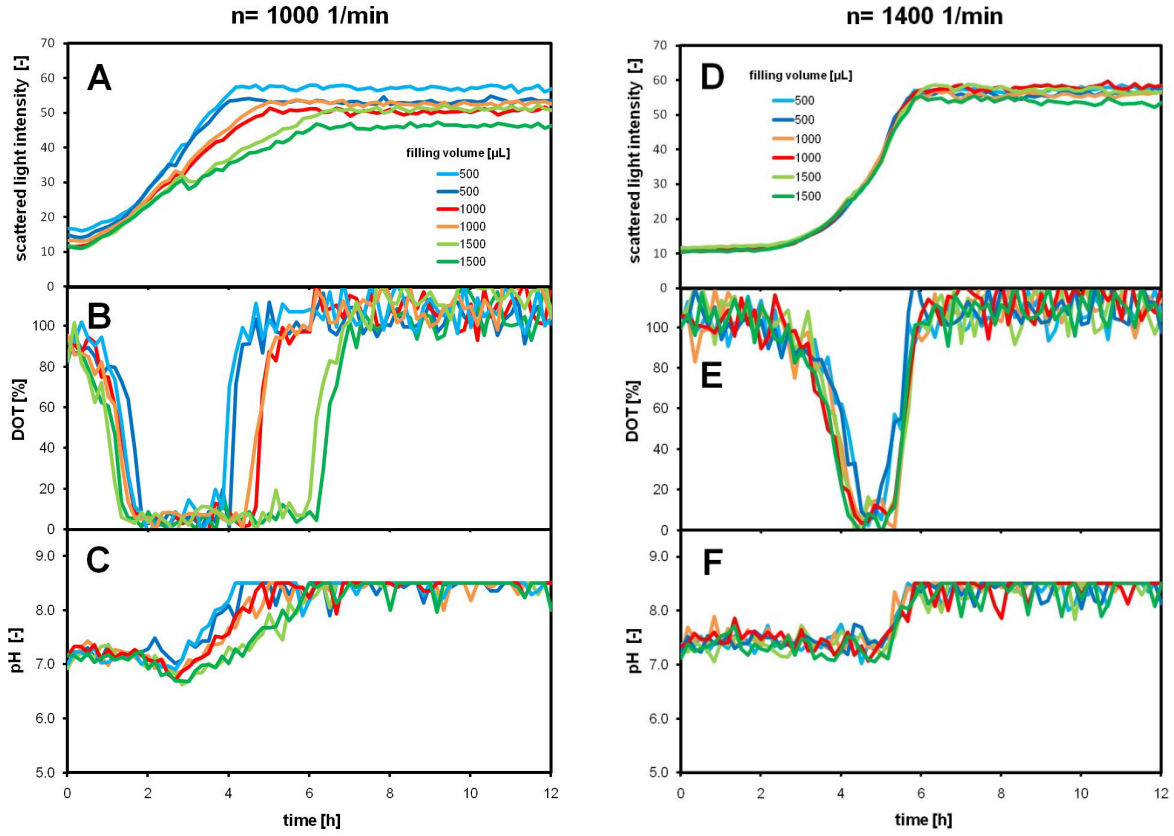


Figure 6.4: *E. coli* fermentation in TB medium at 1000 and 1400 1/min at three filling volumes.

(A-C) Online-Monitoring of biomass (scattered light), *DOT* and *pH* of *E. coli* fermentations in complex TB medium with 500, 1000 and 1500 µL at 1000 1/min; (D-F) Online-Monitoring of biomass (scattered light), *DOT* and *pH* of *E. coli* fermentations in complex TB medium with 500, 1000 and 1500 µL at 1400 1/min. Experimental conditions: 48-well Flowerplate (MTP-48-BOH) covered with a gaspermeable membrane from Abgene (AB-0715), 37 °C, strain *E. coli BL21(DE3) pGLOW-T^{XN}*, 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, Gain: 20), *pH* (Gain: 50) and *DOT* (Gain: 50).

Since the primary carbon source glycerol had been consumed faster at higher oxygen transfer rates and the cultures then switched to the secondary carbon source, the peptones. The second growth phase is characterized by the increase in the *pH* which is a typical phenomenon for TB medium. In this second growth phase, the peptones are consumed and a deamination of peptides takes place. This growth shift is also recognizable by the small decline in the scattered light signals in the middle of the curves (Figure 6.4A). The *pH* measurement is very sensitive near the detection limit of the *pH*-optodes of *pH*= 8.5. Thereby, all *pH* signals over the detection limit are set mathematically to a maximum of 8.5 to avoid excessive signal noise.

Table 6.2: Offline *OD* and *pH* analysis at the end of the fermentation (with TB medium)

	1000 1/min		1400 1/min	
Filling volume [µL]	OD_{600nm} [-]	pH_{end} [-]	OD_{600nm} [-]	pH_{end} [-]
500	32.33	8.09	25.50	8.20
1000	28.33	8.20	30.50	8.20
1500	18.67	8.25	32.33	8.44

The results of the fermentations at 1400 1/min are presented in Figure 6.4D-F. These fermentations were performed to demonstrate the importance of the oxygen transfer conditions. At these oxygen transfer conditions which are elevated at this higher shaking frequency, there is obviously no visible difference in growth among the cultivations at all filling volumes. The *DOT* curves show only slight deviations here. All *DOT* curves slightly touch the 0% baseline, but do not affect the growth significantly. That means that the fermentations at 1400 1/min were operated at almost unlimited oxygen transfer rates. The *pH* curves of all cultures behave similarly and follow the same slope. Unlike to the observations at 1000 1/min, here no prominent decrease in the *pH* values is observable. The *pH* curves rise sharply near by the middle of the exponential growth phase. In this case, the diauxic shift resulting from the shift from glycerol to peptones as main carbon source is not as prominent as that under oxygen-limited conditions. The differences in the OD values in

Table 6.2 for the experiment at 1000 1/min represents roughly the observations with the online monitored scattered light intensities (Figure 6.4A). In contrast, the OD values of the experiment at 1400 1/min does not represent the findings with the scattered light intensites (Figure 6.4D) which all had the same level. This discrepancy between the OD and scattered light intensities might be traced back to the high dilution factor (1:200) applied in the OD measurement and probable pipetting errors.

These experiments in complex TB medium demonstrated that biology can be strictly controlled by mass transfer conditions. It is very important to design fermentation experiments especially at small scales under adequate oxygen transfer conditions. Only when the oxygen transfer rates are designed to exceed the oxygen uptake rate (OUR) of the applied biological system to be studied, can the researcher expect real kinetic data. The online availability of the most common fermentation parameters evoke new insights into the studied biological systems and can accelerate their exploration.

6.3.4 Influence of different initial *pH* values on cell growth and process parameters

In fermentation development, the variation of *pH* is often of great interest in respect to optimal cell growth and protein production. Therefore the realization of *pH* variation and *pH* monitoring in microscale fermentations could be a great help in fermentation development. In the following experiment, the *pH* variation was realized by adjusting different initial *pH* values from 5.8 to 7.4 and applying biological buffers. The experiment was conducted with the yeast *Hansenula polymorpha wt* in the synthetic medium Syn6-red.

Figure 6.5 presents the corresponding scattered light signals, the riboflavin signals, the *pH* and the *DOT*, each as a function of time. Here, the different pH values significantly affected growth. By means of the online scattered light signals four individual exponential growth curves were achieved. With increasing initial pH values, the yeast cell metabolism was hindered and the growth rates were reduced. The riboflavin curves corresponded very well to the scattered light curves. The biogenic

fluorescence of riboflavin in the yeast cells was an additional indicator of growth in this case, but in other cases it could also be a good indicator of the internal cell pool of riboflavin as a derivative of the metabolic coenzymes FAD and FMN and, therefore, could provide insights into the respiratory chain of the microorganism (Marose et al., 1998).

According to Figure 6.5, the *DOT* curves well represent the growth activities of the yeast and, thus, the different oxygen consumptions at the various *pH* values. The faster the cells grew, the more the *DOT* signals decrease. From the *DOT* diagram, it is obvious that the fermentations did not become oxygen-limited. The minimal *DOT* values lie at 45%. Consequently, the applied shaking conditions are well suited for this kind of experiment. According to the *pH* graphs, the cell activity is initially low during the first 5 hours of fermentation and, therefore, all *pH* values keep their initial values. With continuous cell growth, the *pH* values decrease. The higher the initial *pH* values are, the better the *pH* is buffered. Due to the pK_a value of 7.2 (25 °C) of the applied phosphate buffer, the buffering capacity of this buffer is more effective at *pH* values near the pKa value. This was demonstrated by the different shifts in *pH* during the complete fermentation.

Whereas the fermentation at an initial *pH* value of 7.4 decreased only by 0.4 *pH* units, the maximal shift downwards from the initial *pH* of 6.5 was even 1.1 *pH* units. It is recognizable that the fermentations at lower initial *pH* values (6.5 and 5.8) experienced an additional *pH* increase after the biomass growth entered the apparent stationary phase. It might be possible that the citric acid in the medium was consumed in this phase and, therefore, the *pH* increased. At the lower *pH* values more citric acid is dissociated and, therefore, the transport into the cell is easier.

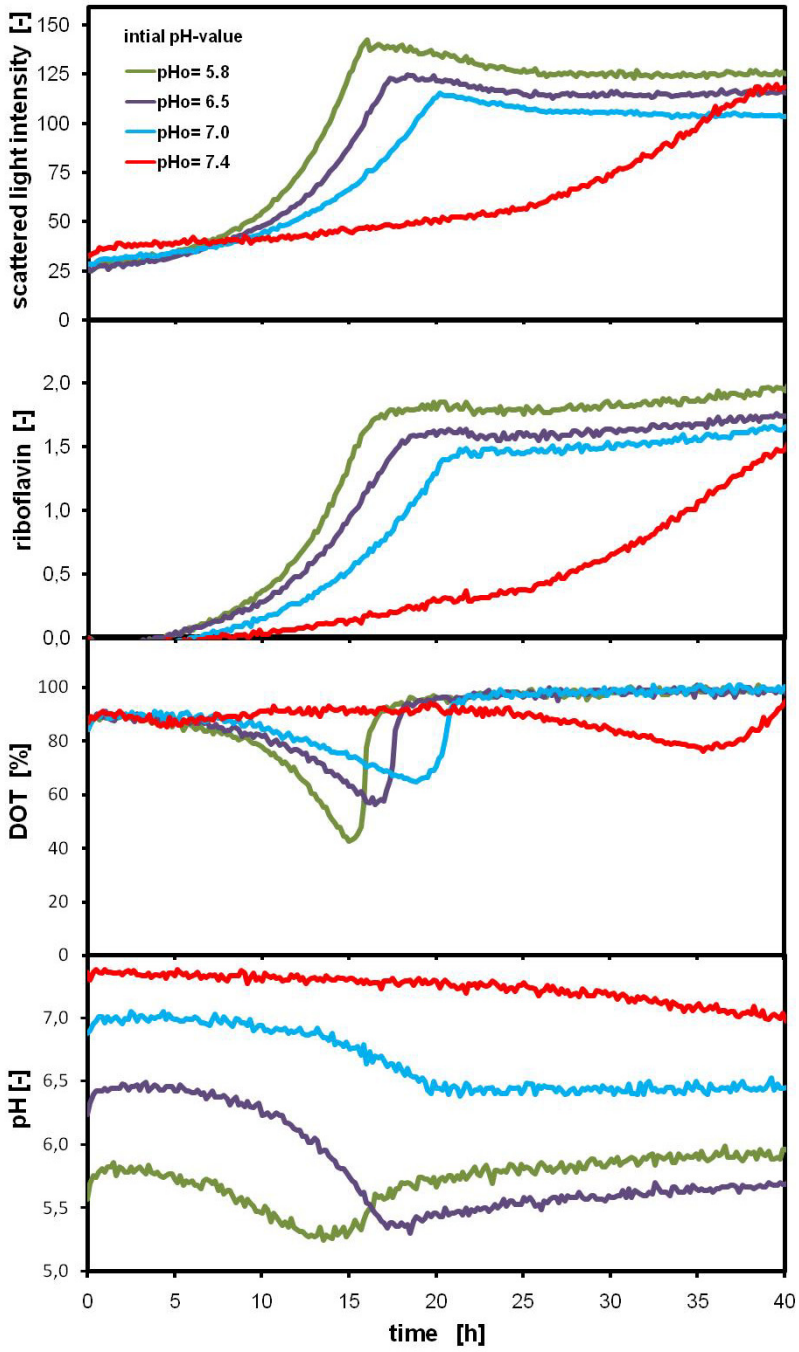


Figure 6.5: Influence of different media *pH* on cell growth and process parameter.

Online monitoring of biomass (scattered light), biogenic riboflavin, *DOT* and *pH* of *Hansenula polymorpha* fermentations in synthetic Syn6-red medium; Experimental conditions: 48-well Flowerplate (MTP-48-BOH) covered with a gaspermeable membrane from Abgene (AB-0715), 37 °C, strain *Hansenula polymorpha wt*, 1000 µL, 800 1/min, 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, Gain: 20), riboflavin (ex: 485 nm/em: 510 nm, Gain: 70), *pH* (Gain: 50) and *DOT* (Gain: 50).

The results of this experiment demonstrated that *pH* variations are possible by applying buffers and different initial *pH* values. The *pH* could be stabilized in a range

between 0.4 and 1.1 *pH* units from the initial *pH* value to the end of fermentation depending on the matching of the applied *pH* values and the pKa of the buffer. The different applied *pH* values showed a significant influence on the growth characteristics of the yeast cells. In addition to the growth monitoring, the *DOT* curves showed different consumptions of oxygen that correspond very well to the detected growth behavior. These findings infer that the BioLector technology together with intelligent design of experiments can facilitate *pH* studies by narrowing the *pH* application range to achieve best growth and protein production as well as protein stability.

6.3.5 Protein stabilization by peptone supplementation to synthetic media

One major aspect in bioprocess development is the protein stability in the bioprocess. Very often proteins are well produced by the expression host. However, at the same time specific proteases of the host degrade the target protein. If the protease is known, molecular biologists can knock out the protease synthesis in the host. That, on the other hand, can lead to an incapability of the host to grow or can massively reduce growth and/or protein production. If the protease function is not well known, another possibility to omit a strong proteolysis is to select specific fermentation parameters so that the protease shows a reduced activity or to supplement the media with special ingredients that protect the target protein or inhibit the protease (Enfors, 1992).

In the following experiment, the supplementation of additional media ingredients, in this case peptones, was studied. In a first trial, 20 different peptones were added to the synthetic medium. From this first trial seven peptones, that did show protein stabilization effects, were selected for a second trial. The results of the second trial are reported here. Figure 6.6 presents the growth curves of the fermentations with different peptone supplements and the sampling procedure as well as the Western Blot analysis of the target protein SAK.

Samples were taken at three different times during the fermentation process to monitor the kinetics of protein expression and stability: **1)** before the stationary

phase, **2)** 2 hours after the entry in the stationary phase, and **3)** 5 hours after the entry in the stationary phase. The fermentations were performed in triplicates and at each respective sampling time, the content of a full well was harvested (sample of 800 µL). Please note that the spontaneous drop of the scattered light signals is the result of sampling; during sampling, 800 µL were taken from the wells and the little remaining filling volumes in the wells result in very low scattered light signals. The spontaneous drop in scattered light signals can be used as an indicator of the sampling in the respective well.

Figure 6.6 shows the different growth kinetics of the yeast clone. It is recognizable that the peptones support growth; e.g. the supplementation of peptone no. 20 results in the fastest growth, whereas peptone no. 17 supports only slightly the growth of the yeast in comparison to the fermentation without peptone supplementation which exhibits the lowest growth. The final achieved biomass concentrations do not seem to be significantly influenced by the peptone supplementation. All curves depict similar scattered light signals at the end of fermentation. The Western Blot analysis on the right side of Figure 6.6 qualitatively illustrates the amount of expressed SAK protein. If protein lanes become more purple, the expression level increases. If the lane color intensity weakens over fermentation time, the protein has probably been proteolyzed.

The Western Blot analysis of the samples taken before the stationary phase gives a clear impression of the expressed protein levels depending on the supplementations. Peptones no. 1 and no. 17 supported the protein production best, whereas peptone no. 9, no.12 and no.16 resulted in a moderate protein production and peptone no. 5 and no. 20 did not improve protein expression. The non-supplemented culture showed a moderate protein production level at this time point. With advancing fermentation time, it became obvious on the Western Blots that the fermentations without and with peptone no. 5 and no. 20 suffered a strong proteolysis.

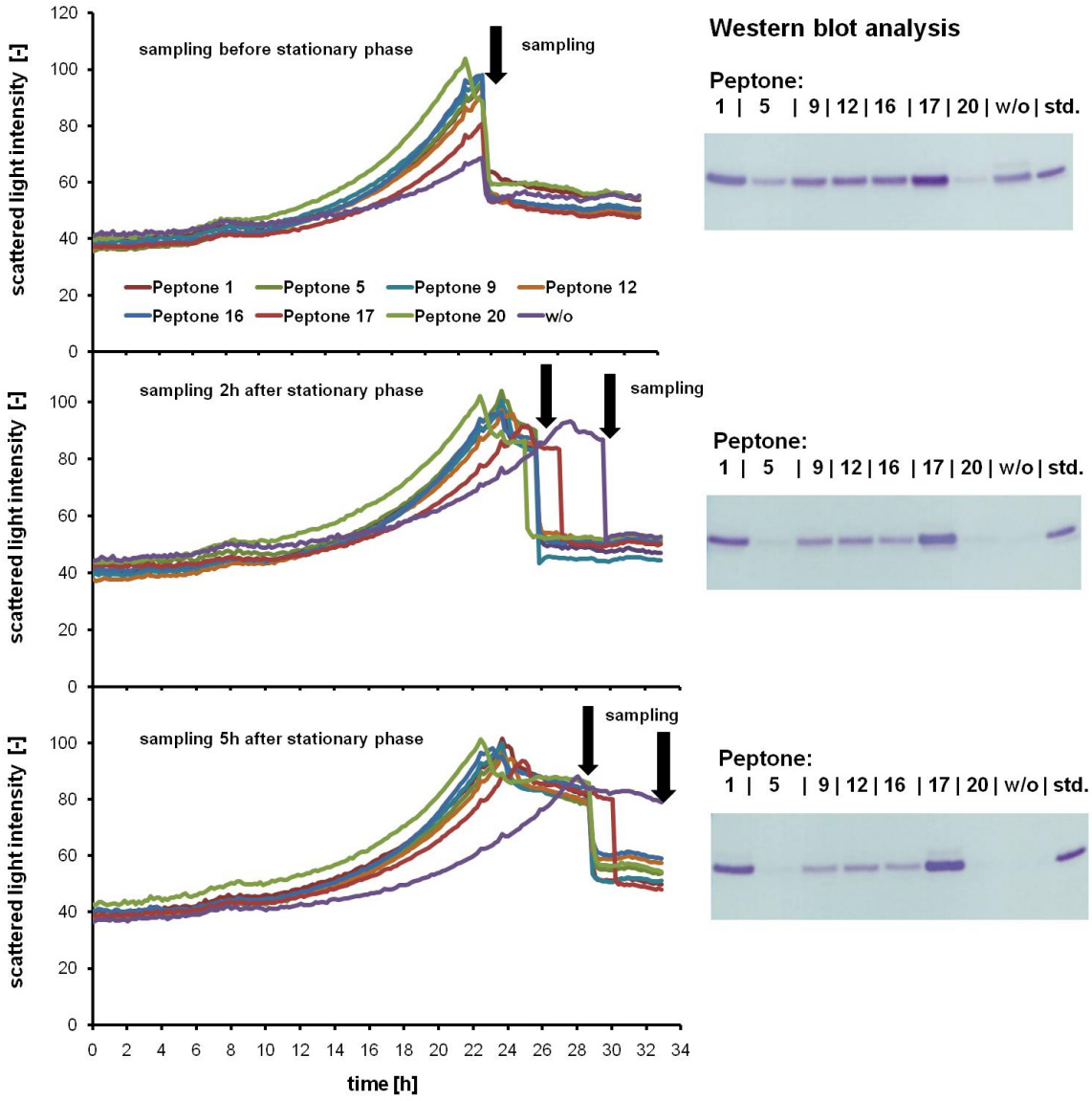


Figure 6.6: Kinetics of protein stability due to different peptone supplements.

Online monitoring of biomass (scattered light) and manual signal-triggered sampling of 8 parallel *Hansenula polymorpha* fermentations with different peptone supplements to synthetic Syn6-red medium; Sampling was performed at the end of the exponential growth phase, 2 hours and 5 hours after the entry in the stationary phase; Experimental conditions: 48-well Flowerplate (MTP-48-BOH) covered with a gaspermeable membrane from Abgene (AB-0715), 30 °C, strain *Hansenula polymorpha RB11/pFPMT MFα+ SAK-A*, 1000 µL, 800 1/min, 3 mm shaking diameter, scattered light (ex: 620 nm/em: -, Gain: 20).

In addition, the peptones no. 9, no. 12 and no. 16 did not stabilize the SAK protein, because rather the color of the protein lanes faded with time. Peptones no. 1 and no. 17 significantly improved the protein production level and secondarily, stabilized the protein resulting in very intensively colored protein lanes in the Western Blot. The

Western Blot analysis showed that peptone no. 17 (peptone from wheat) probably best supports the production of SAK protein under the applied conditions.

The reported study of different supplementations for optimizing of protein production is only one possible example where online fermentation monitoring demonstrates its great potential in contrast to offline experimentation. Due to the online monitoring of growth in this presented research, sampling can be classified to different growth phases and, hence, the process of protein proteolysis and stabilization can be studied in detail. The insight into the fermentation kinetics is a huge advantage of this online monitoring technology in contrary to black box systems such as shake flasks or microtiter plates that are not hooked up to measurement devices. The combination of the BioLector technology with robotic liquid-handling systems (such as the recently reported “RoboLector” (Huber et al., 2009)) and automated high-throughput protein analysis (such as the LabChip GXII from Caliper Life Sciences, Inc., Hopkinton, MA, USA) makes this a very powerful platform in bioprocess development.

6.4 Conclusions

This study finalizes a series of introductions to the BioLector technology. First introduced by Samorsky et al. (2005), this technology has offered the combination of scattered light measurements with fluorescence measurements in shaken microtiter plates. Kensy et al. (2009) has presented an improved version of the BioLector system. The authors have given several application examples in basic research and early bioprocess development with this technology. The aforementioned paper was followed by another paper introducing an automated version of the BioLector called the “RoboLector”, which allows the automated manipulation of the microwell fermentations with a standard liquid-handling system. Several examples of induction-profiling in protein production and growth synchronization of *E. coli* cultures have been presented with this technology (Huber et al., 2009). Recently an inevitable aspect in fermentation science has been reported: the successful scale-up of the microscale fermentations in the BioLector to stirred tank fermenters was demonstrated for the widely used protein expression systems *E.coli* and *Hansenula polymorpha* (Kensy et al., 2009b).

This current study now adds new online parameters to the BioLector technology. The widely applied optode technology for non-invasive *pH* and *DOT* measurements is combined with scattered light and fluorescence measurements of the BioLector. With this combination, it is now possible to monitor all common fermentation parameters, namely biomass concentration, fluorescence of biogenic metabolites or fluorescent proteins (e.g. *GFP*), *pH* and *DOT*, in each well of a 48-well microtiter plate. Thus, the throughput of fermentation experiments is dramatically increased. Additionally, the online information of all 48 parallel fermentations provides more knowledge about fermentation kinetics. The application of the recently introduced Flowerplate in the BioLector adds additional benefits to the system (Funke et al., 2009). Fermentation scientists now have the possibility to choose different oxygen transfer conditions from a broad range of k_La values (10-800 1/h). The different k_La values can be realized by simple changes of the filling volume or the shaking frequency as presented here. In other words, this combined system, i.e. BioLector + Flowerplate, is a very well suited scale-down model for a broad range of fermentations. The importance of oxygen transfer conditions for microbial growth is demonstrated in this study. Intelligent design of experiments makes the BioLector technology a very efficient and powerful tool in screening and early bioprocess development. Variations of fermentation parameters, such as *pH* variation as illustrated here, narrows the parameter range for further process optimization in laboratory fermenters. We believe that this BioLector technology is ideally suited for process optimization approaches such as Quality by Design (QbD) and Process Analytical Technology (PAT) introduced by the U.S. FDA (Madenius et al., 2009), because the combination of online analysis, high-throughput and automation paves the way for better process understanding with less research effort.

7 Summary

This thesis treated three main aspects of fermentation science: 1) the characterization of mass transfer conditions, 2) online-monitoring of the most relevant fermentation parameters, and 3) the scale up from microtiter plates to stirred tank fermenters. All these aspects were analyzed in respect to the application of microtiter plates as a microbioreactor platform in upstream bioprocessing.

First it was demonstrated that, in standard round 48-well microtiter plates, much higher oxygen transfer rates could be achieved than those in standard round 96-well MTPs, considered to be the predominant format in biotechnology. The overall OTR_{max} of 96-well MTPs of 0.030 mol/L/h with 200 µL filling volume (for the maximum shaking frequency of all shaking diameters before spillage) was doubled in round 48-well MTPs. Furthermore, at extreme shaking conditions, OTR_{max} values, respectively of 0.09 mol/L/h at a shaking diameter of 25 mm (n= 550 1/min, V_L= 300 µL) and 0.25 mol/L/h at a shaking diameter of 3 mm (n= 1450 1/min, V_L= 300 µL) could be determined. Even though these extraordinary high OTR_{max} values are well suited to mimic a stirred tank bioreactor with respect to oxygen transfer, they are achieved at extreme operation conditions, i.e. very small filling volumes and high shaking frequencies. Difficult here was that the reaction medium rose up and clung to the well walls thereby hindering online optical measurements. Another drawback of the 48-well MTP was that these plates are only available in completely transparent plastics. To avoid well-to-well crosstalk of the optical measurements, it would be more suitable to work with black-walled MTPs with a transparent bottom.

The integration of *pH*- and *DOT*-optodes into microtiter plates was the first step towards generating more process information from microfermentations. Here *pH*- or *DOT*-sensitive fluorescent dyes were immobilized individually on the bottom of a 24-well MTP. Due to the size of the detecting optics (consisting of excitation and emission filter, LED and photodiode), only one optode could be applied in each well. This technique was applied to understand the physiological states of various *E. coli* fermentations. The online monitored signals for *pH* and *DOT* of two separate fermentations of the same culture could be overlapped in one diagram, and they

correlated well to those of parallel shake flask cultures in the Respiration Activity Monitoring System. The online curves of the *pH* and *DOT* measurement signals provided insight into the respiration activity and the acid-base equilibration of the different media during *E. coli* fermentation. The diagrams clearly pointed out that oxygen uptake rates of the cultures above 0.04 mol/L/h led to an oxygen limitation in the applied 24-well MTPs (at V_L= 600 µL, n= 250 1/min, d_0= 25 mm). Even though further reduction in filling volume or increase in shaking frequency could improve the oxygen transfer rate, it would reduce, however, the liquid coverage of the optode in the middle of each well. This would be counterproductive, because the measurement noise increases dramatically up to a point where the optode runs dry. The determined OTR_{max} of 24-well MTP lay at 0.04 mol/L/h and is not sufficient for most microbial fermentations. This very moderate OTR_{max} value can only fulfill the requirements of cells growing in very minimal media such as LB medium or synthetic medium with carbon sources having concentrations approximately below 5 g/L.

The next step towards a universal online monitoring tool for microfermentations was the development and evaluation of a fiber-optical measurement technique. This new technology could be used to detect in sequence biomass concentrations in suspensions by means of scattered light and fluorescences from fluorescent proteins, fusions thereof, fluorescent metabolites or fluorescent substrates. The wells of a MTP were scanned by a X-Y mover with an optical glass-fiber bundle placed under the bottom of the MTP. The non-invasive optical measurement was performed during the continuous shaking of the MTP. It could be demonstrated that this technology was able to linearly resolve biomass concentrations of bacteria and yeast cultures up to cell dry weights of 50 g/L. Thus, the online detection of biomass concentrations could be realized quantitatively without any dilution of the culture broth as it is normally necessary for optical density measurements. Besides the biomass detection, fluorescence of green fluorescent protein (*GFP*), flavin mononucleotide (FMN)-based fluorescent protein (*FbFP*) and NADH could all be detected online during fermentation. These signals could identify different high *GFP* producers and optimal growth and protein production conditions without the need for further offline analysis. Moreover, the online signals allowed the researcher to evaluate biomass/substrate and product/biomass yields (with *GFP* as model protein) over time. The application of

a soluble fluorescent *pH*-indicator provided additional online process information, but it was disadvantageous that this sensor could not be pre-calibrated and, thus, not universally be applied.

Microscale results are especially valuable when they can be used to predict those on a larger scale. Therefore, it was inevitable to prove the scalability from microtiter plate experiments to standard laboratory fermenters. For this scalability, one prerequisite for aerobic fermentations is to guarantee similar oxygen transfer conditions at both scales. Therefore, the OTR_{max} values for 96-well MTPs were characterized for different filling volumes (V_L= 100 µL-260 µL, n= 995 1/min, d_0= 3 mm). Due to more practical considerations, 200 µL filling volume was applied in *E. coli* and in *Hansenula polymorpha* fermentations. This condition provided an OTR_{max} of approximately 0.03 mol/L/h, which could slightly be oxygen-limiting for the applied bacteria and yeast fermentations. However, the results of this scalability study illustrated a huge comparability of the growth and protein expression kinetics in both fermentations, i.e. bacteria and yeast, and on both scales, i.e. the 200 µL in MTP and 1.4 L in a stirred tank bioreactor. The bioprocess kinetics were evaluated by optical online measurements of biomass and protein concentrations (*GFP*) exhibiting the same characteristic fermentation times and maximum signal deviations of < 10% between the scales. Even with the 96-well MTP which offers very poor applicability in aerobic fermentations due to the limited OTR_{max} (0.03 mol/L/h with 200 µL), an excellent scalability between the shaken microtiter plate and the stirred tank bioreactor was demonstrated. It can be concluded that for common microbial expression systems with water-equivalent viscosities, the scalability from microtiter plate to stirred tank bioreactors is proven. Using microtiter plates with improved oxygen mass transfer (such as the Flowerplate (Funke et al., 2009), which was not available at the time of this study) promises a broad application range for microscale fermentations in shaken microtiter plates and considerably facilitates bioprocess development.

This thesis is finalized combining online monitoring with improved oxygen transfer rates in shaken microtiter plates. The online monitoring technology using scattered light and fluorescence was amplified by the aforementioned optode-based *pH* and

DOT measurements. For this purpose *pH*- and *DOT*-optodes were immobilized at the bottom of each well of a MTP and, additionally, a detection window for the scattered light and fluorescence measurements in the suspension was kept free next to the optodes. The different detection windows on each well bottom were scanned sequentially by the optical detector. The reading time of all four parameters on a 48-well MTP lasted 4 minutes for one cycle, which could be considered a good time resolution for quasi real-time monitoring of microbial and cell culture fermentations. This new measurement technology was combined with the recently developed 48-well Flowerplate (Funke et al., 2009), which provides OTR_{max} values of up to 0.14 mol/L/h. The full-parameter monitoring technique was applied in E. coli and yeast fermentation and gave further understanding of fermentation kinetics. The improved oxygen transfer rate of the Flowerplate verified oxygen-unlimited growth of *E. coli* cells under high shaking frequency (1400 1/min) for media with strong demand of oxygen (OUR > 0.08 mol/L/h). A study of different *pH*-values showed the influence of *pH* on yeast growth and oxygen consumption. Finally, the applied technology was successfully proven by optimizing a synthetic fermentation medium through supplementation of plant peptones.

In conclusion, this thesis presented a powerful online monitoring technology which has been established for the first time and then has been expanded to a full-parameter monitoring system for all relevant fermentation parameters. Its combination with microreactor arrays that provide high-oxygen transfer, such as the Flowerplate, makes this technology an universal and very efficient platform in screening and early bioprocess development.

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