Process Design Aspects
for Small-Scale Fermentation Systems

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Unverständlichkeit ist noch lange kein Beweis für tiefe Gedanken.
Marcel Reich-Ranicki (1920 - 2013)
Abstract

Filamentous or biopolymer producing microorganisms are currently gaining importance due to their huge potential regarding worldwide sustainability issues. An economic industrial screening of these organisms is essential for determining the best producing microbial strains or fermentation conditions. It is known that filaments and biopolymers massively influence viscosity of the fermentation broth, thereby affecting the oxygen supply for microorganisms. In literature, the influence of viscosity is well documented for the oxygen transfer in stirred tank fermentations, however, little is known about the influence of viscosity on gas/liquid oxygen transfer in shake flask cultures. Even the effective shear rate in shake flasks was never systematically investigated, although it influences the apparent viscosity, mixing as well as mass and heat transfer. Since unknown oxygen transfer rates and unknown effective shear rates pose the risk of screening and producing under unfavorable conditions, this work addresses this lack of knowledge.

Four objectives were pursued within this study. First, measurements and numerical simulations of the oxygen transfer in liquid films adhering on shake flask walls were conducted as a function of viscosity and film thickness. Thereby, the suitability of the widely applied film theory of Higbie was studied. It was demonstrated that Higbie’s film theory does not apply for cultivations which occur at viscosities up to 10 mPa·s. For the first time, it was experimentally shown that the maximum oxygen transfer capacity $OTR_{max}$ counter-intuitively increases in shake flasks when viscosity is increased from 1 mPa·s to 10 mPa·s, leading to an improved oxygen supply for microorganisms. Additionally, the $OTR_{max}$ at viscosities of up to 80 mPa·s is not significantly lower than the $OTR_{max}$ at waterlike viscosities. This is contrary to stirred tanks, where the oxygen supply is steadily reduced to only 5% at 80 mPa·s.

Second, a first shear rate correlation for shake flasks – valid for a wide range of pseudo-plastic flow behaviors, shake flask sizes and operating conditions – was developed as a function of viscosity on the basis of Buckingham’s $\pi$-theorem and experimental data. It was found that effective shear rates in shake flasks commonly cover a range from 20 s$^{-1}$ to 2000 s$^{-1}$. The precise applicability of the developed shear rate correlation was demonstrated for three different shake flask fermentations. Depending on the broth’s
flow behavior, the effective shear rate in shake flasks is at least 1.55 times higher than that in stirred tank reactors operated at the same volumetric power input, leading to a potentially 50% lower apparent viscosity in shake flasks.

Third, a novel concept of microtiter plates (MTP) in 96-well, 48-well and 24-well format was developed preventing spill-out of the rotating liquid even at high shaking frequencies and high filling volumes. In spite of the fact that high filling volumes per well are often desirable for offline analytics during screening procedures, as yet, low filling volumes per well have to be adjusted to prevent spill-out and to overcome insufficient oxygen transfer in conventional MTPs. In prototypes of the newly developed MTP, preliminary measurements of the $OTR_{max}$ were conducted using a sulfite system. With respect to the $OTR_{max}$, the advantages of the novel MTP could clearly be displayed for ranges of filling volume and shaking frequency where conventional MTPs spill out: Whereas extreme shaking conditions are needed for the novel 96-well type to reach a higher $OTR_{max}$ compared to the conventional one, the novel 48-well and 24-well formats are already advantageous at moderate shaking conditions. This is due to the influence of the surface tension which becomes less dominant the larger the well diameter is. The biocompatibility of the novel MTP type was proven by *Escherichia coli* fermentations. Due to higher achievable oxygen transfer rates and, thus, faster C-source consumption compared to cultures in the conventional MTP, the new MTP even showed the potential of shortening the fermentation time. Hence, a suitable MTP concept was found for applications in high-throughput screenings where high sample volumes are required.

Fourth, a scale-down was conducted requested by an industrial collaboration partner. To achieve time and cost efficient high-throughput for strain screening, an established shake flask protocol was scaled down into MTP. An approach based on an oxygen-consuming sulfite system was applied to ensure equal $OTR_{max}$-values in MTPs and shake flasks. Obtained sulfite datasets were used to identify operating conditions leading to the same oxygen supply for the model organism *Trichoderma reesei* in shake flasks and 24-well MTPs. For 24-well MTPs, the shake flask $OTR_{max}$ of 20 mmol/L/h of the industrial protocol was obtained under the following optimal operating conditions: 1 mL filling volume per well, 200 rpm shaking frequency and 50 mm shaking diameter. With the identified operating conditions almost identical oxygen transfer rates and product
concentrations were measured in shake flasks and 24-well MTP cultures as a function of fermentation time.

The proposed sulfite approach is a fast and accurate means to scale-down established screening procedures into MTPs to achieve high-throughput. The obtained insights into oxygen transfer and effective shear rates in viscous systems are valuable for explaining existing deviations in screening and production results. Ultimately, by means of consistent scale-up and scale-down procedures, economic bioprocess development is facilitated with the results of this study.
Kurzfassung


Erstens sollten Messungen und numerische Simulationen des Sauerstofftransfers in chemische und mikrobielle Modellflüssigkeiten durchgeführt und die Anwendbarkeit der weitverbreiteten Filmtheorie von Higbie untersucht werden. Hierbei wurde gezeigt, dass die Higbie’sche Filmtheorie nicht für Schüttelkolbenfermentationen mit einer Viskosität von bis zu 10 mPa·s anwendbar ist. Es konnte erstmalig beobachtet werden, dass die Sauerstofftransferkapazität $O_{TR_{max}}$ in Schüttelkolben – entgegen der Intuition – erhöht, wenn die Viskosität von 1 mPa·s auf 10 mPa·s steigt. Das ist gleichbedeutend mit einer verbesserten Sauerstoffversorgung für die Mikroorganismen. Des Weiteren wurde demonstriert, dass die $O_{TR_{max}}$ bei Viskositäten von bis zu 80 mPa·s kaum geringer ist als bei wasserähnlichen Viskositäten. Dies ist gegensätzlich zu Rührreaktoren, da sich die Sauerstoffversorgung hier auf bis zu 5% bei 80 mPa·s stetig verschlechtert.

Zweitens sollte auf Basis des Buckingham’schen $\pi$-Theorems und experimenteller Daten eine erste Scherratenkorrelation für Schüttelkolben entwickelt werden, die für ein großes Spektrum an pseudo-plastischem Fließverhalten, Schüttelkolbengrößen und
Schüttelbedingungen Gültigkeit besitzt. Es wurde festgestellt, dass Scherraten in Schüttelkolben unter üblichen Fermentationsbedingungen einen Bereich von 20 s\(^{-1}\) bis 2000 s\(^{-1}\) abdecken können. Die Anwendbarkeit der entwickelten Scherratenkorrelation wurde an drei verschiedenen Schüttelkolbenfermentationen demonstriert. Abhängig vom Fließverhalten der Fermentationsbrühe ist die effektive Scherrate im Kolben im Vergleich zu einem Rührreaktor mit gleichem Leistungseintrag mindestens 1.55 mal so groß. Dies kann bei üblichem Fließverhalten in einer bis zu 50% geringeren Viskosität im Schüttelkolben resultieren.

Drittens sollte im Rahmen dieser Arbeit ein Mikrotiterplattenkonzept entwickelt werden, das das Überschwappen der rotierenden Flüssigkeit auch bei hohen Drehzahlen und Füllvolumina verhindert. Bislang sind geringe Füllvolumina pro Well erforderlich, um akzeptable Sauerstofftransferraten zu erzielen, was häufig im Gegeninteresse dazu steht, dass ausreichend Kulturflüssigkeit benötigt wird, um Offline-Analysen im Kontext eines Screening durchführen zu können. Mit einem Sulfit-System wurden erste Messungen der \(OTR_{max}\) in Prototypen des neuen Mikrotiterplattenkonzeptes durchgeführt und die erlangten Messdaten mit denen aus konventionellen Mikrotierplatten (MTP) verglichen. Hierbei konnte die vorteilhafte Anwendbarkeit des neuen Konzeptes für Bereiche, in denen konventionelle MTP überschwappen, klar aufgezeigt werden. Diese Bereiche hängen vom Plattenformat (96-well, 48-well oder 24-well) ab, da der Einfluss der Oberflächenspannung mit sinkendem Welldurchmesser steigt. Im Fall des 96-well Formates ist das neue Konzept gegenüber der konventionellen MTP nur bei sehr hohen Drehzahlen und großen Füllvolumina überlegen. Hingegen schwappen konventionelle 48-well MTP und 24-well MTP bereits bei moderaten Schüttelbedingungen über, sodass das neue Konzept hier bereits bei geringeren Schüttelfrequenzen vorteilhaft ist. Um auch die Biokompatibilität sicherzustellen, wurde *Escherichia coli* zum direkten Vergleich im neuen Prototyp und in einer konventionellen MTP kultiviert. Neben der vorhandenen Biokompatibilität wurde hierbei außerdem gezeigt, dass Fermentationen im neuen Konzept durch das Erzielen höherer Sauerstofftransferraten verkürzt werden können. Zusammenfassend wurde also ein MTP-Konzept entwickelt, das sich besonders für Hochdurchsatzscreenings eignet, bei dem hohe Volumina an Überstand für Offline-Analysen benötigt werden.
Viertens sollte ein Scale-down-Verfahren angewendet werden, das auf einem Sulfit-System basiert und zur Einstellung gleicher $OTR_{max}$-Werte in Mikrotiterplatten und Schüttelkolben geeignet ist. Generierte Sulfitdatensätze wurden verwendet, um Schüttelbedingungen zu identifizieren, die für den Modelorganismus *Trichoderma reesei* die gleiche Sauerstoff-versorgung in Schüttelkolben und 24-well Mikrotiterplatten sicherstellen. In 24-well Mikrotiterplatten wurde die in einem industriellen Schüttelkolbenprotokoll erzielte $OTR_{max}$ von 20 mmol/L/h mit folgenden Schüttelbedingungen erreicht: 1 mL Füllvolumen pro Well, 200 rpm Schüttelfrequenz und 50 mm Schütteldurchmesser. Mit diesen Schüttelbedingungen wurden nahezu identische Sauerstofftransferraten und Produktkonzentrationen in Schüttelkolben und 24-well Mikrotiterplatten als Funktion der Fermentationszeit gemessen.

Das angewendete Sulfitverfahren stellt eine schnelle und genaue Möglichkeit dar, um durch das Scale-down eines etablierten Schüttelkolbenprotokolls in eine Mikrotiterplatte Hochdurchsatz zu erzielen. Die gewonnenen Erkenntnisse über den Sauerstofftransfer und die effektive Scherrate in viskosen Systemen sind wertvoll, um Unterschiede in Screening- und Produktionsergebnissen zu erklären. Konsistente Ergebnisse während verfahrenstechnisch fundierten Scale-up- und Scale-down-Prozessen ermöglichen schließlich eine ökonomische biotechnologische Prozessentwicklung.
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Nomenclature

Latin

\( a \)  Volumetric mass transfer area [m²/m³]
\( a_B \)  Volumetric mass transfer area of the bulk liquid [m²/m³]
\( a_F \)  Volumetric mass transfer area of the liquid film [m²/m³]
\( A_F \)  Mass transfer area of the liquid film [m²]
\( C_{O_2,L} \)  Dissolved oxygen concentration in the liquid phase [mol/m³]
\( C_{O_2,L}^* \)  Dissolved oxygen concentration at the gas/liquid interface [mol/m³]
\( C_{O_2,B} \)  Dissolved oxygen concentration in the bulk phase [mol/m³]
\( C_{PVP} \)  Concentration of polyvinylpyrrolidone (PVP) in solution [mol/m³]
\( d \)  Maximum shake flask diameter [m]
\( d_0 \)  Shaking diameter [m]
\( D_{O_2} \)  Diffusion coefficient for oxygen [m²/s]
\( f \)  Proportionality factor [-]
\( h_{max} \)  Maximum liquid height [m]
\( k_L \)  Mass transfer coefficient [m/s]
\( (k_L)_B \)  Mass transfer coefficient for the bulk liquid [m/s]
\( (k_L)_F \)  Mass transfer coefficient for the liquid film layer at the shake flask wall [m/s]
\( k_L \alpha \)  Volumetric mass transfer coefficient [s⁻¹]
\( k_1 \)  First order reaction rate constant [s⁻¹]
\( K \)  Flow consistency index [Pa·sᵐ]
\( K_{MO} \)  Metzner and Otto constant [-]
$K_s$  
Half saturation (Monod) constant [mol/m³]

$L$  
Proportionality factor for power input concept [-]

$L_{O_2}$  
Oxygen solubility [mol/m³/bar]

$m$  
Flow behavior index [-]

$M$  
Torque [N·m]

$n$  
Shaking/Stirring frequency [s⁻¹]

$\dot{n}_{O_2}$  
Oxygen flux across the gas/liquid interface at a certain time point during $t_{exp}$ [mol/s]

$\bar{n}_{O_2}$  
Average of flux-values during $t_{exp}$ [mol/s]

$\bar{n}_{O_2,\infty}$  
Average of flux-values during $t_{exp}$ in case of a thick liquid film [mol/s]

$Ne$  
Newton number [-]

$Ne'$  
Modified Newton number [-]

$OTR$  
Oxygen transfer rate [mol/m³/s] or [mmol/L/h]

$OTR_{max}$  
Maximum oxygen transfer capacity [mol/m³/s] or [mmol/L/h]

$OTR_{max,F}$  
Maximum oxygen transfer capacity of the liquid film [mol/m³/s] or [mmol/L/h]

$OTR_{max,B}$  
Maximum oxygen transfer capacity of bulk liquid [mol/m³/s] or [mmol/L/h]

$OTR_{max,medium}$  
$OTR_{max}$ of a fermentation [mmol/L/h]

$OTR_{max,sulfite}$  
$OTR_{max}$ of a sulfite experiment [mmol/L/h]

$OTR_{max,react}$  
$OTR_{max}$ at reduced oxygen headspace concentration [mmol/L/h]

$OTR_{max,21\%}$  
$OTR_{max}$ at an oxygen mole fraction of 0.21 in the headspace [mmol/L/h]
\( p_{O_2,G} \) Oxygen partial pressure in the gas phase [bar]
\( p_{O_2,L} \) Oxygen partial pressure in the liquid phase [bar]
\( P \) Power input [kW]
\( Ph \) Phase number [-]
\( r \) Reaction rate [mol/m³/s]
\( Re \) Reynold’s number [-]
\( t_{exp} \) Exposure time of a liquid film with gas phase [s]
\( t_{exp,max} \) Exposure time of liquid film with gas phase at max. liquid height [s]
\( V_L \) Liquid volume [m³]
\( V_{L,F} \) Liquid film volume [m³]
\( x \) Penetration depth [μm], Section 2 and 4.1
\( x \) Exponent of the Geometric number [-], Section 4.2
\( y \) Exponent within the \( Ne'/Re \) relationship [-]
\( \gamma_{O_2,headspace} \) Mole fraction of oxygen in the flask/MTP headspace [-]
\( \gamma_{O_2,0.21} \) Oxygen mole fraction of 0.21 in the flask/MTP headspace [-]
\( X \) Biomass concentration [g/m³]
\( Y_{X/O_2} \) Biomass yield for oxygen [g/mol]

**Greek**

\( \dot{\gamma} \) Shear rate \([s^{-1}]\)
\( \dot{\gamma}_{eff} \) Effective shear rate \([s^{-1}]\)
\( \eta_{app} \) Dynamic apparent viscosity \([\text{Pa s}]\)
\( \mu_{max} \) Maximum growth rate \([s^{-1}]\)
\( \rho \) Liquid density \([\text{kg/m³}]\)
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1 INTRODUCTION

Elevated viscosities are caused by filamentous organisms or the production of biopolymers; both are upcoming topics e.g. in sustainability and biofuels literature. However, elevated viscosities pose the risk of screening and producing under unfavorable conditions.

Since an industrial screening of these organisms is essential for an economic selection of the best producing microbial strains and fermentation conditions, this work addresses the following four aspects within small-scale process design:

- **Liquid Films at Shake Flask Walls.** The thickness of liquid films adhering at shake flask walls is influenced by the apparent viscosity. The liquid film has a considerable impact on the oxygen supply to microorganisms.
- **Effective Shear Rates in Shake Flasks.** A shear rate correlation based on the volumetric power input is developed allowing the determination of the apparent viscosity of pseudo-plastic fermentation broths in shake flasks.
- **A New Microtiter Plate Concept.** A new microtiter plate concept is developed enabling high-throughput screening at operation conditions not accessible with conventional microtiter plates.
- **Scale-Down from Shake Flask to Microtiter Plate.** A systematic scale-down of *Trichoderma reesei* considering consistent oxygen supply is demonstrated using an oxygen-consuming 1 M sodium sulfite system.

You find these four aspects as subsections in the Introduction, in the Materials and Methods as well as in the Results part.

**Liquid Films at Shake Flask Walls**

Shake flasks are widely used for screening of microbial strains as well as for optimizing media and shaking parameters. For bioprocess development, it is crucial to characterize the gas/liquid oxygen transfer in shake flasks (Veglio et al., 1998; Büchs, 2001). An undefined oxygen supply or an undesired oxygen limitation may result in an incorrect selection of microbial strains for the subsequent large scale production (Zimmermann et
INTRODUCTION

Within the last forty years, some progress has been made to correlate gas/liquid oxygen transfer in shake flasks as a function of shaking parameters and physicochemical properties of the liquid (Suijdam et al., 1978; Henzler and Schedel, 1991; Maier and Büchs, 2001).

For the first time, Gaden (1962) mentioned the existence of a liquid film at the inner wall of a rotating shake flask in the context of gas/liquid oxygen transfer. Since such a liquid film provides a large interface for gas/liquid oxygen transfer, Maier et al. (2004) introduced a model to account for the different mechanisms of oxygen transfer into the film and the bulk liquid. The liquid film has been described in analogy to a falling film reactor. The authors investigated whether Higbie’s film theory (Higbie, 1935), which is a widely applied model for gas penetration in technical liquid films, is also appropriate for describing oxygen transfer into liquid films in rotating shake flasks. By numerical simulations of oxygen diffusion in a 1 M sodium sulfite system during one rotation, Maier et al. (2004) demonstrated that the applicability of Higbie’s theory depends on the liquid film thickness. Even though it was proven that liquid film thickness and viscosity are strongly correlated for many technical applications like falling film reactors (Wasan et al., 1972) and coating technologies (White and Tallmadge, 1965; Groenveld, 1970; Spiers et al., 1974; van Rossum, 1958), the influence of medium viscosity on oxygen transfer in shake flasks has not been systematically investigated as yet. Published correlations between shaking parameters and maximum oxygen transfer capacity $OTR_{max}$ (Maier and Büchs, 2001; Seletzky et al., 2007) are not applicable for microbial cultures which become increasingly viscous during fermentation.

In literature, bacterial- and plant cell cultures reached maximum viscosities between 11 mPa·s and 110 mPa·s in shake flasks (Peña et al., 2011; Rodríguez-Monroy and Galindo, 1999; Wilming et al., 2013; Rodríguez-Monroy et al., 2004; Henzler and Schedel, 1991; Lotter and Büchs, 2004). A selection of such microorganisms, producing valuable products like biopolymers, antibiotics or enzymes, is listed later in this study (Table II, page 39). In contrast to shake flasks, stirred tank reactors have been well characterized with respect to the influence of viscosity on oxygen transfer (Perez and Sandall, 1974; Yagi and Yoshida, 1975; Ranade and Ulbrecht, 1978; Zlokarnik, 1978; Höcker et al., 1981; Nishikawa et al., 1981; Henzler and Obernosterer, 1991; Zlokarnik, 2001). Thus, one aim of this current study is to investigate the mechanism of oxygen
transfer in increasingly viscous cultures in shake flasks. For this purpose, the applicability of Higbie’s film theory to liquid films in rotating shake flasks was investigated with numerical simulations of Fick’s law of diffusion for two different sodium sulfite systems and a Corynebacterium glutamicum culture medium. The simulated liquid film thickness and exposure times were varied, as such changes have to be expected during changes in viscosity and shaking frequency, respectively. In extension to the work of Maier et al. (2004), the influence of the liquid film thickness on the $OTR_{max}$ was studied experimentally. The $OTR_{max}$ was measured using a Respiration Activity Monitoring System (RAMOS) (Anderlei and Büchs, 2001; Anderlei et al., 2004). In these experiments, the viscosities of the above mentioned three liquids were artificially varied with polyvinylpyrrolidone (PVP).

In case of fermentations with increasingly viscous culture broths, shake flasks with baffles have been suggested as suitable fermentation systems (Delgado et al., 1989). However, the reproducibility for baffled shake flasks is in general lower than for unbaffled shake flasks with their well-defined liquid flow pattern (McDaniel et al., 1965; Freedman, 1969; Büchs, 2001). Additionally, the probability of the so called “out-of-phase phenomenon” (Büchs et al., 2000a; Büchs et al., 2000b), meaning that the liquid does not follow the rotating movement of the shaker, is increased by baffles (Büchs et al., 2001). This phenomenon reduces the specific power consumption, mixing and the gas/liquid mass transfer. Thus, this whole study focusses on the investigation of unbaffled shake flasks.

**Effective Shear Rates in Shake Flasks**

The flow behavior of a fermentation broth of elevated viscosity is usually pseudo-plastic (or shear-thinning), meaning that its apparent viscosity decreases with increasing shear rate (Kemblowski and Kristiansen, 1986). Unknown shear rates and apparent viscosities pose the risk of screening and producing under unfavorable fermentation conditions leading to incorrect conclusions (Peter et al., 2004). Although broth viscosity is known to influence mixing (Tan et al., 2011) as well as gas/liquid mass and heat transfer (Büchs et al., 2001), the effective shear rate in shake flasks has never been systematically investigated before. Due to this lack of knowledge, engineering-based scaling between shake flasks and stirred tanks with consistent apparent viscosities on both scales is not
yet possible, although shake flasks are the predominant screening tool employed for microbial strains, culture media and process parameters (Kumar et al., 2004). Peter et al. (2004) introduced a method to determine the effective shear rate in shake flasks and conducted preliminary measurements within a small spectrum of operating conditions. The authors concluded: “Much more efforts have to be dedicated to this area to introduce a general model, which is valid for all kinds of pseudo-plastic fluids and operating conditions”.

One aim of the present study is to quantify and correlate the effective shear rates in shake flasks for a wide range of pseudo-plastic flow behaviors and operating conditions. The volumetric power input into shake flasks, filled with Newtonian or pseudo-plastic model liquids, is measured using a shaker with power input measurement (Büchs et al., 2001). Equal power input into a Newtonian and a pseudo-plastic liquid at one specific operating condition is presumed to be the result of the same apparent viscosity (Metzner and Otto, 1957; Peter et al., 2004). The viscous flow behavior of the pseudo-plastic liquids is then determined via a rheometer enabling the effective shear rate to be determined for the specific shake flask operating condition. This approach allows formulating a correlation based on the power input concept postulated by Henzler and Kauling (1985). In this study, an effective shear rate correlation is newly derived for shake flasks employing a Dimensional analysis and Buckingham’s π-theorem. The resulting correlation is applied for calculating the effective shear rates in shake flasks and for demonstrating the impacts of the nominal flask volume, filling volume, shaking frequency and flow behavior, respectively. On the basis of the developed shear rate correlation, three different exemplary fermentation systems generating elevated viscosities are analyzed over time with respect to apparent viscosities and Phase numbers. Additionally, comparative calculations of the effective shear rate in shake flasks and stirred tank reactors are conducted.

**A New Microtiter Plate Concept**

Attainable $OTR_{\text{max}}$-levels in common microtiter plates (MTP) are lower than those of most bioreactors like shake flasks and stirred tanks (Funke et al., 2009; Maier et al. 2004). Low $OTR_{\text{max}}$-levels lead to long fermentation times, which are disadvantageous, especially during high-throughput procedures. In the past, different MTP well-
geometries have been investigated with respect to their influence on the \( OTR_{\text{max}} \) (Funke, 2010). For instance, wells with a square cross section area can double the attainable \( OTR_{\text{max}} \)-values. However, this shape causes extensive splashing of the fermentation broth resulting in cross-contamination, wet sealing tapes and undefined oxygen supply (Duetz, 2007). These problems have been massively reduced with a “flower shaped” cross section area: The “so-called” FlowerPlate® in 48-well format delivers an increased \( OTR_{\text{max}} \) with only minor splashing (Funke et al., 2009). Nevertheless, in all kinds of available MTPs the shaking frequency has to be high and the filling volume has to be low to achieve acceptable oxygen supply. However, for offline analytics during screening procedures sufficient liquid volume is essential which, in turn, raises the risk of liquid spill-out out of the rotating MTP.

In this work, a new MTP concept was developed and 96-, 48- und 24-well prototypes were manufactured. The new MTP type should prevent spill-out at both, high relative filling volumes and high shaking frequencies, accessing operating conditions and \( OTR_{\text{max}} \)-levels not achievable in conventional MTPs. An oxygen-consuming sulfite system was applied together with a RAMOS device to conduct preliminary comparisons of the \( OTR_{\text{max}} \) in the newly developed and the conventional MTP type. Biological compatibility test were conducted to investigate the applicability as a fermentation system.

**Scale-Down from Shake Flask to Microtiter Plate**

In biotechnological industry, the increasing cost- and time pressure has led to a strong interest in small-scale, high-throughput screening setups. To reduce process costs and development time, screening fermentations are increasingly performed in MTPs (Hilton, 1999; Kumar et al., 2004). Once a number of suitable strains has been identified by MTP screening, only a few shake flask experiments with a clearly reduced number of strains have to be performed. In the next scale-up step, the best performing strain is commonly fermented in a laboratory-scale stirred tank reactor with controlled oxygen partial pressure. In contrast, the monitoring of oxygen transfer in the small MTP wells is much more difficult (Kumar et al., 2004) and often even not considered (Suresh et al., 2009). In MTPs, cultures grow under a completely unknown oxygen supply, and the best strain can easily be overlooked already on this small scale (Zimmermann et al., 2006).
Therefore, a consistent screening procedure, especially with regard to oxygen supply, is essential to identify the best performing strain for later industrial processes.

In the biorefining, textile, paper and laundry detergent industries, the cellulose-secreting fungus *Trichoderma reesei* has become an important workhorse (Miettinen-Oinonen and Suominen, 2002; Schuster and Schmoll, 2010; Saranraj et al., 2012). *T. reesei*’s filamentous morphology leads to culture broths with elevated viscosity. This viscosity affects liquid mixing and gas/liquid oxygen transfer (Kawase and Moo-Young, 1988; Gabelle et al., 2012). Hardly any *Trichoderma* fermentation procedure described in literature is suitable for high-throughput. The procedures are either based on shake flasks (Cianchetta et al., 2010; Uzbas et al., 2012) or stirred tanks (Ramot et al., 2004; Gabelle et al., 2012). For the rarely described high-throughput procedures, Petri dishes with agar (King et al., 2012) or unshaken MTPs (Thronset et al., 2010; Cianchetta et al., 2012) are applied to screen *T. reesei*. Since these conditions lead to surface growth and accumulation of the biomass on the gas/agar or gas/liquid boundary area, the oxygen and nutrient supply is completely different than under submerged conditions like in shake flasks or stirred tanks – leading to an inappropriate scaling procedure.

As previously described in literature, the transfer of sufficient oxygen into the culture medium in a MTP well, is in general a challenging task (Duetz et al., 2000; John et al., 2003; Duetz and Witholt, 2004; Funke et al., 2009). Since there are no publications about submerged high-throughput screening of *T. reesei*, one aim of this current study is to introduce a systematic scale-down approach based on an oxygen-consuming sulfite system. Applying this approach intends both, an equal $OTR_{\text{max}}$ for *T. reesei* in shake flask and MTP as well as sufficient volume of supernatant from culture broth for later analytical procedures. For this purpose, an established industrial shake flask screening procedure for *T. reesei* screening was optimized with regard to oxygen supply and enzyme productivity, first. Thereafter, the $OTR_{\text{max}}$ of shake flasks (Maier et al., 2004; Seletzky et al., 2007) and 24-well MTPs was investigated with a RAMOS device. A 1 M sodium sulfite system was applied in a wide range of different filling volumes and shaking frequencies. With the obtained dataset, shaking conditions could finally be selected to gain at least 700 µL of supernatant for later analytics and to equal the $OTR_{\text{max}}$ of shake flasks and 24-well MTPs. Consequently, the fungus *T. reesei* is supplied with the same amount of oxygen in both screening scales.
2 THEORETICAL BACKGROUND

2.1 Gas/Liquid Mass Transfer in Shake Flasks

2.1.1 Oxygen transfer capacity in shake flasks

The oxygen transfer rate $OTR$ refers to the transport of oxygen from the gas into the liquid phase. As described in Equation 2.1, the $OTR$ is influenced by the mass transfer coefficient $k_L$, the volumetric transfer area $a$, the concentration in the liquid phase $C_{O_2,L}$ as well as by the dissolved oxygen concentration at the gas/liquid interface $C_{O_2,L}^*$. The driving concentration gradient $(C_{O_2,L}^* - C_{O_2,L})$ is equal to the oxygen solubility $L_{O_2}$ multiplied by the oxygen partial pressure difference in the gas and the liquid phase.

$$OTR = k_L a (C_{O_2,L}^* - C_{O_2,L}) = k_L a L_{O_2} (p_{O_2,G} - p_{O_2,L}).$$  \hspace{1cm} 2.1$$

An expression via partial pressures is often beneficial as these values are easy to measure by respective sensors (compare Section 3.4.2). The parameter $p_{O_2,G}$ represents the oxygen partial pressure in the gas phase and $p_{O_2,L}$ the oxygen partial pressure in the liquid phase.

The driving concentration gradient and the $OTR$ reach their maximum when the dissolved oxygen concentration $C_{O_2,L}$ approaches 0 mol/m³. This is defined as the maximum oxygen transfer capacity $OTR_{\text{max}}$. If a culture is oxygen-limited, $C_{O_2,L}$ is in the range of the Monod constant which is usually very small, approximately 0 mol/m³. This consequently leads to $OTR = OTR_{\text{max}}$. The oxygen headspace mole fraction at the $OTR_{\text{max}}$ is defined to be the oxygen mole fraction in air of 0.21. Due to oxygen consumption and the transport resistance of the sterile barrier, e.g. a cotton plug, the headspace mole fraction $y_{O_2,\text{headspace}}$ will be lower than in air (Mrotzek et al., 2001), resulting in a slightly reduced maximum oxygen transfer capacity $OTR_{\text{max,react}}$ during the measurement. Therefore, Equation 2.2 has to be applied to receive $OTR_{\text{max}}$-values at the reference mole fraction of $y_{O_2,0.21} = 0.21$. 


Figure 2.1 A. “Side view of a shake flask illustrating the applied liquid distribution model. B: Top view of part A (slice cut a-b of liquid distribution). C: Schema of the oxygen concentration in the liquid film adhering to the inner glass wall of the flask. D: Hypothetical division of a shake flask reactor into a falling film reactor and a completely mixed stirred tank reactor (“two sub-reactor” model) published by Maier et al. (2004). The three equations shown in this figure represent the boundary conditions for numerical integration of oxygen diffusion in the liquid film. The term $t_{\text{exp}}$ is the exposure time between the film and the gas phase and is a function of the liquid height. The term $t_{\text{exp,max}}$ is the maximum exposure time (time for a complete rotation), occurring at the top of the rotating liquid $h_{\text{max}}$; $d_o$ indicates the shaking diameter of the shaker. This figure was developed together with my former colleague A. Azizan. Reprint from Giese et al. (2014a) with permission from Wiley-Blackwell.
To model the gas/liquid oxygen transfer in a rotating shake flask, a detailed understanding of the liquid distribution is necessary. This distribution is illustrated in Figure 2.1 A and B. Maier and Büchs (2001) and Maier et al. (2004) demonstrated the existence of a liquid film at the wall of a shaken flask. This film participates on the gas/liquid oxygen transfer (Figure 2.1 B and C). To model the oxygen transfer into the bulk and film liquid, Maier et al. (2004) postulated a “two-sub-reactor model”, which is discussed in the following section.

2.1.2 Higbie’s film theory as a model for oxygen transfer into a liquid film

In the “two-sub-reactor model”, demonstrated in Figure 2.1 D, the bulk liquid of a shake flask is modeled as a homogeneously mixed broth in a stirred tank. Like during an oxygen limitation, the bulk liquid concentration \( C_{O_2,B} \) is assumed to be 0 mol/m\(^3\). For the liquid film in shake flasks, a falling film reactor analogy was chosen. In this analogy, an oxygen-free liquid film becomes enriched with oxygen during a certain falling time \( t_{exp} \), which represents the exposure time in a shake flask for one rotation. In this “two-sub-reactor model”, the \( \kappa_{L} \alpha \)-value consists of the two components \( (k_{L})_{B} a_{B} \) and \( (k_{L})_{F} a_{F} \), which denote the volumetric transport coefficients into the bulk liquid and the liquid film layer, respectively. Based on Equation 2.1, the \( OTR_{max} \) is defined as:

\[
OTR_{max} = OTR_{max,B} + OTR_{max,F} = [(k_{L})_{B} a_{B} + (k_{L})_{F} a_{F}] C_{O_2,L}^{*}. \tag{2.3}
\]

To describe \( (k_{L})_{B} \), Maier et al. (2004) applied and validated an approach by Gnielinski (1975) and Kawase and Moo-Young (1990). The transport coefficient of the liquid film \( (k_{L})_{F} \) was modeled by Higbie’s penetration theory (Higbie, 1935), a simple approach that is widely applied to describe oxygen transfer into liquid films:

\[
(k_{L})_{F} = 2 \frac{D_{O_2}}{\pi t_{exp}}. \tag{2.4}
\]
Here, $D_{O_2}$ denotes the diffusion coefficient of oxygen into the liquid phase. The factor $\pi$ derives from Fick’s law of diffusion and the respective boundary conditions (Taylor and Krishna, 1993; Bird et al., 2007). The term $t_{exp}$ describes the exposure time between the gas phase and liquid film. It is a function of the shaking frequency and liquid film height (Maier and Büchs, 2001). The liquid film can be divided into differential horizontal slices with a corresponding exposure time. At the maximum film height $h_{\text{max}}$, the exposure time peaks at $t_{\text{exp,max}}$. Only at this maximum height, the liquid film is in contact with the gas phase for one complete rotation until it is “renewed” again by the bulk liquid. This means $t_{\text{exp,max}}$ is equal to the inverse shaking frequency $n^{-1}$. Figure 2.1C qualitatively illustrates the dependency between exposure time $t_{\text{exp}}$ and penetration depth $x$. The longer the exposure time, the greater is the penetration depth.

Higbie’s theory (Equation 2.4) is mathematically derived from a semi-infinite space assumption, which means that the model is applicable when the penetration depth $x$ is thin compared to the total liquid film thickness $\delta$ (Higbie, 1935). Under this assumption, the oxygen transfer in the liquid film takes place only close to the gas/liquid interface. Thus, the oxygen concentration $C_{O_2,L}(x = \delta)$ at the flask wall remains 0 mol/m³. To prove whether Higbie’s theory can be applied to model the oxygen transfer into a liquid film in a shake flask, the oxygen concentration profile in the liquid film needs to be investigated.

### 2.1.3 Fick’s diffusion law for simulation of gas transfer inside a liquid film

Fick’s law can be applied to model gas diffusion inside a liquid film. The differential Equation 2.5 describes oxygen concentration as a function of penetration depth $x$ and time $t$. It includes the reaction rate $r(x)$, which is specified in Equation 2.6 for a first-order reaction with the reaction constant $k_1$.

$$\frac{\partial C_{O_2,L}}{\partial t} = D_{O_2} \frac{\partial^2 C_{O_2,L}}{\partial x^2} - r(x) \quad 2.5$$

$$r(x) = k_1 C_{O_2,L} \quad 2.6$$

In the case of culture medium with oxygen-consuming microorganisms, the reaction rate in Equation 2.5 can be modeled utilizing the Monod kinetics (Monod, 1949) as follows:
THEORETICAL BACKGROUND - Gas/Liquid Mass Transfer in Shake Flasks

\[ r(x) = \mu_{\text{max}} \frac{X}{Y_{X/O_2}} \frac{c_{O_2,L}}{K_s + c_{O_2,L}}. \]  \hspace{1cm} 2.7

Equation 2.7 comprises the maximum growth rate \( \mu_{\text{max}} \), the biomass concentration \( X \), the biomass yield for oxygen \( Y_{X/O_2} \) and the Monod coefficient for oxygen \( K_s \). Together with the three boundary conditions listed below (see also Figure 2.1), the inhomogeneous differential Equation 2.5 can be solved e.g. by the software “gPROMS” (Process Enterprise Ltd., London, UK).

\[ C_{O_2,L}(x = 0) = C_{O_2,L}^* \]

\[ \left[ \frac{\partial C_{O_2,L}}{\partial x} \right]_{x = \delta} = 0 \]

\[ C_{O_2,L}(t_{\text{exp}} = 0, x) = C_{O_2,B} \approx 0 \]  \hspace{1cm} 2.8

The latter equation \( C_{O_2,L}(t_{\text{exp}} = 0, x) \approx 0 \) describes the concentration of oxygen in the liquid film directly after the film was formed by the bulk liquid. At this moment, when the bulk liquid forms a new liquid film, the oxygen concentration \( C_{O_2,L} \) in the liquid film is equal to the bulk liquid concentration \( C_{O_2,B} \) (0 mol/m³). The oxygen flux across the gas/liquid interface \( \dot{n}_{O_2} \) [mol/s] can be calculated with Equation 2.9 for any given time point \( t \) within the total exposure time \( t_{\text{exp}} \) during one shake flask rotation.

\[ \dot{n}_{O_2}(x = 0, t) = \left[ \frac{\partial C_{O_2,L}}{\partial x} \right]_{x = 0, t} A_F D_{O_2} \]  \hspace{1cm} 2.9

The term \( A_F \) indicates the mass transfer area of the liquid film. Equation 2.10 gives the mean oxygen flux value over the whole liquid film surface during the exposure time \( t_{\text{exp,max}} \). In this study, the exposure time at any liquid height of the film was assumed to be equal to the exposure time \( t_{\text{exp,max}} \) at the maximum liquid height \( h_{\text{max}} \) (Figure 2.1 A). This simplification was chosen to circumvent a complex function of liquid film height, bulk liquid geometry and exposure time. Especially the geometry of the bulk liquid is influenced by a number of parameters like shaking frequency, shaking diameter, filling volume and viscosity. The simplification \( t_{\text{exp}} = t_{\text{exp,max}} \) leads to lower values for
the calculated oxygen flux compared to the real oxygen flux. This is due to the fact that longer exposure times allow longer accumulation of oxygen in the liquid film, until the liquid film is renewed by the bulk liquid. The resulting reduced average oxygen concentration gradient between gas and liquid reduces the oxygen flux.

\[
\bar{n}_{O_2} = \int_{t=0}^{t=\text{exp,max}} \bar{\omega}_{O_2}(x = 0, t) dt \tag{2.10}
\]

Oxygen flux and maximum oxygen transfer capacity of the liquid film \(OTR_{max,F}\) are related by \(\bar{n}_{O_2} = OTR_{max,F} \cdot V_{L,F}\). Herein, the term \(V_{L,F}\) indicates the liquid film volume.

Calculations based on the theory described in Section 2.1 were conducted by my former colleague A. Azizan (Giese et al., 2014a) with the use of a “gProms”-model previously developed at AVT.BioVT (Maier et al., 2004).

## 2.2 Power Input to Shake Flasks and Stirred Tanks

### 2.2.1 Newton, Reynolds and Phase numbers in bioreactors

Büchs et al. (2001) evaluated the power input into shake flasks during the shaking process, and found a correlation between the modified Newton number \(Ne'\) and the Reynold’s number \(Re\), as defined by Equation 2.11. The modified \(Ne'\) number was derived by Büchs et al. (2000b) representing the power input \(P\) into shake flasks. The definitions of the \(Ne'\) and \(Re\) numbers for shake flasks are given by Equation 2.12.

\[
Ne' = 70 Re^{-1} + 25 Re^{-0.6} + 1.5 Re^{-0.2} \tag{2.11}
\]

\[
Ne' = \frac{P}{\rho \ n^3 \ a^4 \ V_{L,F}^{1/3}} ; \quad Re = \frac{\rho \ n \ a^2}{\eta_{app}} \tag{2.12}
\]

The three \(Re\) number terms in Equation 2.11 are related to the laminar \((Re^{-1})\), transitional \((Re^{-0.6})\) and turbulent \((Re^{-0.2})\) flow regimes, respectively, which are indicated in Figure 2.2 (dashed curve). In contrast to shake flasks and un baffled stirred tanks, the \(Ne\) number of baffled stirred tanks remains constant within the turbulent flow regime (Figure 2.2). In the case of a baffled stirred tank equipped with Rushton turbines,
turbulence is indicated by a Re number larger than $10^4$ (Zlokarnik, 2001). By contrast, the critical Re number for turbulent fluid flow in unbaflled shake flasks and unbaflled stirred tanks is higher. With approx. $6 \cdot 10^4$ (Peter et al., 2006) and $5 \cdot 10^4$ (Zlokarnik, 2001) these critical Re numbers are in a similar range.

Figure 2.2. Qualitative impact of the Re number on the power input into baflled and unbaflled stirred tanks with Rushton turbines (solid lines; Rushton et al., 1950; Zlokarnik, 2001), as well as into unbaflled shake flasks (dotted line; Büchs et al., 2000b). The power input is represented by the conventional Ne number for stirred tanks and the modified Ne’ number for shake flasks, respectively. The critical Re number for turbulence is $10^4$ in baflled stirred tanks, $5 \cdot 10^4$ in unbaflled stirred tanks (Rushton turbines; Zlokarnik, 2001), and $6 \cdot 10^4$ in unbaflled shake flasks (Peter et al., 2006). Reprint from Giese et al. (2014c) with permission from Wiley-Blackwell.

For shake flasks, Büchs et al. (2001) observed the so-called “out-of-phase” phenomenon, which leads to reduced power input into the shaken liquid. In the so-called "in-phase" mode the liquid bulk circulates "in-phase" with the shaker drive; in the "out-of-phase" mode, the largest part of the liquid bulk remains at the shake flask’s bottom. Such "out-of-phase" operating conditions are unfavorable for fermentations because mixing and mass transfer are strongly reduced. According to Büchs et al. (2001) a Phase number ($Ph$) larger than 1.26 is an indicator for "in-phase" operation. The $Ph$ number can be
calculated by the use of Equation 2.13, which involves the maximum shake flask diameter $d$, and the shaking diameter $d_0$ as the most influential parameter.

\[ P_h = \frac{d_0}{d} \left[ 1 + 3 \log_{10} \left( \frac{Re \cdot \pi}{2} \left[ 1 - \sqrt{1 - \frac{4}{\pi (V_L \frac{1}{3} / d)^2}} \right] ^2 \right) \right] > 1.26 \quad 2.13 \]

### 2.2.2 Effective shear rates in bioreactors

The effective shear rate in a stirred tank is commonly described using the stirrer frequency concept devised by Metzner and Otto (1957) and Calderbank and Moo-Young (1959) (Equation 2.14). For laminar flow regimes ($Re < 10^1$) Metzner and Otto found a linear relation between stirrer frequency $n$ and the average shear rate $\dot{\gamma}_{eff}$ in a stirred tank, which are correlated via the Metzner and Otto constant $K_{MO}$.

\[ \dot{\gamma}_{eff} = K_{MO} \cdot n \quad 2.14 \]

Due to the fact that transitional and turbulent flow regimes are commonplace in industrial applications, the Metzner and Otto stirrer frequency concept was also widely applied to these process conditions, although the justification of this questionable approach was never proven. As discussed below, this leads to deficient predictions (Rieger and Novak, 1974) with two major problems: First, the Metzner and Otto constant $K_{MO}$ does not consider the rheological properties of the fermentation broth, despite their obvious influence on the effective shear rate in bioreactors (Ayazi Shamlou and Edwards, 1985; Sánchez Pérez et al., 2006). Second, the volumetric power input and not the stirrer frequency characterizes gas/liquid mass and heat transfer in a bioreactor (Sumino et al., 1972; Henzler and Kauling, 1985). Consequently, the Metzner and Otto stirrer frequency concept applied for the transitional or turbulent flow regime results in discrepancies during scale-up procedures (Rieger and Novak, 1974). This is illustrated for a baffled stirred tank by Equation 2.15 in the following example: If the stirrer frequency $n$ is considered to be equal on two different scales – as demanded by the Metzner and Otto stirrer frequency concept – and both, geometric similarity ($V_L \sim d^3$) and
turbulent flow conditions ($Ne = \text{const.}$, see Figure 2.2) are prevailing, the volumetric power input increases squared with tank diameter:

$$P = Ne \cdot \rho \cdot n^3 \cdot d^5 \sim d^5 \rightarrow \frac{P}{V_L} \sim d^2. \quad 2.15$$

As can be seen from Equation 2.15, the assumption of constant stirrer frequency leads to predictions of unattainable high power inputs in large scale stirred tank reactors. For instance, when a 50 L stirred tank ($d = 0.317$ m) operated at 1 kW/m$^3$ is scaled-up to 100 m$^3$ ($d = 4$ m), a constant stirrer frequency would result in a utopic volumetric power input of 159 kW/m$^3$. A similar example, disclosing the discrepancies occurring during scaling procedures, was presented by Böhme and Stenger (1988).

Henzler and Kaiserling (1985) consistently described effective shear rates in stirred tanks and bubble columns introducing the so-called power input concept. It implies that rather than stirrer frequency, the volumetric power input and the resulting shear rate are the decisive parameters when mass and heat transfer phenomena are scaled up (cited in Kawase and Kumagai, 1991; Flores Candia and Deckwer, 1999; Herbst et al., 1992; Merchuk and Garcia Camacho, 2013). In contrast to the Metzner and Otto stirrer frequency concept, the power input concept does not result in a change of the apparent viscosity when a process is scaled up. As shown in Equation 2.16, the power input concept is the outcome of a Dimensional analysis based on the assumption that the decisive shear rate is a function of only the viscosity and the volumetric power input.

$$\dot{\gamma}_{eff} = L \left( \frac{P}{V_L \eta_{app}} \right)^\frac{1}{2} = L \left( \frac{P}{V_L \eta_{app}} \right)^\frac{1}{2} \left( \frac{P}{V_L K} \right)^\frac{1}{m+1} \quad 2.16$$

If the viscosity is replaced by the Ostwald-de Waele law (Equation 3.2), the power input concept is applicable for pseudo-plastic fluids by using the fluid consistency index $K$ and the flow behavior index $m$. In a set of model liquids, the constant factor $L$ was found to be approx. 1 (Henzler, 2007).

When replacing the stirrer power by $P = Ne \cdot Re \cdot n^3 \cdot d^5$, Equation 2.16 can be converted to Equation 2.17. Since the product $Ne \cdot Re$ is constant under laminar flow conditions (Figure 2.2), interestingly the power input concept simplifies to the Metzner and Otto stirrer frequency concept because $\dot{\gamma}_{eff}$ is proportional to the stirrer frequency in this
case, as stated in Equation 2.17. Therefore, the validity of the Metzner and Otto stirrer frequency concept is verified for the laminar flow regime, whereas for the transitional and turbulent regime the more general power input concept has to be used.

\[
\dot{\gamma}_{\text{eff}} = L \left( \frac{d^3}{V_L} \cdot Ne \cdot Re \right)^{\frac{1}{2}} \cdot n \tag{2.17}
\]

As yet, no study has conducted a systematic investigation of the effective shear rate in shake flasks. Due to this lack of knowledge, shear rates such as 12 s\(^{-1}\) or 68 s\(^{-1}\) have been selected by scientists in the past, solely based on the default settings of the applied viscometers (Burns et al., 1994; Byrne and Ward, 1987; Madi et al., 1997; Peña et al., 1997). By observing transition to the “out-of-phase” operating conditions, Peña et al. (2007) were able to identify an effective shear rate of 90 s\(^{-1}\) at a certain time point during their fermentation. Peter et al. (2004) introduced a practical approach for effective shear rate estimation in shake flasks. For a small spectrum of operating conditions (500 mL shake flask, 70 mm shaking diameter, 30 - 50 mL filling volume and 180 - 340 rpm shaking frequency) effective shear rates of between 116 s\(^{-1}\) and 553 s\(^{-1}\) were found.
3 MATERIALS AND METHODS

3.1 Liquid Films on Shake Flasks Walls

3.1.1 Applied sodium sulfite system
Sodium sulfite is used to simulate the oxygen consumption of a microbial system and to determine the $OTR_{max}$ as a lumped parameter of oxygen transfer into bulk and liquid film. The method is based on the following oxidation reaction of sulfite to sulfate; catalyzed by cobalt ions:

$$SO_3^{2-} + 0.5 \text{O}_2 \rightarrow SO_4^{2-}.$$

To measure the $OTR_{max}$ with the sulfite system, the reaction kinetics have to be adjusted to assure a “non-accelerated” reaction regime. This regime is characterized by reaction kinetics allowing only negligible reaction of oxygen in the liquid boundary layer (Hermann et al., 2001). Like in biological cultures, the by far predominant part of oxygen is consumed in the mixed bulk liquid. The “non-accelerated” regime can be achieved by using a cobalt sulfate concentration of $10^{-7}$ M. Further details regarding the sulfite oxidation are presented e.g. by Linek and Vacek (1981), Hermann et al. (2003) and Maier et al. (2004).

A 0.35 M sodium sulfite solution ($\geq$98%, Roth, Karlsruhe, Germany) is used as a chemical model for laboratory experiments. The solution was adjusted to pH 8 with H$_2$SO$_4$ ($\geq$30%, Applichem, Darmstadt, Germany). A 0.012 M phosphate buffer prepared in deionized water was used (Na$_2$HPO$_4$ $\geq$99%; NaH$_2$PO$_4$ $\geq$98%; Roth). The reaction was catalyzed by $10^{-7}$ M cobalt sulfate from Roth.

Several viscosities ranging from 1 mPa·s to 225 mPa·s were adjusted using polyvinyl-pyrolidone (PVP) with concentrations of 0 g/L to 100 g/L (Luviskol, BASF AG, Ludwigshafen, Germany). The experiments were conducted in 250 mL shake flasks with the Respiration Activity Monitoring System (RAMOS) described by Anderlei and Büchs (2001) and Anderlei et al. (2004). Filling volumes of 20, 30 and 40 mL as well as shaking frequencies of 200, 250 and 300 rpm were applied. Temperature and shaking diameter were constant at 25°C and 50 mm, respectively.
3.1.2 Microbial strain, medium and fermentation conditions

Corynebacterium glutamicum fermentation

*Corynebacterium glutamicum* DM1730 is a recombinant strain derived from ATCC 13032. It contains mutations described in Ohnishi et al. (2002) and Georgi et al. (2005). Experiments were conducted in RAMOS with 250 mL shake flasks. The pre-cultures were prepared with a complex medium (see below) at a shaking frequency of 350 rpm and a filling volume of 12.5 mL. The main cultures were inoculated with 5% pre-culture in the exponential growth phase and cultivated in minimal medium (see below) at various shaking conditions. Temperature and shaking diameter were kept constant at 30°C and 50 mm, respectively. For each experiment a specific viscosity was adjusted with PVP.

The complex medium used for the pre-culture of *C. glutamicum* contained: 20 g/L glucose, 10 g/L yeast extract, 10 g/L peptone (both Roth, Karlsruhe, Germany), 2.5 g/L NaCl and 0.25 g/L MgSO₄·7H₂O. The minimal medium for the main culture of *C. glutamicum* contained: 15 g/L glucose, 10 g/L (NH₄)₂SO₄, 2 g/L urea, 2 g/L K₂HPO₄, 1 g/L KH₂PO₄, 0.25 g/L MgSO₄·7H₂O, 30 mg/L (HO)₂C₆H₃COOH [3,4-dihydroxybenzoic acid], 10 mg/L CaCl₂, 0.2 mg/L C₁₀H₁₆N₂O₃S [D(+)-biotin], 21 g/L C₇H₁₅NO₄S [MOPS]. Trace elements: 10 mg/L MnSO₄·H₂O, 10 mg/L FeSO₄·7H₂O, 1 mg/L ZnSO₄·7H₂O, 0.2 mg/L CuSO₄, 0.02 mg/L NiCl₂·6H₂O. The initial pH-value was adjusted with 5 M NaOH to a pH of 7. The following components were filter-sterilized: trace elements (adjusted to pH 1 with H₂SO₄), urea, (HO)₂C₆H₃COOH (dissolved in 10% of NaOH), CaCl₂ and C₁₀H₁₆N₂O₃S (dissolved in 50% C₃H₈O).

*Experiments described in Sections 3.1.1, 3.1.2 and in the following Section 3.1.3 were conducted by my former colleague A. Azizan (Giese et al., 2014a).*

3.1.3 Determination of viscosity and oxygen solubility

In the 0.35 M sodium sulfite solution as well as in the culture medium, the viscosity and, thus, the liquid film thickness were varied with different concentrations of PVP. The rheological behaviors of the chemical and microbial systems were determined with a cone-and-plate rheometer (Rheoplus MC1, Paar Physica, Stuttgart, Germany) at 25°C.
MATERIALS AND METHODS - Liquid Films on Shake Flasks Walls

Figure 3.1 A. Measured fluid consistency index \( K \) (triangles) and flow behavior index \( m \) (circles) of Ostwald-de Waele law at 25°C (range for determination: 100 s\(^{-1}\) to 3000 s\(^{-1}\)). B: Measured oxygen solubility for PVP-enriched 0.35 M sulfite system (squares) with least square fit (dashed line) as well as apparent viscosity of PVP-enriched 0.35 M sulfite system and culture medium (solid curve) using the parameters of Figure 3.1 A at a shear rate of 300 s\(^{-1}\). Data points in Figure 3.1 A were generated from the culture medium as well as the 0.35 M sodium sulfite system, and each curve represents a least square fit of all measured data determined by both liquid systems. This figure was developed together with my former colleague A. Azizan. Reprint from Giese et al. (2014a) with permission from Wiley-Blackwell.
MATERIALS AND METHODS - Liquid Films on Shake Flasks Walls

To describe the rheological behavior of the non-Newton fluids applied in this study, the Ostwald-de Waele law, shown in Equation 3.2, was used (Blanch and Bhavaraju, 1976; Goudar et al., 1999). The fluid consistency index $K$ and the flow behavior index $m$, depicted in Figure 3.1 A, were determined between shear rates $\dot{\gamma}$ of 100 s$^{-1}$ to 3000 s$^{-1}$.

$$\eta = K \dot{\gamma}^{m-1}$$  \hspace{1cm} 3.2

The apparent viscosity was calculated for a shear rate of 300 s$^{-1}$. As Figure 3.1 B illustrates, there is an exponential increase in the viscosity from 1 mPa·s to 225 mPa·s at increasing concentrations of PVP up to 100 g/L.

The standard iodometric titration method developed by Winkler (1888) was used to determine the oxygen solubility $L_{O_2}$ in the 0.35 M sodium sulfite system at a temperature of 25°C. As shown in Figure 3.1 B, the PVP-enriched sodium sulfite system resulted in a linear decreasing oxygen solubility with increasing PVP-concentration $C_{PVP}$.

3.1.4 Apparatus for liquid film thickness estimation

Up to now, no study is published which quantifies the liquid film thickness at shake flask walls. To obtain a first rough estimate of the liquid film thickness $\delta$ during the shaking process, non-invasive fluorescence measurements were performed by Hermann (2001) with the measuring setup shown in Figure 3.2. The fluorescence intensity of fluorescein-sodium salt (Sigma-Aldrich, Frankfurt, Germany), emitted by the liquid film on a shake flask wall, was measured in a rotating shake flask. To stabilize the fiberoptic cable, a clamp was used to connect the cable to the flask wall. During shaking, a blue LED was used to induce fluorescence. The emission signal was conducted to a photomultiplier.
Figure 3.2. Noninvasive optical estimation of liquid film thickness (Hermann, 2001) at the wall of a shaken flask. Fluorescence was induced by a blue LED with a fiber optic cable. A blue band-pass filter (BG 12, Schott Glas, Mainz, Germany) with a transmission range of 350 nm to 500 nm was applied. Fluorescence emission was conducted to a photomultiplier tube (PMT) (custom-made, Fa. PreSens, Regensburg, Germany) and measured above 570 nm with an edge-pass filter (OG570, Schott Glas). The measured signal was amplified (SA830DSP, Scientific Instruments, Gilching, Germany) and recorded by a PC with a sampling frequency of 0.2 ms. A Hall effect sensor was used to monitor the shaker table position during shaking. Reprint from Giese et al. (2014a) with permission from Wiley-Blackwell.
3.2 Effective Shear Rates in Shake Flasks

3.2.1 Method for experimental determination of the effective shear rate

For the experimental determination of the effective shear rate in shake flasks, Peter et al. (2004) introduced a practical approach which is illustrated in Figure 3.3. In the first step, the volumetric power input was measured for shake flasks filled with a Newtonian liquid ($m = 1$) and those filled with a pseudo-plastic liquid ($m < 1$). The volumetric power input was measured for various shaking frequencies with a fixed filling volume and a fixed shaking diameter. Figure 3.3 (below) shows typical schematic power input curves as a function of shaking frequency for the two different liquids. In comparison to that of the Newtonian liquid, the volumetric power input of the pseudo-plastic liquid increases to a lesser extent. Thus, if suitable liquids are chosen, an intercept point can be identified at which shaking frequency and volumetric power input are equal.

Since all process parameters are equal at the determined intercept point for the power input, the apparent viscosities of both hypothetical liquids must also be equal (Metzner and Otto, 1957; Peter et al., 2004). In the second step, the apparent viscosity was determined by flow curve determination in a rheometer (Figure 3.3, top). If the flow curves of the Newtonian and the pseudo-plastic liquid intercept, the apparent viscosity is equal to the shear-independent Newtonian viscosity. Since only one intercept point can exist between a Newtonian and a pseudo-plastic liquid, the apparent viscosity and the effective shear rate are herewith identified for one specific shaking frequency and the corresponding volumetric power input. A large number of experiments involving a huge variety of different viscosities, shaking frequencies, shaking diameters and nominal flask volumes must be conducted in order to achieve comprehensive insight as to their impact on the effective shear rate.

3.2.2 Online volumetric power input measurement

The online power input into shake flasks was measured using a shaker with power input measurement as illustrated in Figure 3.3. The power input shaker was described in detail by Büchs et al. (2000a). A motor drive (Visco-Pakt rheo-110, Hitec Zang GmbH, Herzogenrath, Germany) enables both, the adjustment and control of the shaking frequency as well as automated and continuous torque readout. Torque was recorded at a
sampling rate of 5 s\(^{-1}\) during each experiment. Prior to each experiment, reference measurements with liquid replaced by weights were conducted in order to determine the influence of mechanical friction losses and wind resistance. Hence, the pure torque values, resulting from the friction between liquid and shake flask wall, could be determined. The online torque signal \(M\) was converted into the online volumetric power input \(P/V_L\) using \(P = M \cdot (2\pi \cdot n)\).

![Diagram illustrating the determination of the effective shear rate in shake flasks.](image)

Figure 3.3. Schema illustrating the determination of the effective shear rate in shake flasks. The schema illustrates the viscosity measurement of a Newtonian fluid (solid lines) and a pseudo-plastic fluid (dashed lines) carried out using a rheometer (above). The online volumetric power input into shake flasks is measured via a shaker with power input measurement (below; Büchs et al., 2000a). The associated intercept points for viscosity and power input together provide one data point for the function between shear rate and shaking frequency (rightmost). To cover a broad spectrum of shear rates and shaking frequencies, the intercept points for various liquids must be investigated. *Reprint from Giese et al. (2014c) with permission from Elsevier.*
3.2.3 Rheometer measurements and viscous model solutions

The viscous flow behaviors of the analyzed model liquids and fermentation broths were measured in Physica Rheolab MC1 portable and Physica MCR 301 rheometers (both from Anton Paar Physica, Stuttgart, Germany) in a range of shear rates between 0.1 s\(^{-1}\) and 3000 s\(^{-1}\). The Physica Rheolab MC1 portable device was equipped with cone 91/6 (cone angle 1°, cone truncation 25\(\mu\)m) and the Physica MCR 301 with cone 189779 (cone angle 0.467°, cone truncation 54 \(\mu\)m). Viscous fermentation broths with filaments were analyzed using the Physica MCR 301 rheometer equipped with a beaker-and-cylinder device (beaker: #CC27 with 28.92 mm diameter, cylinder: #CC25 with 25 mm diameter) or a plate-and-plate device (#PP50/TG with 49.95 mm plate diameter). The measurements were performed at the respective fermentation temperature or at 25°C in case of the model solutions.

Two polymers and four oils were employed to obtain several pseudo-plastic and Newtonian model solutions with different flow behaviors and viscosities. The pseudo-plastic flow behavior was varied using different concentrations of xanthan gum (#95465, Fluka, Basel, Switzerland and #G1253, Sigma-Aldrich, St. Louis, USA). Xanthan gum is a biopolymer consisting of molecules with various chain lengths (Galindo, 1994). As mole masses differ depending on lot and manufacturer (Torres et al., 1993), viscosity was measured prior to each experiment. The viscosity of Newtonian liquids was adjusted using polyvinylpyrrolidone (PVP) (Luviskol, BASF AG, Ludwigshafen, Germany). Completely Newtonian flow behavior was observed for olive oil (Bertolli, Inveruno, Italy), linseed oil (Bio-Zentrale GmbH, Wittibreut-Ulbering, Germany), safflower oil (Bröckelmann+Co, Hamm, Germany) and sunflower oil (Rewe, Cologne, Germany).

3.2.4 Microbial strains, media and fermentation conditions

*Trichoderma reesei* QM9414 was fermented in medium with 40 g/L glucose as a carbon source. The remaining medium components, inoculation procedure and strain maintenance are described in Section 3.4.3. Process conditions were as follows: 250 mL shake flask, 25 mL filling volume, 200 rpm shaking frequency and 50 mm shaking diameter and 28°C. Under these conditions, *T. reesei* QM9414 grew filamentous, forming small agglomerates. Alginate producing *Azotobacter vinelandii* (ATCC9046) was fermented in a modified Burk’s medium with 20 g/L sucrose at 29°C. Medium,
MATERIALS AND METHODS - Effective Shear Rates in Shake Flasks

inoculation procedure and strain maintenance are described in Peña et al. (1997) and Peña et al. (2007). The cultures were shaken in 500 mL shake flasks with 100 mL medium at 200 rpm and 25 mm shaking diameter. The xanthan-producing Xanthomonas campestris pv. campestris B100 (Vorhölter et al., 2008) was fermented in 500 mL shake flasks with 20 mL filling volume at 200 rpm, 25 mm shaking diameter and 30°C. The pre-culture was performed with 20 mL filling volume at 300 rpm and 50 mm shaking diameter for 24 h with the pre-culture medium containing per liter: 15 g glucose, 10 g yeast extract, 3 g malt extract and 3 g peptone (all from Roth, Karlsruhe, Germany). A ratio of 1:20 was used to inoculate the main culture. The main culture medium contained per liter: 25 g yeast extract, 20 g sucrose, 5 g glucose, 2 g K$_2$HPO$_4$, 0.1 g MgSO$_4$ and 50 g MOPS buffer (acid form, C$_7$H$_{15}$NO$_4$S) (all from Roth). For strain maintenance X. campestris was stored in glycerol (50% v/v) at -80°C.

All cultures were fermented on a power input shaker (Büchs et al., 2000a). T. reesei and X. campestris cultures were additionally monitored using a RAMOS device to measure the oxygen transfer rate $OTR$. For offline viscosity measurements, cultures were also performed in a number of parallel flasks. Withdrawn parallel flasks for sampling were not placed back onto the shaker. Experiments were performed in at least duplicates.
3.3 A New Microtiter Plate Concept

3.3.1 Applied conventional microtiter plates for comparison

For comparison of the $OTR_{max}$ in the newly developed and the conventional MTPs, the following widely applied commercial MTPs were used:

- 96-Well-MTP Rotilabo®-Mikrotest, flat-bottom, hydrophobic (#9293.1, Roth, Karlsruhe, Germany)
- 48-Well-MTP Cellstar® suspension culture plate, flat-bottom, hydrophobic (Greiner bio-one, Kremsmünster, Austria, #677102)
- 24-Well-MTP Costar®, flat-bottom, hydrophobic (#3738, Corning, USA)
- 24-Well-MTP Costar®, flat-bottom, hydrophilic (#3524, Corning, USA)

3.3.2 Applied sodium sulfite system

A 1 M sodium sulfite solution (≥98%, Roth, Karlsruhe, Germany) with a 0.1 M phosphate buffer was used for the determination of the $OTR_{max}$. The residual solution was prepared as described in Section 3.1.1. The temperature was adjusted to 22.5°C.

3.3.3 Microbial strain and medium

*Escherichia coli* BL21 pRset eYFP-IL6 was used for fermentation in the new and in the conventional MTP types. The strain is described in detail by Samorski et al. (2005) with an additional gene dLys. In case of these microbial fermentations, the MTPs were covered with a gas-permeable adhesive sealing tape (#AB-0718, Thermo Scientific, Waltham, USA). The TB medium used for pre-culture and main culture contained: 24 g/L yeast extract, 5 g/L glycerin, 12 g/L tryptone (all from Roth), 12.54 g/L $K_2HPO_4$, 2.31 g/L $KH_2PO_4$ (both Merck, Darmstadt, Germany) and 0.1 g/L Na-Ampicillin.

The pre-culture was fermented in shake flasks with 10 mL filling volume at 350 rpm and 50 mm shaking diameter. It was stopped in the exponential growth phase at 60 mmol/L/h. In case of biocompatibility experiments, the pre-culture was centrifuged. The obtained pellet was resuspended and concentrated in fresh medium (pellet from 80 mL pre-culture medium in 60 mL fresh medium).

Most data points were measured in duplicates during one experimental run.
3.4 Scale-Down from Shake Flask to Microtiter Plate

3.4.1 Applied sodium sulfite system

A 1 M sodium sulfite solution (≥98%, Roth, Karlsruhe, Germany) with a 0.1 M phosphate buffer was used to simulate the oxygen consumption of \( T.\ reesei \) and to determine the \( OTR_{\text{max}} \) of shake flasks and 24-well MTPs. The residual solution was prepared as described in Section 3.1.1. During the experiments, the temperature was adjusted to 22.5°C.

\[
f = \frac{OTR_{\text{max, medium}}}{OTR_{\text{max, sulfite}}} \tag{3.3}
\]

The proportionality factor \( f \), shown in Equation 3.3, is defined as the quotient of the \( OTR_{\text{max}} \) of a microbial culture (\( OTR_{\text{max, medium}} \)) and the \( OTR_{\text{max}} \) of a sodium sulfite system (\( OTR_{\text{max, sulfite}} \)). In order to determine \( f \), the \( OTR_{\text{max, medium}} \) and the \( OTR_{\text{max, sulfite}} \) have to be measured under equal shaking conditions. The factor \( f \) is different from unity, since the oxygen solubility and the oxygen diffusivity depend on the chemical composition of the liquid. Since the oxygen solubility and diffusivity are specific for the applied culture media, the proportionality factor \( f \) usually ranges between 1 and 2.8 for common fermentation systems (Klöckner and Büchs, 2011).

3.4.2 Experimental set-up

The fermentations and sodium sulfite experiments were conducted in a RAMOS device. Commercial shake flask devices are available from Kühner AG (Birsfelden, Switzerland) and Hitec Zang GmbH (Herzogenrath, Germany). The RAMOS-principle was adapted to MTPs and has been successfully used for several investigations described in Hermann et al. (2001), Kenty et al. (2005) and Scheidle et al. (2010). This microtiter plate RAMOS device is commercially available from Kühner AG (Birsfelden, Switzerland). Figure 3.4 shows the assembly of one MTP with the special measuring lid. The MTP and lid form the boundary for material balancing. It must be pointed out that \( OTR, CTR \) and \( RQ \) are measured for the whole plate and not for each individual well. Therefore, the microtiter plate RAMOS device is not a tool for screening of each individual well, but is very
helpful for identifying suitable operating conditions in MTPs – e.g. to adjust the oxygen supply.

The experiments were conducted in a RAMOS device equipped with 24-well MTPs (Costar® #3526; Corning, USA). In cases of microbial fermentations, the MTPs were covered with gas-permeable adhesive sealing tapes (#AB-0718, Thermo Scientific, Waltham, USA). Filling volumes and shaking frequencies were varied. Temperature and shaking diameter were kept constant at 22.5°C and 50 mm, respectively. All experiments were conducted at least in duplicates. Sulfite $OTR_{max}$-results for shake flasks have been obtained under the same conditions and were already presented by Maier et al. (2004) and Seletzky et al. (2007).

Figure 3.4. Schematic sketch of a microtiter plate RAMOS device. The sealed MTP is covered by a special lid containing the $pO_2$-sensor, the gas inlet- and outlet-valves as well as a ventilator with an eccentric weight which is driven by the orbital shaking motion. This experimental set-up measures the $OTR$ of the MTP as a whole. The RAMOS principle with its gassing method is described by Anderlei and Büchs (2001) and Anderlei et al. (2004). Reprint from Giese et al. (2014b) with permission from Elsevier.

### 3.4.3 Microbial strain, medium and fermentation conditions

The recombinant fungal strain *Trichoderma reesei* GA8 expressing native glucoamylase was provided by Genencor International B.V. (today: DuPont Industrial Biosciences) and was fermented in this study. To construct this strain, the *T. reesei* host strain GICC20000150 derived from strain RLP3740 by sequential deletion of the genes encoding the four major secreted cellulases (cel7a, cel6a, cel7b, cel5a) (Schneider et al.,
2012) was transformed with a PCR fragment amplified from the expression vector pTTTpyrG-GA. The latter was obtained by cloning of the *T. reesei* *gla1* gene into the destination vector pTTTpyrG13 (Aehle, W., et al., patent WO 2010141779 A1, 2010) via Gateway recombination cloning (Life technology, Carlsbad, USA). The PCR fragment encompassed the *Aspergillus nidulans* gene *pyrG* together with the *T. reesei* glucoamylase gene under the control of *T. reesei* Cbh1 promoter and terminator sequences. *T. reesei* transformants were selected on minimal medium for uridine prototrophy and stable ones were checked for production of glucoamylase directly secreted into the culture medium.

For inoculation, 5·10⁶ scraped spores/mL from agar plates were mixed into the culture medium. The agar plates were prepared with 26.5 g/L potato-dextrose bouillon for yeast and fungal cultures (Roth, Karlsruhe, Germany, CAS 9002-18-0) and 16 g/L agar powder for bacteriology and mycology (Roth, #CP74). Submerged fermentation experiments were conducted in a RAMOS device equipped with 250 mL shake flasks or 24-well MTPs (Costar® #3526) at 28°C and a shaking diameter of 50 mm. The microbial experiments were run at least in quadruplicates. For strain maintenance *T. reesei* GA8 spores were stored on a 1 cm² agar piece immerged in glycerol (50% v/v) at -80°C. Spores for maintenance and fermentations were taken from agar plates which had been placed under a conventional 60 W incandescent light bulb at 28°C for 10 days (Ellison et al., 1981).

The culture medium 1 for standard fermentation contained per liter: 9.4 g casamino acids (Bacto, Mt Pritchard, Australia), 5.2 g (NH₄)₂SO₄, 4.7 g KH₂PO₄, 1.0 g MgSO₄·7H₂O, 1.0 g CaCl₂·2H₂O, 34.3 g PIPPS buffer (Piperazine-N,N′-bis(3-propanesulfonic Acid), Merck, Darmstadt, Germany) and 2.6 mL trace elements (see below). A volume of 800 mL was adjusted to pH 5.5 with NaOH (4 mol/L) and filled up to 900 mL with deionized water. The medium was filter-sterilized and completed with 35.5 mL heat-sterilized saccharide-syrup. Among other sugars, the saccharides-syrup contains 562 g/L glucose and 15.6 g/L sophorose, leading to a concentration of 20 g/L glucose and 0.55 g/L sophorose in medium 1. Culture medium 2 was modified compared to medium 1 as follows: 61.5 mL saccharide-syrup was used, resulting in 36 g/L glucose and 1 g/L sophorose in medium 2. Furthermore, the concentration of (NH₄)₂SO₄ was doubled. The trace elements contained per liter: 175 g citric acid (anhydrous), 200 g
FeSO₄·7H₂O, 16 g ZnSO₄·7H₂O, 3.2 g CuSO₄·5H₂O, 1.4 g MnSO₄·H₂O and 0.8 g H₃BO₃. The trace elements were filter-sterilized and stored at 4°C.

3.4.4 Assays for enzyme activity and qualitative concentration

Glucoamylase activity was measured with a pNPG-assay (4-Nitrophenyl α-D-glucopyranoside). For each sample, merged culture broth from four wells of a 24-well MTP or one shake flask was centrifuged. A volume of 5 µL supernatant or 5 µL of purified glucoamylase standard was transferred into each well of a 96-well flat-bottom MTP (Roth, #9293.1). Supernatant and glucoamylase standard were buffered with 20 µL of 50 mM NaAc buffer (pH 4.3). As an artificial substrate, 25 µL of 25 mM pNPG (Sigma Aldrich, St. Louis, USA) in 100 mM NaAc (pH 4.3) was applied. The dilutions were incubated for 30 min at 45°C and 900 rpm on a Thermomixer HLC MHR 23 (HLC Ditabis AG, Pforzheim, Germany). The reaction was stopped with 100 µM Borax (sodium tetraborate decahydrate, Sigma Aldrich). A volume of 100 µL was transferred to each well of a 96-well flat-bottom MTP. Absorbance was measured at 450 nm in a microtiter plate reader (BioTek Synergy 4 Hybrid, BioTek, Winooski, USA). The pNPG-assay was performed in duplicates. Since water evaporates during fermentation, measured active glucoamylase concentrations were recalculated to the original volume to take evaporation into account.

A volumetric SDS-gel electrophoresis was applied to compare entire protein concentrations in shake flasks and 24-well MTPs. The SDS-device (Invitrogen, Carlsbad, USA) was filled with 40 mL NuPage MES SDS Running Buffer and 760 mL deionized water. The SDS-samples were prepared by mixing 80 µL pure supernatant, 110 µL Tris-Glycine SDS Sample Buffer and 10 µL Dithiothreitol (all from Invitrogen). The samples were shaken on a Thermomixer HLC MHR 23 (HLC Ditabis AG, Pforzheim, Germany) at 70°C and 900 rpm for 10 min. A volume of 20 µL prepared SDS-sample or 15 µL Roti-Mark Standard (Roth) was filled in each slot of a NuPage 4-12% Bis-Tris Gel (Invitrogen). The SDS-device was run at 200 V and 0.25 W. Overnight, the gel was stained with Simply Blue Safe Stain solution (Invitrogen) at 37°C. Thereafter, the gel was scanned with a photo scanner (Epson, Perfection V700).
3.4.5 Determination of viscosity and morphology

The viscosities of the *T. reesei* shake flask broths were determined with a rheometer (Rheoplus MC1, Paar Physica, Stuttgart, Germany) at 28°C. The rheometer was equipped with a beaker-and-cylinder device (beaker: #CC27 with 28.92 mm diameter, cylinder: #CC25 with 25 mm diameter). In case of small particles and low particle numbers, a plate-and-plate device was installed to avoid turbulent flow regimes (#PP50/TG with 49.95 mm plate diameter). To describe the non-Newtonian rheological behavior, the Oswald-de Waele law (Equation 3.2) was used. The fluid consistency index $K$ and the flow behavior index $m$ were determined between shear rates $\dot{\gamma}$ of 100 s$^{-1}$ to 1000 s$^{-1}$. The apparent viscosity was then calculated with a shear rate correlation for shake flasks derived later in the study at hand (Equation 4.3).

The morphology of *T. reesei* offline samples was evaluated during shake flask experiments by measuring the size and number of the particles. A particle sizing and counting analyzer (Multisizer 4, Beckman Coulter, Brea, USA) was applied with an adjusted measuring range of 2 - 28 µm.
4 RESULTS

4.1 Liquid Films on Shake Flask Walls

To investigate the applicability of the widely applied Higbie’s film theory to liquid films on shake flask walls, in this section, the numerical simulation of the oxygen diffusion inside a liquid film based on Fick’s law is presented. Since Maier et al. (2004) simulated diffusion only in a 1 M sodium sulfite system, in this study, also calculations for a 0.35 M sodium sulfite system and a microbial culture medium were conducted; the latter systems are comparable with regard to their oxygen solubility. In addition, $OTR_{max}$-levels of the 0.35 M sulfite solution and the culture with C. glutamicum were experimentally measured with a RAMOS device. Hereby, the viscosities of the liquids were varied artificially, generating changes in the liquid film thicknesses.

4.1.1 Numerical simulations of the dissolved oxygen concentration profile in liquid films

Higbie’s theory (Equation 2.4) for the calculation of $(k_L)_{F}$ is only valid if the oxygen concentration at the shake flask wall $C_{O_2,L}(x = \delta)$ equals 0 mol/m³. Equations 2.5 to 2.8 and the constants shown in Table I were implemented in the simulation program and the dissolved oxygen concentration profile in a liquid film layer was calculated applying Fick’s law of diffusion. In the first simulation, shown in Figure 4.1, a fixed liquid film thickness of 50 µm was applied. The calculations were performed for a 1 M, 0.35 M sodium sulfite system and for culture medium (Section 3.1.2). Figure 4.1 shows the dissolved oxygen concentration $C_{O_2,L}$ as function of penetration depth $x$ and exposure time $t_{exp}$, which was set in the range of 0.15 s - 0.6 s. This reflects shaking frequencies between 400 rpm - 100 rpm. In accordance to the qualitative illustration in Figure 2.1 C, all curves in Figure 4.1 demonstrate the decreasing dissolved oxygen concentration in the liquid film layer with decreasing exposure time and increasing penetration depth. Longer exposure times provide more time for oxygen diffusion into the liquid film. With the method of Winkler (1888), a clearly lower oxygen concentration $C_{O_2,L}^*$ at the gas/liquid interface was put into the model for the 1 M sodium sulfite system compared to the concentration $C_{O_2,L}^*$ of the 0.35 M system. This is depicted in Figure 4.1 A and B. Due to differences in solubility, this concentration is 0.109 mol/m³ compared to...
0.183 mol/m³, respectively. With 0.21 mol/m³ (Figure 4.1 C), the concentration $C_{O_2,L}$ of
the culture medium is only slightly higher than the value for the 0.35 M sulfite system.

Table I. Input values for ‘gPROMS’ simulation. *Reprint from Giese et al. (2014a) with
permission from Wiley-Blackwell.*

<table>
<thead>
<tr>
<th>Explanation</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{O_2,L}$ Dissolved oxygen concentration at the gas-liquid interface</td>
<td>0.10924 mol/m³ (1 M sodium sulfite system) (Maier et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>0.183 mol/m³ (0.35 M sodium sulfite system) (Schumpe et al., 1982)</td>
</tr>
<tr>
<td></td>
<td>0.21 mol/m³ (culture medium)</td>
</tr>
<tr>
<td>$C_{O_2,b}$ Dissolved oxygen concentration in the bulk liquid</td>
<td>0 mol/m³</td>
</tr>
<tr>
<td>$D_{O_2}$ Diffusion coefficient of oxygen in the liquid phase</td>
<td>1.22 x 10^{-9} m²/s (1 M sodium sulfite system) (Maier et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>2.63 x 10^{-9} m²/s (0.35 M sodium sulfite system and culture medium) (Akita, 1981)</td>
</tr>
<tr>
<td>$k_1$ First order reaction rate constant</td>
<td>0.52 s⁻¹ (1 M sodium sulfite system) (Maier et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>0.66 s⁻¹ (0.35 M sodium sulfite system and culture medium) (Hermann et al., 2001)</td>
</tr>
<tr>
<td>$\delta$ Total liquid film thickness</td>
<td>25 - 125 µm</td>
</tr>
<tr>
<td>$\partial t$ Minimal time step</td>
<td>1 x 10^{-16} s</td>
</tr>
<tr>
<td>$X$ Biomass concentration in culture medium</td>
<td>10 g/L</td>
</tr>
<tr>
<td>$Y$ Yield coefficient in culture medium (<em>C. glutamicum</em>)</td>
<td>41.6 g biomass/mol O₂</td>
</tr>
<tr>
<td>$\mu_{max}$ Maximum specific rate of reaction in culture medium (<em>C. glutamicum</em>)</td>
<td>0.4 h⁻¹ (experimental value)</td>
</tr>
<tr>
<td>$K_s$ Saturation constant for culture medium (<em>C. glutamicum</em>)</td>
<td>0.0001 mol/m³</td>
</tr>
</tbody>
</table>
RESULTS - Liquid Films on Shake Flask Walls

Figure 4.1. Simulation of the dissolved oxygen concentration $C_{O_2,L}$ in the liquid film at the shake flask wall on basis of Fick’s diffusion law as a function of penetration depth $x$ and exposure time $t_{exp}$. A penetration depth of 0 µm refers to the position of the gas/liquid interface. The assumed maximum penetration depth $x = 50$ µm refers to the position of the glass wall of the flask and is equivalent to the total film thickness $\delta$. The shaking frequencies specified in parenthesis refer to $t_{exp,max}$ for one full rotation. This is valid for the liquid at its highest position $h_{max}$ (see Figure 2.1 A). If exposure times averaged over liquid height would be considered, the corresponding shaking frequencies would be higher. A: 1 M sodium sulfite system, B: 0.35 M sodium sulfite system and C: culture medium. This figure was developed together with my former colleague A. Azizan. Reprint from Giese et al. (2014a) with permission from Wiley-Blackwell.
RESULTS - Liquid Films on Shake Flask Walls

Regarding the 1 M sodium sulfite system (Figure 4.1 A), at low exposure times of 0.15 s and 0.2 s the oxygen concentration $C_{O_2,I}(x = \delta)$ near the flask wall remains close to 0 mol/m$^3$. Thus, for the depicted 50 µm liquid film, exposure times of up to 0.2 s (300 rpm) result in concentration profiles which can be treated as semi-infinite. For exposure times longer than 0.2 s, the 1 M sodium sulfite system shows dissolved oxygen concentrations $C_{O_2,I}$ at $x = \delta$ which are higher than 0 mol/m$^3$. In contrast to the 1 M sodium sulfite system, for the 0.35 M sulfite system and the culture medium, the concentration of dissolved oxygen at $x = \delta$ exceeds 0 mol/m$^3$ for all simulated exposure times. Thus, for these two latter solutions Higbie’s semi-infinite space assumption, based on $C_{O_2,I}(x = \delta) = 0 \text{ mol/m}^3$, is generally not fulfilled at the chosen conditions. The calculations were conducted for the maximum exposure time $t_{exp,max}$ at $h_{max}$. If exposure times averaged over liquid height would be considered, the shaking frequencies, corresponding to the exposure times listed in Figure 4.1, would be higher. In other words: A certain frequency would relate to a shorter exposure time and, thus, a more detailed model would favor the applicability of Higbie’s model.

Figure 4.2 illustrates the numerical simulations performed using Equations 2.5 to 2.8 at various liquid film thicknesses. The dissolved oxygen concentration profiles are shown for a 1 M sodium sulfite system (A and B), a 0.35 M sulfite system (C and D) as well as for the culture medium (E and F). The profiles are depicted with respect to five different liquid film thicknesses and two chosen exposure times 0.2 s (A, C, E) and 0.6 s (B, D, F). For the 1 M sulfite system with an exposure time of 0.2 s, a liquid film of at least 50 µm thickness is necessary to fulfill semi-infinite conditions; for an exposure time of 0.6 s even 100 µm is required. In the case of the 0.35 M sulfite system and the culture medium at an exposure time of 0.2 s, a liquid film of at least 75 µm thickness is necessary for the applicability of Higbie’s model. For an exposure time of 0.6 s, the semi-infinite assumption is not valid for layers thinner than 125 µm.
Figure 4.2. Simulated dissolved oxygen concentration $C_{O_2,L}$ in the liquid film on the basis of Fick’s diffusion law as a function of penetration depth $x$ for varying total liquid film thicknesses $\delta$. A, C and E (exposure time $t_{\text{exp}}$ of 0.2 s) and B, D and F (exposure time $t_{\text{exp}}$ of 0.6 s) refer to a 1 M sodium sulfite system, a 0.35 M sulfite system and the culture medium described in the material and methods section, respectively. The positions of the glass wall are indicated in the figures by the corresponding symbols. The exposure times 0.2 s and 0.6 s reflect shaking frequencies of 300 rpm and 100 rpm if a complete rotation at $h_{\text{max}}$ ($t_{\text{exp}} = t_{\text{exp,max}}$) is considered. This figure was developed by my former colleague A. Azizan. Reprint from Giese et al. (2014a) with permission from Wiley-Blackwell.
Figure 4.3. Numerical simulations of the relative average oxygen flux as a function of total film thickness $\delta$ based on Fick’s diffusion law. Relative oxygen flux: $\bar{n}_{O_2}/\bar{n}_{O_2,\infty}$. The oxygen flux $\bar{n}_{O_2,\infty}$ describes the gas/liquid oxygen transfer into a liquid film, adhering at the shake flask wall, for an infinite total liquid film thickness $\delta_\infty$. The oxygen fluxes $\bar{n}_{O_2}$ and $\bar{n}_{O_2,\infty}$ are averaged over one full rotation. Shaking frequencies and total liquid film thicknesses $\delta$ were varied at constant filling volume (30 mL in a 250 mL shake flask) and constant shaking diameter (50 mm). The oxygen flux into the bulk liquid was not considered here. *Reprint from Giese et al. (2014a) with permission from Wiley-Blackwell.*
In all those cases where the dissolved oxygen concentration at the flask wall has increased to values higher than 0 mol/m³, the oxygen flux through the gas/liquid interface is reduced due to the impaired total driving concentration gradient. This is shown via the simplified calculation of oxygen fluxes based on Fick’s diffusion (Equations 2.9 and 2.10). Figure 4.3 illustrates the dimensionless flux \( \tilde{n}_{O_2}/\tilde{n}_{O_2,\infty} \) as a function of total liquid film thickness and shaking frequency for the three investigated solutions. The flux \( \tilde{n}_{O_2,\infty} \) relates to an infinite thick liquid film with a penetration depth much smaller than the total film thickness. In this case \( C_{O_2,L}(x = \delta) \) is always 0 mol/m³. The relative flux equals 1, if the oxygen concentration at the wall is 0 mol/m³ for a certain liquid film thickness. By contrast, the relative flux is less than 1 if elevated oxygen concentrations at the flask wall occur, meaning \( C_{O_2,L}(x = \delta) > 0 \) mol/m³. Consequently, in these cases \( \tilde{n}_{O_2} \) is lower than \( \tilde{n}_{O_2,\infty} \).

The simulation results clearly indicate the range of applicability for Higbie’s film theory: Relative fluxes equal to 1 imply a valid semi-infinite space assumption and, thus, Higbie’s model applies for these cases. At conditions where the relative flux is less than 1, Higbie’s model would overestimate the actual oxygen flux calculated with Fick’s diffusion. If the curves at equal shaking frequencies are compared, Figure 4.3 reveals that for the 1 M sodium sulfite solution (A) the semi-infinite space assumption is valid for thinner films in comparison to the 0.35 M sulfite solution (B) and the culture medium (C). Both, Figure 4.2 and Figure 4.3 illustrate that generally lower shaking frequencies require thicker liquid films to fulfill the semi-infinite space assumption. For instance, at 200 rpm and a thickness of 25 \( \mu \)m, Figure 4.3 C shows a relative flux of 0.76. At the same frequency, an increase of the liquid film thickness to 50 \( \mu \)m, for example as a result of elevated viscosity, approaches a relative flux of 1, which is equal to an oxygen flux increase by 30%.

Especially in fungal- and plant cell cultures or in cultures of biopolymer-secreting organisms, the viscosity can significantly increase during fermentation. Hence, during such fermentations also the liquid film thickness will increase as a result of increasing viscosity. For stirred tanks with dedicated impeller design and at high power input, maximum viscosities of up to 1000 mPa·s have been reported in literature (Johansen et al., 1998; Peña et al., 2000; Neves et al., 2000; Bhargava et al., 2004; Gabelle et al., 2012). Table II shows microorganisms that increased the viscosity of their culture broth.
during fermentation in shake flasks. Freshly inoculated culture broths usually have waterlike viscosities of about 1 mPa·s. Maximum viscosity values between 11 mPa·s (Beta vulgaris) to 110 mPa·s (Xanthomonas campestris) were measured in these shake flasks cultures. A shear rate \( \dot{\gamma} \) of 300 s\(^{-1}\) was used, which was previously postulated to be the mean shear rate at usual operating conditions of shake flasks (Peter et al., 2004). Further relevant viscosity studies of shake flask cultures, conducted at shear rates differing from 300 s\(^{-1}\), are presented in Byrne and Ward (1987), Burns et al. (1994), Madi et al. (1997) and Peña et al. (1997). Such an increase in viscosity in combination with the presented increase in liquid film thickness (Figure 4.1 to Figure 4.3) may lead to a striking change in oxygen supply during viscous shake flask fermentations.

Table II. Selected viscous shake flask fermentations of various microorganisms with respective viscosity ranges. The apparent viscosities were determined at a shear rate for shake flasks of 300 s\(^{-1}\). Detailed fermentation conditions are given in the listed literature. Reprint from Giese et al. (2014a) with permission from Wiley-Blackwell.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Product</th>
<th>Apparent viscosity [mPa·s]</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Azotobacter vinelandii</em></td>
<td>alginate</td>
<td>1 - 35</td>
<td>Peña et al. (2011)</td>
</tr>
<tr>
<td><em>Beta vulgaris</em></td>
<td>betalain</td>
<td>1 - 11</td>
<td>Rodríguez-Monroy and Galindo (1999)</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em></td>
<td>( \gamma )-polyglutamic acid</td>
<td>1 - 58</td>
<td>Wilming et al. (2013)</td>
</tr>
<tr>
<td><em>Solanum chrysotrichum</em></td>
<td>saponin</td>
<td>1 - 42</td>
<td>Rodríguez-Monroy et al. (2004)</td>
</tr>
<tr>
<td><em>Streptomyce tendae</em></td>
<td>nikkomycin</td>
<td>1 - 44</td>
<td>Henzler and Schedel (1991)</td>
</tr>
<tr>
<td><em>Xanthomonas campestris</em></td>
<td>xanthan</td>
<td>1 - 110</td>
<td>Lotter and Büchs (2004)</td>
</tr>
</tbody>
</table>
4.1.2 Experimental studies: Influence of viscosity on the maximum oxygen transfer capacity

4.1.2.1 Experimental estimation of film thickness

The aforementioned numeric simulations demonstrate the strong influence of exposure time and liquid film thickness on the oxygen mass transfer into a liquid film. It was shown that Higbie’s theory to calculate $(k_L)_F$ has a limited range of applicability. An erroneous estimation of $(k_L)_F$ directly leads to a wrong calculation of the $OTR_{max}$ (Equation 2.3). To study the influence of liquid film thickness on $OTR_{max}$, the liquid film thickness was varied experimentally by modifying the viscosity with PVP. The apparatus shown in Figure 3.2 was constructed by Hermann (2001) in order to obtain a rough understanding for the relationship between liquid film thickness and viscosity. It should be noted that this experimental setup was just capable to clarify the order of magnitudes of the liquid film thickness. For instance, the measuring signal for the liquid film thickness is highly influenced by the vertical mounting height of the fiber cable shown in Figure 3.2. It was found that liquid film thicknesses of approx. 50 µm and 500 µm correspond to viscosities of 1 mPa·s and 100 mPa·s, respectively (data not shown). Thus, a 100-fold change in viscosity caused a 10-fold change in the film thickness.

4.1.2.2 Experiments with the 0.35 M sodium sulfite system

With the RAMOS device, the $OTR_{max}$ was measured as the sum of oxygen transfer into both the bulk and liquid film. Figure 4.4 shows $OTR_{max}$ results of the 0.35 M sodium sulfite system as a function of viscosity. Independent of the liquid viscosity, higher shaking frequencies and lower filling volumes generally tend to yield higher $OTR_{max}$-levels. However, if one follows a specific $OTR_{max}$ curve at a viscosity ranging from 1 mPa·s to 10 mPa·s – a range typical for viscous microbial fermentations (Table II) – at any given shaking frequency and filling volume, the $OTR_{max}$ increases markedly. For instance, at a filling volume of 20 mL and a shaking frequency of 300 rpm the $OTR_{max}$ is equal to 39 mmol/L/h at 1 mPa·s (Figure 4.4 C). At 10 mPa·s, the $OTR_{max}$ increased to 47 mmol/L/h which is an increase by 20%. This striking phenomenon was observed for all shaking conditions applied in this experiment.
Figure 4.4. Maximum oxygen transfer capacity $OTR_{\text{max}}$ as a function of viscosity $\eta$ for a 0.35 M sodium sulfite system. The inserts provide a detailed view into the maximum $OTR_{\text{max}}$-ranges of viscosities from 1 mPa·s to 30 mPa·s. The viscosity was adjusted by the addition of PVP at varying shaking frequencies of 200, 250 and 300 rpm using filling volumes of 20, 30 and 40 mL. Measurements were performed at a shaking diameter of 50 mm and a temperature of 25°C in a 250 mL shake flask with RAMOS. This figure was developed together with my former colleague A. Azizan. Reprint from Giese et al. (2014a) with permission from Wiley-Blackwell.
Between 10 mPa·s and approx. 20 mPa·s the $OTR_{\text{max}}$ remains almost constant and, for all cases, decreases gradually with further increase in viscosity. Until approx. 80 mPa·s the $OTR_{\text{max}}$-values are not significantly lower than the initial $OTR_{\text{max}}$ at 1 mPa·s.

4.1.2.3 Experiments with *Corynebacterium glutamicum* medium

To confirm the experimental results generated for the sodium sulfite system, *C. glutamicum* DM1730 was cultivated in 250 mL shake flasks. The initial viscosity was artificially increased through PVP addition in a range between 1 mPa·s to 77.9 mPa·s. As *C. glutamicum* does not produce a biopolymer, the viscosity remained constant during the whole fermentation time (data not shown).

Figure 4.5 depicts these fermentations shaken at 350 rpm at a constant filling volume of 30 mL. During the exponential growth phase, all fermentations showed a simultaneous increase in oxygen consumption. At approximately 5.5 h, the cultures reached their $OTR_{\text{max}}$-levels, resulting in a plateau indicating oxygen limitation. Between 8 h and 9 h, the respiration activities sharply decreased due to carbon source depletion.

![Experimental values for the oxygen transfer rate OTR as function of fermentation time. *Corynebacterium glutamicum* DM1730 in 30 mL culture medium supplemented with different amounts of PVP which resulted in the indicated viscosity values. Measurements were performed in a 250 mL shake flask at a 50 mm shaking diameter and at 350 rpm. The experiments were run in triplicates at 30°C using a RAMOS device. The average standard deviation of the measured OTR values was 0.18 mmol/L/h. This figure was developed by my former colleague A. Azizan. Reprint from Giese et al. (2014a) with permission from Wiley-Blackwell.](#)
The plateaus represent different $OTR_{\text{max}}$-levels depending on the viscosity of the medium. In the culture broth with a viscosity of 1 mPa·s, the $OTR_{\text{max}}$-plateau was at 30.5 mmol/L/h. With increasing viscosity up to a value of 10.9 mPa·s, the heights of the plateaus rose, resulting in a maximum $OTR_{\text{max}}$ of 34.5 mmol/L/h. This level is equal to a 13% higher oxygen supply for the microorganisms compared to the 1 mPa·s-culture. Experiments at even higher viscosities led to a decreasing $OTR_{\text{max}}$-level, approaching again the initial $OTR_{\text{max}}$-level measured at 1 mPa·s. These microbial experiments agree well with the experimental findings generated with the chemical model system (Figure 4.4). The dependency between $OTR_{\text{max}}$ and viscosity was further investigated for additional shaking conditions. *C. glutamicum* was cultivated in filling volumes of 30 mL and 40 mL at 250 rpm as well as in 20, 30 and 40 mL at 350 rpm. As illustrated in Figure 4.6, the liquid viscosity was varied again between 1 mPa·s and 77.9 mPa·s. Similar to the 0.35 M sodium sulfite model system (Figure 4.4), for all shaking conditions the $OTR_{\text{max}}$ rose at viscosities ranging from 1 mPa·s to 10 mPa·s. An approx. constant $OTR_{\text{max}}$ could be observed between viscosity values of 10 mPa·s to 20 mPa·s. Up to a viscosity of 80 mPa·s, the $OTR_{\text{max}}$ did not significantly undermatch the initial $OTR_{\text{max}}$ at 1 mPa·s.

In Table II (page 39) a *Beta vulgaris* culture has been shown with a maximum viscosity of 11 mPa·s. With the findings of Figure 4.4 and Figure 4.5, this maximum viscosity of 11 mPa·s implies an increasing oxygen supply during the entire fermentation time. For *Azotobacter vinelandii, Beta vulgaris, Bacillus licheniformis, Solanum chrysotrichum* and *Streptomyces tendea* the oxygen supply at any time point during fermentation must be assumed to be higher compared to the initial oxygen supply at 1 mPa·s – although the viscosity increased to values of up to 58 mPa·s. The maximum viscosity of *Xanthohomonas campestris* culture broth was determined to reach values of about 110 mPa·s. As can be seen in Figure 4.4, the oxygen supply at this elevated viscosity was still approx. 90% of the initial oxygen supply.
RESULTS - Liquid Films on Shake Flask Walls

Figure 4.6. $OTR_{\text{max}}$ vs. viscosity $\eta$: Left axis (A, B and C): Experimental values of *Corynebacterium glutamicum* DM1730 culture at shaking frequencies of 250 and 350 rpm and filling volumes of 20, 30 and 40 mL in a 250 mL shake flask. Measurements were performed in triplicates at a shaking diameter of 50 mm and a temperature of 30°C with a RAMOS device. The average standard deviation of the measured $OTR$ values was 0.48 mmol/L/h. Right axis (C): Typical trend of $OTR_{\text{max}}$ in dependency of viscosity in a stirred tank reactor. Influence of the viscosity on the oxygen mass transfer in a stirred tank was correlated with $OTR_{\text{max}} \sim \eta^{-0.7}$. All other physical parameters were kept constant. Exponent -0.7 is a mean from literature values listed in Kawase and Moo-Young (1988). Data are related to $OTR_{\text{max}}$ at 1 mPa·s. *Reprint from Giese et al. (2014a)* with permission from Wiley-Blackwell.
To contrast these shake flask results with those of conventional stirred tank reactors the typical dependency $OTR_{max} \sim \eta^{-0.7}$ for stirred tanks is illustrated in Figure 4.6 C. The exponent -0.7 is a mean from literature values listed in Kawase and Moo-Young (1988). Contrary to shake flasks, stirred tank reactors show a continuous decrease in $OTR_{max}$ with increasing viscosity. Whereas in shake flasks the oxygen supply at a viscosity of 10 mPa·s can be up to 20% higher than that at a viscosity of 1 mPa·s, in a stirred tank reactor it may decrease by about 80% within the same viscosity interval. For the stirred tank reactor, the $OTR_{max}$ between 50 mPa·s and 80 mPa·s is only approx. 5% of the initial $OTR_{max}$ at 1 mPa·s. In this viscosity range the $OTR_{max}$ in shake flasks does not markedly fall below the initial value. At an even higher viscosity of 225 mPa·s, which is not untypical for a number of industrial relevant fermentations (Johansen et al., 1998; Peña et al., 2000; Neves et al., 2000; Bhargava et al., 2004; Gabelle et al., 2012), the $OTR_{max}$ in shake flasks, is still 60% of the $OTR_{max}$ at 1 mPa·s (Figure 4.4).

The different $OTR_{max}$-characteristics described can be explained considering several phenomena: In a stirred tank reactor the volumetric gas/liquid exchange area $a$ is reduced with increased viscosity. This is due to reduced bubble breakup and increased coalescence (Lee et al., 1987; Zlokarnik, 2001). In addition, increasing viscosity leads to a reduced diffusion coefficient for oxygen (Wilke and Chang, 1955). In stirred tanks, the power input by stirring remains constant as long as the liquid is in turbulent motion (Figure 2.2; Zlokarnik, 2001). This is in contrast to shake flasks. Here, as long as the liquid rotates “in-phase”, the power input increases with increasing viscosity – in the interval of 1 mPa·s to 80 mPa·s it increases approx. 5 times (Büchs et al., 2000b). This increased power input results in a positive impact on the $k_L$-value (Maier and Büchs, 2001), which counteracts the decreasing diffusion coefficient for diffusion into the bulk and the liquid film of a shake flask. Additionally, in the surface-aerated shake flask system, no negative influence of coalescence and, therefore, the volumetric gas/liquid mass transfer area $a$ occurs. As a result, in a wide viscosity range up to 80 mPa·s, the positive influences of increasing viscosity on gas/liquid oxygen transfer into the bulk and liquid film of a shake flask compensate the negative impacts.
4.1.3 Numerical simulations of diffusion in a liquid film explain experimental results

In this study, the chemical and microbial RAMOS experiments are consistent to each other. Most notably, similar $OTR_{max}$-profiles were measured as a function of viscosity. For several technical applications like falling film reactors or technical coating technologies, many authors reported that the oxygen transfer decreases with increasing viscosity (Wilke and Chang, 1955; Levich, 1962; Popovic et al., 1962). In the case of shake flasks, Figure 4.4 and Figure 4.6 clearly demonstrate that this is only valid for viscosities that exceed approximately 20 mPa·s. The described $OTR_{max}$-drop after 20 mPa·s concurs with the results of Figure 3.1, because oxygen solubility decreases with increasing viscosity. This solubility effect – together with impaired gas/liquid diffusion – becomes predominant. However, the rise in $OTR_{max}$ at viscosities up to 10 mPa·s is a remarkable and somehow counter-intuitive finding which was never reported before. In principle, this phenomenon was predicted already by the fundamental work of Maier et al. (2004). However, in the current study, it was substantiated by experiments as well as by numerical simulations based on Fick’s law of diffusion in liquid films (Figure 4.1 to Figure 4.3) of three different solutions. The simulations showed that at low viscosities and consequently thin liquid films, the oxygen concentration at the shake flask wall is larger than 0 mol/m³. This reduces the driving concentration gradient at the gas/liquid interface, resulting in comparatively low oxygen transfer into the liquid film. The oxygen transfer into the liquid film increases with elevating viscosity, because the thicker liquid film increases the driving oxygen concentration gradient. Because no additional increase of oxygen transfer can be observed at further increasing viscosity, at 10 mPa·s the maximum driving concentration gradient under ambient conditions seems to be reached.
4.2 Effective Shear Rates in Shake Flasks

In this section, the determination of effective shear rates and apparent viscosities in shake flasks is presented. For this purpose, the experimental approach introduced in Figure 3.3 (page 23) as well as a Dimensional analysis based on Buckingham’s π-theorem were applied.

4.2.1 Viscosity and online power input measurements

Figure 4.7 displays two examples of intercept point determination during the online volumetric power input (A, C, E) and viscosity measurements (B, D, F) based on the approach shown in Figure 3.3. Figure 4.7 A presents the online volumetric power input signals as a function of the shaking frequency for a xanthan solution of 10 g/L and different PVP-solutions with concentrations from 62 g/L to 87 g/L. The solutions were shaken on a power input shaker in 500 mL shake flasks with 80 mL filling volume at 50 mm shaking diameter. As the shaking frequency-dependent increase in power input is lower in the case of the xanthan solution than in the Newtonian PVP-solutions, four intercept points are produced in the power input curves at 168, 225, 263 and 325 rpm. Figure 4.7 B presents the respective viscosity values fitted using the Ostwald-de Waele law (Equation 3.2). The xanthan solution (10 g/L) exhibits a clear pseudo-plastic flow behavior, leading to a sharp viscosity decrease with increasing shear rate; a viscosity of 130 mPa·s was measured at 200 s⁻¹, falling to only 35 mPa·s at 1000 s⁻¹. In contrast, the viscosities of the four PVP-solutions remained almost constant with increasing shear rates, resulting in four intercept points between the flow curves of the Newtonian and pseudo-plastic solutions at 210, 440, 530 and 795 s⁻¹. The shear rate at a specific viscosity intercept point is equal to that at the identically numbered power input intercept point (Figure 4.7 A). For instance, at intercept point 1, an effective shear rate of 210 s⁻¹ (at 122 mPa·s) can be identified for the following shaking conditions: 500 mL shake flask, 80 mL filling volume, 50 mm shaking diameter, and a shaking frequency of 165 rpm.

Figure 4.7 C and D present as log./log. diagrams the power input and viscous flow curves of Figure 4.7 A and B, thereby clearly revealing the exponential relationship between volumetric power input and shaking frequency, as well as viscosity and shear rate. Figure 4.7 D also shows data regarding the flow behavior index $m$ (Equation 3.2). Values of between 0.924 and 0.956 confirm nearly Newtonian flow behavior for PVP-solutions, whereas a low value of 0.207 for Xanthan (10 g/L) is a distinct indicator of a clearly pseudo-plastic fluid.
RESULTS - Effective Shear Rates in Shake Flasks

Figure 4.7. Identification of associated intercept points for selected volumetric power input (A, C, E) and viscosity measurements (B, D, F) carried out via the method introduced in Figure 3.3. Newtonian PVP-solutions and different oils are indicated by filled symbols, and pseudo-plastic xanthan solutions by open symbols. The logarithmic plots (C, D, E, F) illustrate the strong exponential relationships between volumetric power input and shaking frequency, as well as between viscosity and shear rate. Measurements shown in A, B, C and D were performed in 500 mL shake flasks with 80 mL filling volume at 50 mm shaking diameter. Measurements shown in E and F were performed in 250 mL shake flasks with 25 mL filling volume and 50 mm shaking diameter. Flow behavior indexes $m$ are shown in D and F. Equally-numbered intercept points are associated with each other and were used to build up the shear rate correlation. Reprint from Giese et al. (2014c) with permission from Elsevier.
A second example of intercept point detection is shown in Figure 4.7 E and F, which refer to the application of 8 g/L and 10 g/L xanthan solutions (different xanthan lot than applied for Figure 4.7 A-D) and two oils (linseed and olive). In both cases the volumetric power input into 250 mL shake flasks with 25 mL filling volume was measured on a power input shaker at 50 mm shaking diameter. The viscosity values reveal that, first, higher xanthan concentrations result in a stronger pseudo-plastic flow behavior and, second, the applied oils are Newtonian ($m = 0.999$). The different flow behaviors of the xanthan solutions and the oils lead to four associated intercept points (points 5, 6, 7, 8). A huge variety of liquid solutions and shaking conditions were analyzed in order to achieve a comprehensive impression of effective shear rates. Various concentrations of the PVP and xanthan polymers, as well as the four oils, were analyzed with respect to the method presented in Figure 3.3 and Figure 4.7. They were analyzed in 50, 100, 250, 500 and 1000 mL shake flasks at shaking diameters of 25, 50, 70, and 100 mm. Based on this approach, a total of 126 associated intercept points were experimentally found at “in-phase” operating conditions.

### 4.2.2 Development of an effective shear rate correlation for shake flasks

The 126 intercept points determined above were used to develop a shear rate correlation for shake flasks via the application of a Dimensional analysis. Five independent variables were assumed to influence the correlation: The effective shear rate $\dot{\gamma}_{eff}$, the apparent viscosity $\eta_{app}$, the power input $P$, the shake flask diameter $d$ and the filling volume $V_L$. The SI-units of the independent variables are listed below:

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\eta_{app}$</td>
<td>$\frac{kg}{m \cdot s}$</td>
</tr>
<tr>
<td>$\dot{\gamma}_{eff}$</td>
<td>$s^{-1}$</td>
</tr>
<tr>
<td>$V_L$</td>
<td>$m^3$</td>
</tr>
<tr>
<td>$P$</td>
<td>$\frac{kg \cdot m^2}{s^3}$</td>
</tr>
<tr>
<td>$d$</td>
<td>$m$</td>
</tr>
</tbody>
</table>

Considering Buckingham’s $\pi$-theorem, the quantity of dimensionless numbers must be equal to the number of independent variables minus the quantity of SI-units (Buckingham, 1915). Here, the numbers of independent variables and different SI-units are 5 and 3, respectively, thus, leading to 2 dimensionless numbers describing the physical circumstances. The dimensionless numbers are commonly derived by a dimensional matrix consisting of a square core matrix and a residual matrix (Zlokarnik, 2001). The core matrix is then linearly transformed into a matrix of unity by matrix calculation. Zlokarnik (2001) described the way
the dimensionless numbers are composed: “After the generation of the matrix of unity, the dimensionless numbers are created as follows: Each element of the residual matrix forms the numerator of a fraction while its denominator consists of the fillers from the matrix of unity with the exponents indicated in the residual matrix.” Thus, with respect to the physical circumstances at hand, the independent variables were categorized into the dimensionless numbers $\Pi_1$ and $\Pi_2$ (Equation 4.1) according to the dimensional matrix below:

<table>
<thead>
<tr>
<th>Variable</th>
<th>$\eta_{app}$</th>
<th>$\dot{\gamma}_{eff}$</th>
<th>$V_L$</th>
<th>$P$</th>
<th>$d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>kg</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>s</td>
<td>-1</td>
<td>-1</td>
<td>0</td>
<td>-3</td>
<td>0</td>
</tr>
<tr>
<td>m</td>
<td>-1</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>kg</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
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<tr>
<td>s</td>
<td>0</td>
<td>-1</td>
<td>0</td>
<td>-2</td>
<td>0</td>
</tr>
<tr>
<td>m</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>1</td>
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<td>kg</td>
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<td>s</td>
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<td>2</td>
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<tr>
<td>m</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1/3</td>
<td>1/3</td>
</tr>
</tbody>
</table>

A power law approach ($1 = L \cdot \Pi_1 \cdot \Pi_2 \cdot \Pi_3^{\gamma} \ldots$) leads to Equation 4.2. Using the Ostwald-de Waele law (Equation 3.2) and some rearrangement, the shear rate correlation for shake flasks (Equation 4.3) follows. This approach is analogous to the stirred tank correlation devised by Henzler and Kauling (1985) (Equation 2.16).

$$\Pi_1 = \frac{P / V_L}{\eta_{app} \left(\dot{\gamma}_{eff}\right)^2}$$  \hspace{1cm} 4.1

$$\Pi_2 = \frac{d}{V_L^{1/3}}$$

$$\frac{P / V_L}{\eta_{app} \left(\dot{\gamma}_{eff}\right)^2} = L \left(\frac{d}{V_L^{1/3}}\right)^x$$  \hspace{1cm} 4.2
\[ \dot{\gamma}_{\text{eff}} = L^{\frac{1}{m+1}} \left( \frac{P}{V_L} \right)^{\frac{1}{m+1}} \left( \frac{V_L^{1/3}}{d} \right)^x \]  

Due to changing liquid distribution, the reactor diameter \( d \) and the filling volume \( V_L \) were assumed to influence the effective shear rate in shake flasks – even in instances of geometric similarity. This influence is considered with the last term shown in Equation 4.3. All found 126 intercept points are plotted in Figure 4.8 (dots). For graphical illustration the axes are in logarithmic scale and the effective shear rates were normalized with respect to \( K, L \) and \( m \). The proportionality factor \( L \) and the exponent \( x \) were determined by fitting Equation 4.3 to the identified intercept points. The plotted area shows this least square fit \((R^2 = 0.901)\) leading to \( L = 2.06 \) and \( x = -0.331 \).

Figure 4.8. Determination of proportionality factor \( L \) and exponent \( x \) used in the developed shear rate correlation for shake flasks (Equation 4.3). Dots represent intercept points found for 50, 100, 250, 500 and 1000 mL shake flasks using the method illustrated in Figure 3.3. Intercept points were identified in the following ranges: Shaking frequency: 130 - 380 rpm, shaking diameter: 25 - 100 mm, effective shear rate: 167 - 1025 s\(^{-1}\), apparent viscosity: 11 - 151 mPa·s, volumetric power input: 0.71 - 15.1 kW/m\(^3\), consistency index \( K \): 49 - 23,233 mPa·s\(^m\), and flow behavior index \( m \): 0.107 - 0.867. The plotted surface area illustrates a least square fit, leading to \( L = 2.06 \) and \( x = -0.331 \). Raw data was partially provided by my former colleagues C. Peter and S. Lotter. Reprint from Giese et al. (2014c) with permission from Elsevier.
RESULTS - Effective Shear Rates in Shake Flasks

Figure 4.9. Linear (A) and logarithmic (B, C) parity plots of measured and calculated effective shear rates. The x-axes in A and B present shear rates determined from the intercept points of online power input and viscosity measurements (dots in Figure 4.8). Values on the y-axes represent shear rates calculated via the new shear rate correlation, which represents a fit for all measured intercept points (Equation 4.3, surface area in Figure 4.8); 76% of all calculated shear rates lie in the marked sector indicating a maximum deviation of +/-35% (A, B, dashed lines). The calculated shear rates on the y-axis in C were determined without direct power input measurement by using the $Ne'/Re$ relationship (Equation 2.11, Büchs et al., 2000b) and the new shear rate correlation; 75% of these calculated shear rates lie in the marked sector indicating a maximum deviation of +/-40% (dashed lines). Modified reprint from Giese et al. (2014c) with permission from Elsevier.
In Figure 4.9 A and B, the effective shear rates calculated using the new correlation (Equation 4.3) are compared to those determined via the experimental method presented in Figure 3.3 and Figure 4.7. The comparison between measured values on the x-axis and calculated values on the y-axis shows the accuracy of the applied approach. Note that whereas Figure 4.9 A has linear axes, those in Figure 4.9 B are logarithmic. The figures illustrate that a percentage of 76% of all calculated effective shear rates differs less than 35% from the measured values. The deviation range of +/-35% is indicated by dashed lines. The figures also demonstrate that these deviations are not dependent on the nominal flask volume, but are instead randomly scattered.

If no shaker with automated power input measurement is available, the power input must be calculated. Using the $Ne'/Re$-relationship derived by Büchs et al. (2000b) (Equation 2.11), and with Equations 2.12 and 3.2, the power input $P$ can be expressed by:

$$P = \rho n^3 d^4 V_L^{1/3} \left[ 70 \left( \frac{\rho n d^2}{K \dot{\gamma}_{eff}^{(m-1)}} \right)^{-1} + 25 \left( \frac{\rho n d^2}{K \dot{\gamma}_{eff}^{(m-1)}} \right)^{-0.6} + 1.5 \left( \frac{\rho n d^2}{K \dot{\gamma}_{eff}^{(m-1)}} \right)^{-0.2} \right].$$

When Equation 4.4 is applied to Equation 4.3, the effective shear rate can be determined via iteration (e.g. via MS Excel Solver). As presented in Figure 4.9 C, the effective shear rates calculated using Equation 4.4 are compared to measured values. Due to the fact that the $Ne'/Re$-relationship is also an empirical fit, the mean deviation between measured and calculated values slightly increases, with 75% of the measured points lying in the +/-40% sector. It should be noticed that the relative angles of the volumetric power input lines representing Newtonian and pseudo-plastic fluids, are in general relatively small (compare e.g. Figure 4.7 C or E). Xanthan was chosen as pseudo-plastic fluid, which has a quite low flow behavior index. In this way, the relative angles described above are maximized as far as possible. However, small deviations of the measured power inputs result in large shifts of the intercept points to smaller or larger shaking frequencies. The accuracy of the applied evaluation method for the effective shear rate as a function of the operating conditions is, therefore, limited. However, this is the only experimental method currently available.
4.2.3 Shear rate and apparent viscosity as a function of shaking parameters

By applying the developed shear rate correlation for shake flasks (Equation 4.3), the effective shear rate, apparent viscosity and Ph number are for the first time accessible for the analysis of pseudo-plastic fermentation broths. Figure 4.10 examines the dependency of these quantities as function of the nominal flask volume, filling volume, and shaking frequency. As an example, the consistency indexes $K$ and flow behavior indexes $m$ were taken from a X. campestris broth after 20 h and 39 h of fermentation, respectively. This fermentation was conducted by Galindo et al. (1989) and, thus, process conditions as well as all $K$ and $m$ values are listed in the latter paper. Figure 4.10 A, B and C display the results calculated for the fermentation broth analyzed after 20 h with $K = 100$ mPa·s$^m$ and $m = 0.65$.

In Figure 4.10 A, the effective shear rate is shown as a function of shaking frequency, with relative filling volumes of 5% illustrated by thin curves and of 40% by bold curves. It is obvious that the effective shear rate increases with increasing shaking frequency. In addition, a low filling volume leads to higher effective shear rates than a high filling volume. This can be explained by the liquid distribution in shaken flasks (Büchs et al., 2007): By the shake flask wall, the shaking power is transferred to the rotating bulk liquid. A low filling volume results in a thin layer of bulk liquid on the wall and a high filling volume results in a thick one. The shear rate decreases with an increasing distance between shake flask wall and gas/liquid surface. Consequently, the representative effective shear rate for the whole bulk liquid falls with rising filling volume. Figure 4.10 A also demonstrates that the effective shear rate increases with nominal flask volume. The circular track of the bulk liquid in a large flask is longer compared to that in a small shake flask. A larger shake flask diameter leads to a higher circular velocity of the bulk liquid and to a higher volumetric power input at constant shaking frequency. In summary, Figure 4.10 A shows that an effective shear rate of between 20 s$^{-1}$ and approx. 2000 s$^{-1}$ should be expected under common shaking conditions.

In order to calculate the apparent viscosity shown in Figure 4.10 B via the Ostwald-de Waele law (Equation 3.2), the determined effective shear rates (Figure 4.10 A) were applied. Since the effective shear rate increases with increasing shaking frequency, the apparent viscosity of the depicted pseudo-plastic broth decreases. The apparent viscosity increases with decreasing nominal flask volume and increasing filling volume.
Figure 4.10. Calculated effective shear rate (A, D), apparent viscosity (B, E) and Ph number (C, F) as a function of shaking frequency for 500 mL shake flasks (dotted curves), 250 mL shake flasks (solid curves) and 100 mL shake flasks (dashed curves) at 50 mm shaking diameter. The results shown in A, B and C were calculated using a consistency index $K = 100 \text{ mPa} \cdot \text{s}^m$ and a flow behavior index $m = 0.65$ obtained for a *Xanthomonas campestris* fermentation broth after 20 h of fermentation (Galindo et al., 1989). The results shown in D, E and F were calculated using the parameters $K = 30,000 \text{ mPa} \cdot \text{s}^m$ and $m = 0.18$, which were determined during the same fermentation but after 39 h (Galindo et al., 1989). Thin curves illustrate shake flasks with 5% and bold curves with 40% relative filling volume. The results were obtained from calculations carried out using the new shear rate correlation (Equation 4.3) and the $Ne'/Re$ relationship (Equation 2.11). (B, E): To generate a quantitative idea of power input into shake flasks, the symbols highlight volumetric power input values for selected shaking conditions. Reprint from Giese et al. (2014c) with permission from Elsevier.
In the chosen example, 1 kW/m³ is transferred at 11 mPa·s and 160 rpm, if a 250 mL shake flask is filled with 5% relative filling volume. If the relative filling volume is increased to 40%, a higher shaking frequency of 270 rpm is necessary to transfer 1 kW/m³ and, the apparent viscosity is slightly higher (12 mPa·s). Further exemplary volumetric power input values are highlighted by symbols in the figure. In Figure 4.10 C, the Ph number is plotted as a function of shaking frequency, nominal flask volume and relative filling volume. As already shown by Büchs et al. (2001), low filling volumes and low shaking frequencies increase the risk of “out-of-phase” operation. In the chosen common spectrum of operating conditions, the determined effective shear rates lead to Ph numbers higher than 1.26, meaning that a broth with \( K = 100 \text{ mPa·s}^m \) and \( m = 0.65 \) is permanently shaken “in-phase” on 50 mm shaking diameter.

Figure 4.10 D, E and F present the calculated results for a broth sample from the same fermentation but obtained after 39 h. The flow consistency and flow behavior index values were determined to be \( K = 30,000 \text{ mPa·s}^m \) and \( m = 0.18 \), respectively (Galindo et al., 1989), thus indicating a more distinctly pseudo-plastic flow behavior compared to the broth analyzed in Figure 4.10 A-C. This results in a lower level of effective shear rates (Figure 4.10 D). For example, at 300 rpm and 5% relative filling volume, the effective shear rates in 250 mL shake flasks are 1400 s⁻¹ and 620 s⁻¹ for the samples taken after 20 h (Figure 4.10 A) and 39 h (Figure 4.10 D), respectively. This leads to markedly higher apparent viscosities for the 39 h-sample (Figure 4.10 E). When this sample is shaken in a 250 mL shake flask at 300 rpm and 5% relative filling volume, a representative apparent viscosity of 150 mPa·s is generated, compared to only 8 mPa·s for the sample taken after 20 h. Figure 4.10 E illustrates the strong impact of the effective shear rate on pseudo-plastic fluids: Whereas at 100 rpm the viscosity in a 250 mL shake flask with 5% relative filling volume is 600 mPa·s, at 400 rpm it is only 100 mPa·s. Figure 4.10 F discloses a striking finding: Even with high performance shaking machines (maximum shaking frequency: 400 rpm at 50 mm shaking diameter), distinctive pseudo-plastic fermentation broths cannot be shaken “in-phase” if the relative filling volume is low. For a relative filling volume of 5% all calculated Ph numbers are below 1.26. Only a significant increase in the relative filling volume and high shaking frequencies allow such broths to be shaken “in-phase”. High filling volumes, however, reduce the \( OTR_{\text{max}} \). It has to be checked, if this is acceptable. It should be noticed that “in-phase” operation is a boundary condition of the new shear rate correlation (Equation 4.3) and for the power input calculation.
Equations 2.11 and 4.3 are not valid at $Ph < 1.26$ and, thus, all calculated effective shear rates resulting in $Ph < 1.26$ may be overestimated.

### 4.2.4 Application of the shear rate correlation to three different shake flask fermentations

Figure 4.11 shows a *T. reesei* fermentation conducted in 250 mL shake flasks with 10% relative filling volume at 50 mm shaking diameter. The culture was shaken at 200 rpm with a shaking diameter of 50 mm. The OTR, shown in Figure 4.11 A, was measured in duplicates. After a lag-phase of 10 h, the culture grew exponentially, until reaching an oxygen limitation at approx. 30 h and an $OTR_{\text{max}}$-level of 17 mmol/L/h. After 48 h, the main carbon sources were depleted and the OTR dropped down. The online volumetric power input was measured with a power input shaker and is illustrated in Figure 4.11 A. A sharp increase could be observed between 24 h and 36 h leading to a maximum volumetric power input of approx. 1.5 kW/m³ detected at 72 h. For the calculation of the effective shear rates (Figure 4.11 B), offline samples were taken from parallel flasks to obtain the consistency indexes $K$ and the flow behavior indexes $m$ at seven distinct time points. At these time points, the respective volumetric power input values were taken, completing all required parameters to calculate the effective shear rates with the new shear rate correlation (Equation 4.3). As shown in Figure 4.11 B, the fermentation process is characterized by effective shear rates of between 1100 s⁻¹ (beginning) and 450 s⁻¹ (end of fermentation). Based on the obtained effective shear rates, the apparent viscosities were determined applying Equation 3.2. Only a slight increase in apparent viscosity could be observed up to 24 h. Up to the depletion of the carbon source at 48 h, a sharp increase to 18 mPa·s was found, with the apparent viscosity thereafter remaining almost constant.

Figure 4.11 C illustrates the $Re$ number, $Ph$ number and exponent $-y$ ($Ne'\sim Re^{-y}$ proportionality, Equation 2.11) as a function of fermentation time. All these parameters are now assessable via the newly-established shear rate correlation. The $Re$ numbers indicate that a turbulent flow regime can be presumed to exist only at the very beginning of fermentation, because the critical value of 60,000 (Figure 2.2) was exceeded solely during the first few hours of the process. Due to the fact that the exponent $y = -0.28$ at $t = 0$ h is close to the “turbulence flow term” (-0.2) (Equation 2.11), turbulence is also confirmed by the $Ne'\sim Re^{-y}$ proportionality.
Figure 4.11. *Trichoderma reesei* QM9414 fermentation in 250 mL shake flasks with 25 mL filling volume at 200 rpm, 50 mm shaking diameter, and 28°C. A: Oxygen transfer rate (thin lines, duplicates) and online volumetric power input (bold line) measured via a power input shaker as a function of fermentation time. B: Effective shear rates (stars) as a function of fermentation time calculated using the newly-developed shear rate correlation (Equation 4.3). For Equation 4.3, $K$ and $m$ and the prevailing volumetric power input values at the sampling time points were applied. The apparent viscosities (squares) were determined using the obtained effective shear rate data (stars) and Equation 3.2. C: The $Ph$ numbers as a function of fermentation time are indicated by open circles and the critical Phase number $Ph_{krit}$ by a dashed arrow. With $Ne'\sim Re^\gamma$ (Equation 2.11), the $Re$ numbers and the exponents $-\gamma$ enable the estimation of the liquid flow regimes occurring during fermentation. *Reprint from Giese et al. (2014c) with permission from Elsevier.*
With increasing apparent viscosity, the $Re$ number dropped down to an approx. constant level of 2,000 between 40 h and 90 h. During this period, the exponents $y$ reached values between $y = -0.40$ and $y = -0.43$, with the $Ne' \sim Re^{-y}$ proportionality revealing an intermediate between transitional and turbulent flow regime (Equation 2.11). The $Ph$ numbers varied between 4.8 at the beginning and 2.6 at the end of fermentation, indicating that the fermentation broth was permanently shaken at “in-phase” conditions.

Figure 4.12 presents results based on an A. vinelandii fermentation conducted by Peña et al. (2007) in 500 mL shake flasks. The culture was shaken at 200 rpm with a shaking diameter of 25 mm. With an initial value of 0.18 kW/m³, the online volumetric power input remained constant for the first 20 h, before increasing exponentially until approx. 42 h to a maximum value of 1.3 kW/m³ (Figure 4.12 A). Thereafter, the volumetric power input slightly decreased. Since broth samples were analyzed by Peña et al. (2007) with regard to both $K$ and $m$, it is now possible using the new shear rate correlation to determine the effective shear rates: During the first 20 h, the effective shear rate decreased only slightly from 420 to 390 s⁻¹, before falling sharply to only 90 s⁻¹ at 40 h. Thereafter, the effective shear rate remained at a level below 90 s⁻¹. At 40 h, Peña et al. (2007) visually observed the “out-of-phase” phenomenon, with the fermentation broth beginning to remain on the shake flask’s bottom. The authors stipulated the critical Phase number $Ph_{crit} = 1.26$ for this specific time point, and recalculated the viscosity. Using this viscosity they estimated an effective shear rate of 90 s⁻¹.

Figure 4.12 B displays two viscosity curves, one representing the viscosity at a constant shear rate of 90 s⁻¹ as already presented by Peña et al. (2007) and the second illustrating the apparent viscosity calculated using the new shear rate correlation. Starting from waterlike viscosity, the apparent viscosity increased exponentially to 50 mPa·s at 40 h, followed by a linear increase until 70 h. The value of 50 mPa·s at 40 h is equal to that recalculated by Peña et al. (2007) using 90 s⁻¹. Due to their visual observation of the “out-of-phase” phenomenon, the authors were able to determine the correct effective shear rate for this particular time point. The correct visual observation is proven in the present study by the $Ph$ numbers calculated based on the effective shear rate correlation, with Figure 4.12 B revealing that the $Ph$ number at 40 h is equal to the critical value of 1.26. Hence, the visual observation of the phenomenon and the calculated $Ph$ number are consistent with each other.
Figure 4.12. *Azotobacter vinelandii* fermentation in 500 mL shake flasks with 100 mL filling volume at 200 rpm, 25 mm shaking diameter, and 29°C (Peña et al., 2007). A: Online volumetric power input and effective shear rates as a function of fermentation time. Effective shear rates (stars) were determined using the new shear rate correlation (Equation 4.3), and online volumetric power input measurement via a power input shaker. B: The effective shear rates were used to determine the apparent viscosities (squares), which are compared to the viscosities at a constant shear rate of 90 s⁻¹ (diamonds). At 40 h the system was visually observed to be “out-of-phase” by Peña et al. (2007). At this time point, the Ph number (circles, Equation 2.13), calculated based on the new shear rate correlation (Equation 4.3) and the resulting apparent viscosity (squares), falls below the critical Ph number of 1.26 (dashed arrow and dashed area). *Modified reprint from Giese et al. (2014c) with permission from Elsevier.*

Figure 4.13 displays the result of a *X. campestris* fermentation conducted in 500 mL shake flasks with 4% relative filling volume. The culture was shaken at 200 rpm with a shaking diameter of 25 mm. After an exponential growth phase the culture experienced an oxygen limitation at 23 mmol/L/h and 12 h of fermentation (Figure 4.13 A). Subsequently, after 16 h a slow but continuous decrease in the OTR indicates another problem: Immediately before
14 h, the online volumetric power input decreased sharply from a maximum of 1.16 kW/m³ to only 0.65 kW/m³ (Figure 4.13 A). The reason for this sharp decrease is shown in Figure 4.13 B, which presents the effective shear rates determined using the new shear rate correlation: The effective shear rates decreased from 1400 s⁻¹ at 0 h to 750 s⁻¹ at 14 h, respectively. The latter value resulted in an apparent viscosity of 8 mPa·s at 14 h, leading to a Ph number of 1.26. Hence, the sharp decrease in the online volumetric power input must be explained by the “out-of-phase” phenomenon ascertained for the period between 14 h and 48 h. This phenomenon is the reason for the slow OTR decrease observed after 16 h. Since the apparent viscosity still increased, the oxygen supply for the culture became worse with time.

Figure 4.13. *Xanthomonas campestris* fermentation in 500 mL shake flasks with 20 mL filling volume at 200 rpm, 25 mm shaking diameter, and 30°C. A: Online volumetric power input and OTR as a function of fermentation time. B: Effective shear rates (stars), determined using the new shear rate correlation (Equation 4.3), apparent viscosities (squares, Equation 3.2) and the Ph numbers (circles, Equation 2.13) as a function of fermentation time. The online volumetric power input was measured via a power input shaker. At 13.5 h, the Ph number falls below the critical value of 1.26 (dashed area). Reprint from Giese et al. (2014c) with permission from Elsevier.
4.2.5 Comparison of effective shear rates in shake flasks and stirred tanks

Figure 4.14 presents a comparison of the effective shear rates and apparent viscosities associated with shake flasks and stirred tanks. If equal volumetric power input is presumed, the quotient of the power input correlations for shake flasks and stirred tanks, Equation 4.3 and 2.16, respectively, leads to:

$$\frac{\dot{\gamma}_{\text{eff,flask}}}{\dot{\gamma}_{\text{eff,tank}}} = \frac{L_{\text{flask}}^{\frac{1}{m+1}}}{L_{\text{tank}}^{\frac{2}{m+1}}} \left(\frac{V^{1/3}}{d}\right)^{\frac{x}{m+1}} = \left(\frac{\eta_{\text{app,flask}}}{\eta_{\text{app,tank}}}\right)^{\frac{1}{m-1}}. \tag{4.5}$$

With $L_{\text{flask}} = 2.06$, $L_{\text{tank}} = 1$ and $x = -0.331$ (Section 2.2.2 and 4.2.2), the quotients of effective shear rates and apparent viscosities were calculated as a function of the flow behavior index $m$.

Figure 4.14. Quotient of effective shear rates (open symbols) as well as the quotient of apparent viscosity (filled symbols) in shake flasks and stirred tanks as a function of the flow behavior index $m$. Calculations were conducted for three different relative filling volumes in shake flasks: 5% (stars), 20% (squares), and 40% (circles). For comparison between shake flasks and stirred tanks, equal volumetric power inputs and equal broths with equal consistency index $K$ were implied. The results are valid for 100, 250 and 500 mL shake flasks. Reprint from Giese et al. (2014c) with permission from Elsevier.
Figure 4.14 illustrates that effective shear rates in shake flasks are in general higher and, therefore, the apparent viscosities of pseudo-plastic broths are lower, compared to those in stirred tanks. This is an extremely important finding for scale-up and -down. It can also be observed that low relative filling volumes in shake flasks lead to higher discrepancies between shake flasks and stirred tanks.

In the case of a Newtonian fluid ($m = 1$), the effective shear rate in a shake flask filled with 40% relative filling volume is 1.55 times higher than that in stirred tanks. If the shake flask is filled with only 5%, the discrepancy is higher at 1.75 times. In the case of a Newtonian broth, these discrepancies do not influence apparent viscosity, which is then equal in both bioreactors. However, the more pseudo-plastic the fermentation broth is, the greater is the discrepancy between the apparent viscosity in shake flask and stirred tank. At $m = 0.2$, the effective shear rate is more than 2.5 times higher in shake flasks with a relative filling volume of 5%. Depending on the filling volume, the apparent viscosity in shake flasks is then approx. half of that in stirred tanks.

Figure 4.15 shows comparisons between effective shear rates in shake flasks and stirred tanks for three applied examples: A waterlike culture medium at a fermentation’s start, a pseudo-plastic $S. tendea$ fermentation broth and a pseudo-plastic $A. vinelandii$ fermentation broth. Shake flasks with 250 mL nominal flask volume and 5% relative filling volume were presumed.

The two left bars represent culture medium at the beginning of a fermentation with waterlike Newtonian viscosity ($K = 1 \text{ mPa}\cdot\text{s}^m$, $m = 1$). Effective shear rates of 3960 s$^{-1}$ for shake flasks (leftmost bar, dark, Equation 4.3) are prevailing, whereas 2240 s$^{-1}$ have to be expected in stirred tanks (left, striped bar, Equation 2.16). Also confirmed by Figure 4.14, the effective shear rate in shake flasks is 1.77 times higher under these “extreme” conditions. Since the minimum reasonable viscosity and filling volume were selected, the effective shear rate of 3960 s$^{-1}$ is the upper boundary achievable in 250 mL shake flasks. In addition, the power input of 5 kW/m$^3$ is equal to 382 rpm which is in the upper range of possible shaking frequencies on high-performance shakers at 50 mm shaking frequency.

The numbered factors of the displayed shear rate correlation for shake flasks (no. 1, 2 and 3 in Figure 4.15) relate to the accordingly numbered arrows highlighted in the shear rate bars. The arrows demonstrate the impact of the respective terms on the resulting effective shear rate.
RESULTS - Effective Shear Rates in Shake Flasks

Figure 4.15. Effective shear rates in 250 mL shake flasks and stirred tanks at an exemplary power input of 5 kW/m³ were calculated based on the shear rate correlations for shake flasks (Equation 4.3) and stirred tanks (Henzler and Kauling, 1985; Equation 2.16). A waterlike culture medium at $t = 0$ h, a *Streptomyces tendea* sample (Henzler and Schedel, 1991) and an *Azotobacter vinelandii* sample (Figure 4.12, $t = 51$ h, originally fermented in a 500 mL flask) are compared. The effective shear rates, the consistency indexes $K$ and the flow indexes $m$ were used to determine the apparent viscosities. The numbered factors of the shake flask shear rate correlation (no. 1, 2 and 3) relate to the accordingly numbered arrows highlighted in the shear rate bars.

The two middle bars represent effective shear rates determined for a *S. tendea* sample with $K = 760$ mPa·s$^m$ and $m = 0.4$ analyzed by Henzler and Schedel (1991). The power input concepts reveal effective shear rates of 1210 s$^{-1}$ and of 530 s$^{-1}$ for shake flasks and stirred tanks, respectively. Hence, the effective shear rate is 2.28 times higher in shake flasks (see also Figure 4.14). Since the investigated fermentation broth shows pseudo-plastic flow behavior, this difference in effective shear rates results in different apparent viscosities for both bioreactors. Whereas the apparent viscosity in shake flasks is only 11 mPa·s, it is 18 mPa·s in a stirred tank.
The two right bars relate to an *A. vinelandii* broth sample with $K = 8416$ mPa·s$^m$ and $m = 0.4$ (obtained after 51 h of fermentation in 500 mL shake flask; Figure 4.12). Such values result in an effective shear rate of $210$ s$^{-1}$ for 250 mL shake flasks with 5% relative filling volume and $93$ s$^{-1}$ for stirred tanks. The high flow consistency index value $K$ and the relatively low effective shear rates indicate a high apparent viscosity. As can be seen from Figure 4.15, values of 355 mPa·s and 574 mPa·s prevail in shake flask and stirred tank, respectively. This is in consistency with Figure 4.14 which predicts an apparent viscosity of only 62% in shake flasks compared to stirred tanks ($m = 0.4$, 5% relative filling volume). As the apparent viscosity influences mixing as well as mass and heat transfer processes, the differences in effective shear rates are valuable to explain existing deviations in production and screening results.
4.3 A New Microtiter Plate Concept

In conventional MTPs, high shaking frequencies and low filling volumes must be applied to achieve sufficient oxygen supply for microorganisms. In spite of that, such low filling volumes may be insufficient for the offline analytics during screening procedures. Therefore, a novel concept for MTPs preventing the spill-out of the rotating liquid even at high filling volumes is presented in this section.

4.3.1 Idea and Geometry

The novel concept was constructed for three formats: 24-well, 48-well and 96-well. Figure 4.16 shows an exploded assembly drawing of the 24-well format illustrating the plastic cuboid with its countersank wells.

![Figure 4.16. Exploded assembly drawing of the new MTP concept in 24-well format. It consists of three components: plastic cuboid (top), bonding sheet (middle) and bottom plate (bottom).](image)

The technical drawing of the novel 24-well MTP is displayed in Figure 4.17, whereas the drawings of the 48-well and 96-well formats are included in the Appendix of this work. As illustrated in Figure 4.17, each well is equipped with a top cover drilled open along the respective cylindrical symmetry axis – the main enhancement of the novel concept. The diameter of the resulting hole in the top cover was selected so that a pipette tip reaches the bottom of each well, allowing a complete emptying of the MTP. Ensuring the compatibility to devices like MTP reader, BioLector® and microtiter plate RAMOS, the outer geometries of the new MTPs were chosen to be consistent with conventional
MTPs. In contrast to the outer MTP geometries, the inner well diameters differ slightly. This is a result of the manufacturing process and is illustrated in Figure 4.18. The countersank wells of the new MTP type are cylindrical whereas the conventional wells, formed by injection molding, are slightly conic (91° - 92°). The well diameters of the new MTP type were manufactured according to the average well diameter of common MTPs. Since common milling tools are not available for odd diameters, these average well diameters were not attained exactly. However, the highest deviation resulted for the 48-well MTPs to only 2.9%; Here, the average well diameter of a conventional MTP is 11.66 mm and the well diameter of the new MTP type is 12 mm.

BioLector® devices require non-transparent well-walls for optical analytics across the transparent well-bottom. In view of potential application in those devices, the new 48-well and 96-well MTPs were manufactured from black polyoxymethylene (POM). The POM cuboid was fixed to a polycarbonate bottom plate by an adhesive double-sided bonding sheet (DuploCOLL® 3702 F, Lohmann, Neuwied, Germany) particularly suitable for the conglutination of these two materials (Figure 4.16). These sheets with a thickness of only 180 µm were laser-cut to the respective MTP geometry (dLS Lichtschneiderei, Aachen, Germany). For potential visual observation of microbial pellet formation, the new 24-well MTP was manufactured out of clear polymethylmethacrylate (PMMA/acrylic glass). To stick PMMA to the polycarbonate bottom plate, the double-sided Duplobond® 3605.2 Plus from Lohmann (thickness: 112 µm) was selected.

### 4.3.2 Spill-out experiments

Operating conditions causing spill-out of conventional MTPs promise the highest potential for the new MTP type. The operating range of conventional MTPs is limited by shaking frequencies and filling volumes leading to spill-out of the fermentation medium. Blotting paper was used to determine the “spill-out shaking frequencies” for common filling volumes. The approach is shown in Figure 4.19: The rows of a conventional MTP were filled with different filling volumes and covered with blotting paper. The shaking frequency was increased stepwise in 5 rpm steps. Once the blotting paper of a row has been wetted, this shaking frequency was determined to be the spill-out shaking frequency for the respective filling volume. The spill-out experiments were conducted with a 1 M sodium sulfite system described in Section 3.3.2 (without the toxic cobalt) to obtain the same surface tension like in the \( OTR_{max} \)-investigations described later.
Figure 4.17. Technical drawing of the novel MTP concept in 24-well format.
Figure 4.18. Detailed well geometries of the new MTP type in comparison to conventional well geometries.
RESULTS - A New Microtiter Plate Concept

Figure 4.19. Exemplary photograph of spill-out experiments in a 24-well MTP at 3 mm shaking diameter. Left (shaking frequency 810 rpm): The liquid filled in a row with 1400 µL touched the blotting paper, whereas with 1200 µL the blotting paper remained dry. Right (shaking frequency 870 rpm): A further stepwise increase of the shaking frequency also revealed the spill-out shaking frequency for a filling volume of 1200 µL.

Figure 4.20 shows the results of the described spill-out experiments. Typical shaking frequencies higher than the critical shaking frequency (Hermann et al., 2003) were chosen at 50 mm and 3 mm shaking diameter, respectively. For better comparability between 24-well, 48-well and 96-well MTPs, the filling volumes are expressed as relative filling volumes, related to the respective complete well volume. The complete well volume is 3473 µL for a 24-well MTP, 1762 µL for a 48-well MTP and 395 µL for a 96-well MTP.

As illustrated by fits (Figure 4.20), the relation between the relative filling volume and the spill-out shaking frequency is approximately linear. Combinations of relative filling volume and spill-out shaking frequency which are located above such a linear fit, cause spill-out and mark the potentially advantageous sector for the new MTP type. At equal shaking frequencies, spill-out in 24-well MTPs occurs at lower relative filling volumes than in 48-well MTPs. Similarly, a 48-well MTP spills out at lower relative filling volume than a 96-well MTP. For instance, a 24-well MTP can only be filled with 28% (972 µL), whereas a 96-well MTP can be filled with 80% (316 µL) before it spills out at 300 rpm and 50 mm shaking diameter. Two aspects must be assumed to be the major
reasons: First, the height/diameter ratio is 1.8 in case of 96-well MTPs, 1.4 for 48-well MTPs and only 1.1 for 24-well MTPs. Intuitively, a low height/diameter ratio abets early spill-out. Second, the impact of the surface tension increases with decreasing well diameter (Doig et al., 2004) counteracting spill-out.

In the case of 24-well MTPs, spill-out experiments were additionally conducted with a surface treated (hydrophilic) MTP. As can be seen from Figure 4.20, the surface tension has a significant influence: Due to a more distinctive wetting of hydrophilic well walls, the spill-out of such hydrophilic wells occurs at lower filling volumes than in conventional hydrophobic wells. However, the influence seems to diminish with increasing shaking frequency. Since surface tension also varies with the MTP material and its surface structure, the translation of the shown spill-out results to others than the here applied MTPs should be treated with caution.

Figure 4.20. Dependency between relative filling volume and spill-out shaking frequency. 96-well, 48-well and 24-well MTPs at 3 mm and 50 mm shaking diameter were considered. The relative filling volume is related to the complete well volume, which is 395 µL for a 96-well MTP, 1762 µL for a 48-well MTP and 3473 µL for a 24-well MTP. In case of 24-well MTPs, a common MTP is compared to a surface treated MTP (hydrophilic). As liquid a 1 M sodium sulfite system (without cobalt) was applied.
4.3.3 \( \text{OTR}_{\text{max}} \) in conventional and new MTP type

Figure 4.21 illustrates very first, preliminary \( \text{OTR}_{\text{max}} \)-measurements with a 1 M sodium sulfite system using the new MTP type. The measurements were conducted in a microtiter plate RAMOS device (Section 3.4.2) without sealing foils at a shaking diameter of 50 mm. \( \text{OTR}_{\text{max}} \)-values are shown for conventional MTPs and the new MTP type as a function of shaking frequency. For 96-well (Figure 4.21 A), 48-well (B) and 24-well MTPs (C) broad ranges of filling volumes were investigated. For the great majority, measuring points identified for the conventional and the novel MTP type could be fitted by exponential fits. Exponential \( \text{OTR}_{\text{max}} \)-characteristics have already been described by Hermann et al. (2003) and Kensy et al. (2005) for conventional MTPs.

In case of 96-well MTPs, \( \text{OTR}_{\text{max}} \)-levels of the new MTP type are in general lower compared to those of conventional MTPs (Figure 4.21 A). With respect to all investigated filling volumes, the \( \text{OTR}_{\text{max}} \)-levels did not increase significantly until a shaking frequency of 300 rpm. At higher shaking frequencies, conventional 96-well MTPs spill out when filled with 260 µL or more. However, at 260 µL an \( \text{OTR}_{\text{max}} \) of only 3.5 mmol/L/h was measured. The new MTP concept eliminates such limitations: Without a spill-out of the liquid, these MTPs can be shaken with every filling volume that is lower than the total well volume at the shaker’s maximum frequency. For the exemplary filling volume of 260 µL, 12.5 mmol/L/h were achieved at 400 rpm. This is an increase by factor 3.5 compared to the maximum of the conventional MTP at the same filling volume. Between 350 rpm and 400 rpm, the low increase of the \( \text{OTR}_{\text{max}} \) for a filling volume of 320 µL cannot be approximated by an exponential fitting. This low \( \text{OTR}_{\text{max}} \)-increase is not yet understood but may be related to a phenomenon discussed by Klöckner (2013): As soon as rotating liquid touches the top cover of a shaken vessel, Klöckner observed linear increases in the \( \text{OTR}_{\text{max}} \). However, in the present study, for the new MTPs, such an \( \text{OTR}_{\text{max}} \)-course was solely observed for 320 µL filling volume.

With regard to Figure 4.21 B, enlarged operating ranges are now accessible with the new 48-well MTP type for filling volumes higher than 600 µL: Whereas with 200 µL the conventional MTP does not spill out until the maximum shaking frequency of 400 rpm, shaking with 600 µL is not feasible at frequencies higher than 300 rpm. With 600 µL, the conventional MTP is limited to 13 mmol/L/h before it spills out. In contrast, using the new MTP type, 32 mmol/L/h are reached at 400 rpm – an enhancement by factor 2.5.
Figure 4.21. Maximum oxygen transfer capacity $OTR_{\text{max}}$ as a function of shaking frequency and different filling volumes at a constant shaking diameter of 50 mm. Conventional MTPs (solid lines) are compared to the new MTP type (dashed lines): 96-well MTPs are shown in part A, 48-well MTPs in part B and 24-well MTPs in part C. As oxygen consumer a 1 M sodium sulfite system was applied. Between two different experiments, reproduction-tests have revealed an average reproducibility error of 5.5% for 24-well MTPs and 17.5% in case of 96-well MTPs. Duplicates in one experiment had an average deviation of 11%.
In case of 24-well MTPs four different filling volumes from 400 µL to 1600 µL were investigated. Applying the new MTP type at 400 rpm, new operating ranges are accessed for 800, 1200 and 1600 µL. Compared to the \( OTR_{\text{max}} \)-values at the spill-out frequencies, this equals an increase in the \( OTR_{\text{max}} \) by 31%, 64% and 88%, respectively.

In conclusion, extreme shaking conditions are needed for the novel 96-well type to be advantageous in comparison to the conventional 96-well MTP. The novel 48-well and 24-well formats are already advantageous at moderate shaking conditions. Since culture media usually have lower surface tension than the here applied sulfite system, advantages of the new MTP type should already be recognizable at even more moderate operating conditions – especially in case of 96-well MTPs where the surface tension plays a dominant role (Hermann et al., 2003).

Figure 4.22 illustrates preliminary \( OTR \) measurements with an \emph{E. coli} culture in the new MTP type. For a first proof of principle with a microbial application, the aim was to inspect both, the biocompatibility of the used materials and the attainable \( OTR_{\text{max}} \)-levels. The fermentations should provide a direct comparison between the new MTP type and conventional MTPs. 96-well and 24-well MTPs were chosen at 50 mm shaking diameter. It was presumed that certain volumes of fermentation broth are requested for later laboratory analytics: 300 µL per well from 96-well MTPs and 1000 µL per well from 24-well MTP. To avoid wetting of the MTP sealing tape, the conventional MTPs were shaken at 250 rpm. This is 60 rpm below the spill-out shaking frequency of both, the conventional 96-well MTP with 300 µL filling volume and the conventional 24-well MTP with 1000 µL filling volume (Figure 4.20). Using the new MTP types, cultures could be shaken at the shaker’s maximum frequency of 400 rpm.

Figure 4.22 A and B illustrate that lag-phases were slightly shorter in the new MTP-type. With respect to all four cultures a small \( OTR \)-peak was observed after 2 h of fermentation – most likely a result of metabolized complex components from the yeast extract or the tryptone. As can be seen from Figure 4.22 A, an \( OTR_{\text{max}} \) of 7.5 mmol/L/h was obtained in the conventional 96-well MTP and a value of 14.5 mmol/L/h in the new MTP type. This is approx. a duplication in the \( OTR_{\text{max}} \). The abnormal \( OTR_{\text{max}} \)-increase after 12 h in the conventional MTP was caused by a progressive wetting and contamination of the sealing tape.
RESULTS - A New Microtiter Plate Concept

Figure 4.22. *Escherichia coli* fermentations in 96-well MTPs (part A) and 24-well MTPs (part B) in TB medium. Oxygen transfer rates are shown as a function of fermentation time. The new MTP type is indicated by open symbols and conventional MTPs are represented by filled symbols. 96-well MTPs were filled with 300 µL inoculated medium (76% relative filling volume). 24-well MTPs were filled with 1000 µL (29% relative filling volume). The conventional MTPs were shaken at 250 rpm (spill-out shaking frequency is 310 rpm for both conventional MTPs, Figure 4.20). The new MTP type allowed shaking at the shaker’s maximum frequency of 400 rpm. The shaking diameter was 50 mm and the temperature was 37°C for all experiments.

Most likely the wetting was caused by gradual changes of the physicochemical properties of the fermentation broth; although a shaking frequency clearly lower than the spill-out frequency (determined with the sulfite system) was adjusted. Spill-out experiments with culture medium would allow a more precise prediction of the spill-out conditions for real microbial fermentations. However, such problems cannot occur at all when using the new MTP type.

Figure 4.22 B illustrates that \( OTR_{\text{max}} \)-levels of 23 mmol/L/h and 43 mmol/L/h are achieved in the conventional and the new 24-well MTP type, respectively. This again is
almost a duplication of the oxygen supply for the microorganisms. With the results discussed, the biocompatibility of the used materials is clearly demonstrated. Contact between the bonding sheet’s glue and the fermentation broth was obviously successfully avoided by using very thin sheets with exact laser-cut geometries. Beside the pure improvement of oxygen supply for microorganisms, additionally, higher $OTR_{\text{max}}$-levels in the new MTP-type reduce the fermentation time. This is an important factor for high-throughput screening systems to be operated economically.

Figure 4.23. $OTR_{\text{max}}$ of *Escherichia coli* in TB-medium as a function of shaking frequency in the new MTP type. Fermentations were conducted in 24-well MTP with 400 µL (triangles), 48-well MTP with 400 µL (diamonds) and 96-well MTP with 180 µL (circles). The dashed lines represent fitting curves from sulfite experiments (Figure 4.21) multiplied by a proportionality factor of 2.4. All experiments were conducted in the new MTP type.

By the experiments illustrated in Figure 4.23, $OTR_{\text{max}}$-values obtained with a culture broth were correlated linearly by a constant factor to the values obtained with the sulfite system. Since the culture medium has a lower surface tension compared to the sulfite system, it is not self-evident that using a constant factor is feasible. Values for the surface tension of 44 to 54 mN/m are common in culture media (Marshall, 1924) and approx. 74 mN/m is reported for a 0.5 M sodium sulfite system (Vázquez et al., 1995).
In the study at hand an even higher difference must be expected because a 1 M sulfite system was applied here. The difference in surface tension between culture medium and sulfite system results in a larger oxygen transfer area for the culture medium when such liquids are shaken. This effect may potentiate with increasing shaking frequency.

For Figure 4.23, the $OTR_{max}$ of *E. coli* cultures in the new MTP type were measured while the shaking frequency was increased (Figure 4.23, preparation described in Section 3.3.3). Each shaking frequency was held for 3 h, leading to six $OTR_{max}$-values per shaking frequency. In Figure 4.23 the respective averaged $OTR_{max}$-values of the culture broths in 24-well, 48-well and 96-well MTPs are indicated by open symbols. The dashed curves result from exponential fits of the sulfite results (partially shown in Figure 4.21) multiplied by a proportionality factor of 2.4. The value of 2.4 was found to be best suited for the correlation of the biological $OTR_{max}$-data applying the sulfite results. It should be stressed again that the dashed fits do not origin from the $OTR_{max}$-data obtained from the *E. coli* fermentations but from the sulfite $OTR_{max}$-results (Figure 4.21).

Figure 4.23 illustrates that the sulfite experiments allow to approximate and predict the $OTR_{max}$ of fermentations in the new MTP type to a certain accuracy. No systematic deviation can be ascertained. The highest deviation is prevailing for the 96-well MTP at 300 rpm. Here, 20 mmol/L/h were measured with the *E. coli* culture and 13 mmol/L/h are predicted by the sulfite system. All other measured $OTR_{max}$-values from *E. coli* fermentations show a mean deviation of only 3.5 mmol/L/h to the fit curve originally obtained from the sulfite experiments. This is a low deviation when taking into account that only one experimental run was conducted. Obviously, the physicochemical effect of different surface tensions is here sufficiently considered by the constant proportionality factor of 2.4. Hence, for the future, the sulfite system is a suitable tool for further and more thorough characterization of the new MTP type.
4.4 Scale-Down from Shake Flask to Microtiter Plate

In this section the scale-down of *T. reesei* from shake flask to MTP is presented. For preparation, an established industrial *T. reesei* shake flask protocol was analyzed and improved with respect to various process parameters. With this information, the MTP format which is most suitable as the future screening system for the filamentous fungus *T. reesei* was selected. The selected MTP was characterized regarding its $OTR_{max}$ at various filling volumes and shaking frequencies. For this task, a microtiter plate RAMOS device together with a 1 M sodium sulfite system was applied. With the obtained dataset, the scale-down itself is conducted: The $OTR_{max}$ of the MTP fermentation was adjusted to the improved shake flask fermentation protocol.

4.4.1 Analysis and improvement of an established *T. reesei* shake flask protocol

Prior to the scale-down, an established standard protocol for an industrial shake flask screening was analyzed with respect to the oxygen supply, dry cell weight, active glucoamylase concentration, pH, viscosity and morphology. In Figure 4.24 the results of a *T. reesei* fermentation under these standard conditions are indicated by the dashed lines. A volume of 50 mL of inoculated medium 1 was shaken at 200 rpm, at a shaking diameter of 50 mm and at a temperature of 28°C. Figure 4.24 A depicts an exponential increase in the $OTR$ within the first 24 h. Thereafter, the culture experiences an oxygen limitation, remaining at an $OTR_{max}$-level of approx. 13 mmol/L/h. At 50 h, the carbon sources were depleted (data not shown) and the $OTR$ decreased. At the same time point, the production of glucoamylase started. After 165 h of fermentation, a glucoamylase concentration of 25 a.u. (arbitrary unit) was determined with a pNPG-assay. Until 50 h, the dry cell weight increased to approx. 18 g/L. After carbon source depletion, the dry cell weight remained almost constant. To improve the standard shake flask screening protocol, the low $OTR$-level at standard conditions implies that the filling volume has to be decreased in order to raise the oxygen supply for the microorganisms. Thus, only 25 mL of the cultures were shaken at 200 rpm, 50 mm and 28°C. To obtain at least the same total amount of glucoamylase at the reduced filling volume, the carbon source and ammonium concentrations were doubled to enhance product formation.
Figure 4.24. Comparison of two different *Trichoderma reesei* GA8 shake flask protocols, both at a shaking frequency of 200 rpm, a shaking diameter of 50 mm and a temperature of 28°C. Standard protocol (dashed lines): 50 mL filling volume of medium 1. Improved protocol (solid lines): 25 mL filling volume of medium 2. A: OTR (lines), dry cell weight (diamonds) and active glucoamylase concentration (circles). B: pH (squares), effective shear rate (open triangles) and apparent viscosity (filled triangles). C: Mean particle size (triangles) and number of particles (stars). Range for particle size measurement was between 2 µm and 28 µm. Particle number only accounts for those particles in that range. The sizes of spores in the inocula were between 2.5 µm and 4 µm (not shown). *Modified reprint from Giese et al. (2014b) with permission from Elsevier.*
RESULTS - Scale-Down from Shake Flask to Microtiter Plate

By solid lines, Figure 4.24 A depicts the OTR, dry cell weight and glucoamylase activity of this improved protocol. The figure shows an exponential increase in the OTR with a slightly shorter lag-phase compared to that of standard conditions. Oxygen limitation under the improved conditions was reached at 24 h at a measured \( OTR_{\text{max}} \)-level of about 18.5 mmol/L/h, which is 1.4 times higher than that of the standard conditions. The further increase in the OTR during oxygen limitation between 24 h and 50 h is probably due to the recently found viscosity effect (Section 4.1). After 50 h, the OTR drops down because of C-source depletion (data not shown). The maximum dry cell weight concentration of 31 g/L was 1.8 times higher than that attained at standard conditions. This maximum dry cell weight was measured at 72 h, which is also the starting time for glucoamylase production. With 98 a.u., the glucoamylase concentration at 165 h was 3.9 times higher under the improved conditions compared to standard conditions. Since the filling volume was halved, approx. the double amount of active glucoamylase was obtained. This is a striking result attained from only small protocol modifications as described above.

Figure 4.24 B illustrates the pH, the effective shear rate and the apparent viscosity over time. A decrease in pH-values from 5.50 to 3.94 within the first 50 h was measured under standard conditions. Until 90 h the pH-value increased and remained almost constant at approx. pH 4.6. Under the improved conditions the pH decreased to a value of 2.73 at 70 h. Since the pKₐ-values of PIPPS buffer used in the culture medium are 3.73 and 7.96 (at 25°C) the measured pH-value of 2.73 represents the lower buffer boundary (Yu et al., 1997). Thereafter, the pH increased continuously to a value of 3.85 at 165 h. The effective shear rates were calculated applying the newly developed shear rate correlation Equation 4.3. Values were ranging between approx. 200 s\(^{-1}\) (begin of fermentation), 45 s\(^{-1}\) (43 h) and approx. 80 s\(^{-1}\) (end of fermentation) under standard and improved conditions, respectively. The effective shear rates were used in order to determine apparent viscosities. The culture which grew under standard conditions reached its measured viscosity maximum of 180 mPa·s at 43 h. Under improved conditions, the maximum viscosity of 167 mPa·s was measured at the same time point. Between 48 h and 130 h clear differences with respect to the viscosity were determined between the standard and the improved protocol. This may be correlated to differences in dry cell weight and fungal cell morphology. The latter is represented in Figure 4.24 C as mean particle sizes and number of particles. Until approx. 130 h, the number of particles...
is generally lower under standard conditions. This is attributed to the lower biomass concentration (Figure 4.24 A) and larger particles (Figure 4.24 C) due to less segmentation. Under the improved conditions, the large number of small particles which interact with one another results in higher viscosities compared to the standard conditions.

The parameters viscosity, mean particle size and number of particles are important for the selection of a suitable MTP type for screening of the filamentous fungi *T. reesei*. To mix the viscous liquid properly, the power input into the liquid has to be high enough. This can be mainly achieved by choosing the appropriate well diameter and shaking frequency, which has already been shown by Zhang et al. (2008) and Büchs et al. (2000b). Due to surface growth caused by the high viscosity of *T. reesei* cultures (Figure 4.24 B), the very common combination of 96-well MTPs and 3 mm shaking diameter was not applied in this study. Instead 24-well MTPs were shaken at 50 mm shaking diameter. In contrast to 96-well and 48-well MTPs, a 24-well MTP offers enough volume per well in order to receive the desired amount of 700 µL supernatant from culture broth needed for the intended analytical procedure during the later industrial high-throughput screening.

### 4.4.2 Preparation for scale-down: Gas/liquid oxygen transfer in shake flasks and 24-well microtiter plates

Datasets of the $OTR_{max}$ under various shaking conditions are necessary for a systematic scale-down from shake flasks to a 24-well MTP. For this purpose, an oxygen-consuming 1 M sodium sulfite system was used. In the case of 250 mL shake flasks, the $OTR_{max}$ was previously determined as a function of filling volume, shaking frequency and shaking diameter (Maier et al., 2004; Seletzky et al., 2007). As shown in Figure 4.25 A, the $OTR_{max}$ increases with decreasing filling volume and increasing shaking frequency. For instance, at a low filling volume of 10 mL, $OTR_{max}$-values of 16 mmol/L/h and 37 mmol/L/h are obtained at 150 rpm and 350 rpm, respectively. Between 150 rpm and 350 rpm, the $OTR_{max}$ at a high filling volume of e.g. 75 mL varies between 2.5 mmol/L/h and 7 mmol/L/h.
RESULTS - Scale-Down from Shake Flask to Microtiter Plate

Figure 4.25. Maximum oxygen transfer capacity $OTR_{\text{max}}$ as a function of filling volume and shaking frequency in a 250 mL shake flask (A) and in a 24-well MTP (B). Experimental conditions: 22.5°C, 1 M sodium sulfite system, $10^{-7}$ M CoSO$_4$, 100 mM phosphate buffer, initial pH 8, shaking diameter 50 mm. The $OTR_{\text{max}}$ of the 250 mL shake flask (A) was characterized by Maier et al. (2004) and Seletzky at al. (2007). In this figure the shaking parameters of experiments presented in Figure 4.26 to Figure 4.28 are indicated by the numbered open circles. Reprint from Giese et al. (2014b) with permission from Elsevier.

The well filling volume of a 24-well MTP was varied between 0.25 mL and 1 mL. At a shaking diameter of 50 mm, the shaking frequency was varied between 150 rpm and 350 rpm. For 24-well MTPs, Figure 4.25 B illustrates the same qualitative dependency between $OTR_{\text{max}}$, filling volume and shaking frequency as shown in Figure 4.25 A for shake flasks. Furthermore, the $OTR_{\text{max}}$-range is in the same order of magnitude as for shake flasks. In the investigated range of shaking frequencies, the $OTR_{\text{max}}$ is e.g. between 19 mmol/L/h and 50 mmol/L/h at a respective filling volume of 0.25 mL and between 4 mmol/L/h and 16 mmol/L/h at a filling volume of 1 mL. The
numbered open circles indicate shaking conditions as illustrated in the following Figure 4.26 to Figure 4.28. The newly generated dataset of Figure 4.25 permits a scale-down from shake flask to 24-well MTP based on the oxygen consumption of the 1 M sodium sulfite system. However, in order to determine the proportionality factor between the $OTR_{max}$ of a 1 M sodium sulfite system and the $T. reesei$ media, microbial fermentations were run in shake flasks and 24-well MTPs under various operating conditions. Based on the generated sulfite data, this proportionality factor will allow predicting the $OTR_{max}$-level of a fermentation culture.

Figure 4.26. Identification of oxygen limitation during shake flask fermentations of *Trichoderma reesei* at various shaking parameters. The shaking diameter was 50 mm and the temperature 28°C. $OTR_{max}$-levels are used for the determination of proportionality factors as described in the text. The different levels of oxygen limitations ($OTR_{max}$) are indicated by horizontal dash-dotted lines. The respective numbers refer to the corresponding numbers in Figure 4.25. A: Medium 1 with 20 g/L glucose, 0.55 g/L sophorose and 5.2 g/L (NH$_4$)$_2$SO$_4$. B: Medium 2 with 36 g/L glucose, 1 g/L sophorose and 10.4 g/L (NH$_4$)$_2$SO$_4$. Reprint from Giese et al. (2014b) with permission from Elsevier.
Figure 4.26 depicts $OTR$-curves of shake flask cultures using medium 1 and medium 2, respectively. The label numbers at specific $OTR$-curves correspond to the respective label numbers in Figure 4.25. They indicate sodium sulfite experiments and biological cultures under exactly the same operating conditions. As explained below, the levels of the horizontal lines indicate the $OTR_{max}$ which is equal to the beginning oxygen limitation of the respective cultures. The $OTR$-curves shown in Figure 4.26 A reveal differences in the duration of the lag-phase, which may result from small deviations in the inoculation solution due to the spores’ ages and concentrations. Figure 4.26 A shows a shake flask culture which was shaken at 350 rpm with 10 mL filling volume (dotted line). It was not oxygen-limited during the whole fermentation time. The two peaks clearly indicate a diauxic growth. The highest $OTR$ of this fungal culture was 51 mmol/L/h, which is even in the range of bacterial cultures (Kunze et al., 2012; Scheidle et al., 2007). All other chosen shaking conditions shown in Figure 4.26 A lead to oxygen-limited cultures. The culture shaken at 200 rpm with 25 mL (solid line) runs into oxygen limitation at 27 h (see also Figure 4.24 A). The obtained $OTR_{max}$-level was 19 mmol/L/h. Higher filling volumes of 37.5, 50 and 75 mL resulted in even lower $OTR_{max}$-levels (Figure 4.26 A).

Figure 4.26 B shows $OTR$-curves from shake flask cultures fermented in medium 2. A shaking frequency of 350 rpm and a filling volume of 10 mL, again, resulted in an oxygen-unlimited culture. This culture reached a highest $OTR$ of 62 mmol/L/h, which is 11 mmol/L/h higher than the highest $OTR$ of the culture grown in medium 1 under the same shaking parameters. This is attributed to the higher carbon source and ammonium concentration in medium 2 compared to those of medium 1. The two $OTR$-curves in Figure 4.26 B represented by solid lines clearly prove that the numbered horizontal dashed/dotted lines mark the beginning of oxygen limitations. Both cultures were shaken at 200 rpm with a filling volume of 25 mL. An ordinary oxygen gassing concentration of 21% (air) resulted in an $OTR_{max,react}$ of 18.5 mmol/L/h (bold curve). The thin curve shows that at a higher oxygen gassing concentration of 43% the limitation was reached at a higher $OTR_{max,react}$ of 40.5 mmol/L/h. This demonstrates that it is not a medium component which acts as the limiting factor, but rather the rate of oxygen which is transferred from the gas into the liquid phase.
A further proof that the numbered horizontal dashed/dotted lines mark the beginning of oxygen limitations is given in Figure 4.27. The *T. reesei* culture of Figure 4.26 A indicated with no. 3 (37.5 mL filling volume, 200 rpm) is compared to an *Arxula adeninivorans* H80 culture. Both cultures were grown under absolute identical fermentation conditions – even the culture medium was the same (medium 1). In contrast to *T. reesei*, the yeast *A. adeninivorans* does not grow filamentous. The yeast culture remained at waterlike viscosities proven by viscosity measurements (data not shown). A clear plateau can be observed for the *A. adeninivorans* culture indicating oxygen limitation. Since all parameters were kept constant and, considering that the level of oxygen limitation is independent from the fermented microorganism, this plateau also indicates the beginning oxygen limitation (*OTR*$_{\text{max}}$) of the *T. reesei* culture. With the results of Section 4.1, subsequent increases of the *OTR*$_{\text{max}}$-level have to be assumed to be the result of increasing viscosity.

![Image](image.png)

Figure 4.27. Check of starting oxygen limitation. *Trichoderma reesei* and *Arxula adeninivorans* fermented in 37.5 mL of medium 1 at 200 rpm and 50 mm shaking diameter. The *OTR*$_{\text{max}}$-plateau of the waterlike yeast broth indicates oxygen limitation at the selected shaking conditions. The numbering “3” relates to the numbering in Figure 4.25 and Figure 4.26.

Figure 4.28 depicts two *OTR*-curves of 24-well MTP cultures. Medium 2, a shaking diameter of 50 mm and a temperature of 28°C were applied. Whereas one culture was shaken with a filling volume of 0.73 mL per well at 300 rpm, the other culture was fermented at a well filling volume of 1 mL and 250 rpm. The chosen shaking conditions resulted in homogenously dispersed submerged cultures. Like in shake flasks, both
*T. reesei* cultures started their exponential growth at approx. 10 h and were oxygen-limited after 24 h. As a result of carbon source depletion, the OTR dropped down at 36 h and 50 h, respectively. The MTP fermentations were oxygen-limited at $OTR_{max}$-levels of 41.5 mmol/L/h and 27.5 mmol/L/h, respectively.

![Graph](image)

Figure 4.28. $OTR_{max}$ of *Trichoderma reesei* fermentations in 24-well MTPs as a function of fermentation time for the determination of the proportionality factor $f$, as explained in the text. Cultivations were carried out with 0.73 mL and 1 mL filling volume per well at shaking frequencies of 300 rpm and 250 rpm, respectively. The shaking diameter was 50 mm and the temperature 28°C. The horizontal dash dotted lines indicate the $OTR_{max}$-levels. *Reprint from Giese et al. (2014b) with permission from Elsevier.*

For equal shaking parameters, all obtained $OTR_{max}$-levels of *T. reesei* fermentations and 1 M sodium sulfite experiments are compared in Table III. The $OTR_{max,react}$-values taken from the $OTR$ measurements (Figure 4.25 to Figure 4.28) were normalized with Equation 2.2 to a headspace concentration of 21% oxygen. With Equation 3.3, the proportionality factor $f$ was determined to be ranging from 1.9 to 2.1 for shake flasks. This is in the range previously described by Klöckner and Büchs (2011). No clear difference in the proportionality factor $f$ could be observed between the slightly varied *T. reesei* media 1 and 2. Both MTP experiments resulted in proportionality factors of 2.3 and 2.4 which are slightly higher compared to those of shake flask experiments. A mean of all measurements lead to an average proportionality factor of 2.1 between the 1 M sodium sulfite system and both *T. reesei* media. Beside the fact that this proportionality factor $f$ enables to determine whether oxygen-limited conditions have to be expected or not, the theoretical $OTR_{max}$-level of a fermentation can be estimated. As a general
example, the oxygen-unlimited shake flask cultures shaken at 350 rpm with 10 mL have their highest measured $OTR$ at 51 mmol/L/h (medium 1) and 62 mmol/L/h (medium 2). Thereafter, the respective carbon sources were depleted (Figure 4.26). However, if more of the carbon sources would have been available, the cultures could have reached an $OTR_{\text{max}}$-level of 85.5 mmol/L/h, which indicate the oxygen limitation under the selected shaking conditions. This value was calculated with Equation 3.3 applying the $OTR_{\text{max}}$ of the sulfite system (Figure 4.25) and, particularly, applying the newly determined average proportionality factor $f$ of 2.1.

Table III. Fermentation conditions and $OTR_{\text{max}}$-data for the calculation of the proportionality factors $f$ (Equation 3.3). The measured $OTR_{\text{max,react}}$-values were taken from the fermentations shown in Figure 4.25 to Figure 4.28. The $OTR_{\text{max}}$ (normalized to an oxygen headspace concentration of 21%) is calculated by Equation 2.2. The required headspace concentration $y_{O_2,\text{headspace}}$ was taken from the respective measured RAMOS data. Reprint from Giese et al. (2014b) with permission from Elsevier.

<table>
<thead>
<tr>
<th>Curve label in Fig. 4.25 to 4.28</th>
<th>Fermentation system</th>
<th>Medium</th>
<th>Filling volume [mL]</th>
<th>Shaking freq. [rpm]</th>
<th>$OTR_{\text{max,react}}$ [mmol/L/h]</th>
<th>$OTR_{\text{max}}$ [mmol/L/h]</th>
<th>Proport. factor $f$</th>
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<td>200</td>
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<td>200</td>
<td>18.5 10.0</td>
<td>20.2 10.4</td>
<td>1.9</td>
</tr>
<tr>
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<td>200</td>
<td>13 7.1</td>
<td>14.3 7.4</td>
<td>1.9</td>
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<tr>
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<td>200</td>
<td>7.0 3.9</td>
<td>7.7 4.1</td>
<td>1.9</td>
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<td>350</td>
<td>- 38.2</td>
<td>85.5** 40.7</td>
<td>2.1**</td>
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<td>41.5 18.7</td>
<td>48.6 20.5</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* Oxygen-unlimited culture. The $OTR_{\text{max}}$ at these conditions is not reached during the experiment.

** This $OTR_{\text{max}}$ was calculated based on Equation 3.3, the sulfite-$OTR_{\text{max}}$ and the average medium factor of 2.1. Figure 4.26 demonstrates that the $OTR$ during these fermentations, by far, does not reach the level of oxygen limitation at $OTR_{\text{max}}$ of 85.5 mmol/L/h.
4.4.3 Scale-down of *T. reesei* from shake flask into 24-well microtiter plate

The improved shake flask protocol (Figure 4.24) was scaled-down to MTP. Hereby, a high-throughput screening was supposed to be accomplished for the filamentous fungus *T. reesei*. To achieve consistent growth conditions between both scales, the scale-down is based on a comparable oxygen supply ($OTR_{\text{max}}$) for the microorganisms. The shaking parameters of the improved shake flask protocol are 200 rpm, 25 mL filling volume and 50 mm shaking diameter (Figure 4.26 A, no. 2). For this parameter set, a shake flask $OTR_{\text{max}}$ of 10 mmol/L/h for the 1 M sulfite system can be taken from Figure 4.25 A. With the new MTP dataset shown in Figure 4.25 B, the respective conditions for the same oxygen supply in a 24-well MTP can be identified. Two combinations of parameters leading to 10 mmol/L/h are found. The combinations are 0.5 mL and 150 rpm as well as approx. 1 mL and 200 rpm. Since 700 µL of supernatant from culture broth are needed for later analytical procedures during the desired high-throughput screening, the combination 1 mL filling volume and 200 rpm shaking frequency was selected in this study (Figure 4.25 B) to achieve the maximum throughput. With the determined mean proportionality factor of 2.1, these parameters should lead to an $OTR_{\text{max}}$ of approx. 21 mmol/L/h in 24-well MTP fermentations – an $OTR_{\text{max}}$-level like in shake flasks.

![Comparison of improved Trichoderma reesei shake flask and 24-well MTP protocol at 200 rpm and at a shaking diameter of 50 mm. Filling volumes in shake flask and 24-well MTP were 25 mL and 1 mL, respectively. OTR for shake flask (solid line) and 24-well MTP (dashed line) show equal respiration activity as function of time as well as comparable $OTR_{\text{max}}$-levels. Equal respiration activity results in the same productivity in shake flask (filled circles) and MTP (open circles). Reprint from Giese et al. (2014b) with permission from Elsevier.](image)
With the shaking parameters identified above, 24-well MTP fermentations were carried out in a microtiter plate RAMOS device. Figure 4.29 shows the resulting OTR and product curves as a function of fermentation time (dashed lines). For comparison, the respective results of the improved shake flask protocol (solid lines) are plotted as well. It can be clearly seen that both cultures started their exponential growth after 10 h. At 24 h, both, the shake flask as well as the MTP cultures, reach their OTR\textsubscript{max}-level at 20 mmol/L/h. This is approximately the same value predicted above with the proportionality factor. During oxygen limitation, the OTR-curves show slightly different shapes. This might be attributed to the effect of viscosity on the gas/liquid oxygen transfer into liquid films adhering on shake flasks walls (Section 4.1). The OTR of both cultures decreased at nearly equal time points after 50 h and 55 h, respectively. The similar oxygen supply for the microorganisms resulted in consistent productivity in both scales. As shown in Figure 4.29, the main enzyme production did not start before 70 h. In the period between 115 h and 120 h, in both scales concentrations of approx. 80 a.u. active glucoamylase were determined with the pNPG-assay. Even after 165 h, almost equal values of 91 a.u and 98 a.u were determined in the shake flask and 24-well MTP, respectively.

The samples taken at 165 h were also examined qualitatively by a SDS-page analysis, shown in Figure 4.30. The leftmost position contains 15 µL of a molecular weight standard. The middle and right position were each filled with 20 µL prepared fermentation sample. The sample taken from the shake flask fermentation is depicted in the middle column and the MTP sample is shown in the right column (Figure 4.30). The glucoamylase with a molecular weight of 62 kDa clearly generates the biggest band in both samples. Only very little amounts of by-product were produced in shake flasks and MTPs. As was already shown with the activity assay, it is also apparent on the SDS-gel that similar amounts of glucoamylase were produced on both scales. These consistent and scale-independent concentrations obtained are very important, since – as long as the demonstrated systematic scaling procedure is applied – the most productive strain cultivated in a MTP is also the most productive strain in a shake flask.
Figure 4.30. Comparison of glucoamylase concentration of improved shake flask (middle column) and newly designed 24-well MTP protocol (right column) via a SDS-gel. Molecular weight of glucoamylase is 62 kDa. The samples were taken at 165 h (compare Figure 4.29). The concentrations were analyzed volumetrically. The leftmost column contained 15 µL glucoamylase standard. The two residual column were filled with 20 µL prepared SDS-sample containing 8 µL centrifuged culture broth. Reprint from Giese et al. (2014b) with permission from Elsevier.
5 CONCLUSION AND OUTLOOK

With Fick’s law of diffusion, oxygen concentration profiles in the liquid film on the wall of a rotating shake flask were numerically simulated. It has been proven that for shaking conditions frequently used for microbial fermentations, the oxygen concentration on the shake flask wall is not always 0 mol/m³. However, this assumption is the prerequisite of the applicability of Higbie’s film theory (Higbie, 1935); a theory which is widely applied to describe gas penetration (e.g. oxygen) into liquid films. At shaking conditions with an invalid “0 mol/m³-assumption” at the flask wall, Higbie’s film theory implies a larger driving concentration gradient than the realistic one. Consequently, Higbie’s film theory is not always applicable: It might overestimate the oxygen flux into the liquid film for thin films and long exposure times. It was experimentally shown by chemical and microbial reaction systems that the $OTR_{max}$ in shake flasks increases with increasing viscosities of up to 10 mPa·s. The oxygen transfer into the liquid film increases with elevating viscosity, because the thicker liquid film increases the driving oxygen concentration gradient as demonstrated via the numerical results. The increasing $OTR_{max}$ means that the oxygen supply for microorganisms is improved in rotating shake flasks at increasing medium viscosities of up to 10 mPa·s. Up to elevated viscosities of approx. 80 mPa·s, the $OTR_{max}$ does not significantly undermatch the $OTR_{max}$ at waterlike viscosities. By contrast, the oxygen supply in stirred tanks decreases to only 5% at 80 mPa·s. In practice, this has to be compensated by increasing agitation or gassing at the expense of energy costs. These results have significant implications for scale-up: The liquid film formation at shake flask walls inherently promotes the oxygen supply to the microorganisms at moderate and elevated viscosities. In future, a correlation between the apparent viscosity and the liquid film thickness at a shake flask’s wall should be developed.

The presented shear rate correlation (Equation 4.3) is the first approach to calculate the effective shear rate in shake flasks as a function of all decisive shaking parameters. The correlation is valid for nominal shake flask volumes ranging from 50 to 1000 mL. The shear rate increases with increasing shaking frequency and nominal flask volume, as well as with decreasing filling volume. Effective shear rates of between 20 s⁻¹ and 2000 s⁻¹ should be expected under common fermentation conditions in shake flasks. The flow regimes and Phase-numbers occurring during fermentations can now be quantified via
the presented shear rate correlation. Even peculiar effects observed in OTR curves of fermentations at elevated viscosity can now be interpreted on the basis of effective shear rates. Depending on the broth’s flow behavior index value, the effective shear rate in shake flasks is at least 1.55 times higher compared to that in stirred tanks operated at the same power input, leading to a possibly 50% lower apparent viscosity. As the apparent viscosity influences mixing as well as mass and heat transfer processes, the attained correlation is a valuable tool to explain existing deviations in production and screening results. These results will help to develop processes by means of consistent scale-up or scale-down procedures.

A novel MTP type was developed in 96-well, 48-well and 24-well format. The manufactured prototypes demonstrated the successful prevention of spill-out of the rotating liquid. Preliminary measurements of the $OTR_{max}$ were conducted in this novel concept and compared to those of conventional MTPs. The novel MTPs displayed advantages compared to the conventional ones at high filling volumes and high shaking frequencies – ranges where conventional MTPs spill out. These ranges were found to be dependent on the MTP format because surface tension gets more important the smaller the well diameter is. Whereas extreme shaking conditions are needed for the novel 96-well type to be advantageous compared to the conventional one, the novel 48-well and 24-well formats are already advantageous at moderate shaking conditions. The biocompatibility of the novel MTP type was demonstrated by E. coli fermentations. These cultures showed the potential to shorten fermentations because higher $OTR_{max}$ – leading to a faster C-source consumption – could be achieved. Conclusively, an efficient MTP concept was developed for applications where high sample volumes are desired. As yet, spill-out characteristics of conventional MTPs were solely determined for the applied sodium sulfite system. In future, such characteristics should additionally be determined for culture medium as this will allow a more precise prediction of the spill-out shaking frequency in cases of microbial fermentations. Since culture media usually have lower surface tension than the here applied sulfite system, advantages of the new MTP type should already be recognizable at even more moderate operating conditions. It has been demonstrated by Sieben (2013) that the constructed well openings of the new MTP, conveniently, will not constrain the $OTR_{max}$. Thus, it will be interesting to evaluate to which extent these openings are capable to reduce liquid evaporation. Ultimately, an automated counterbalancing shaker currently established at
AVT.Bioverfahrenstechnik will be a valuable tool to comprehend the full potential of the novel MTP type. Such a shaker accesses shaking frequencies which are currently not available with the best high performance machines. Due to the investigated spill-out characteristic, it is foreseeable that common MTPs are not suited for these high shaking frequencies. In contrast, the new MTP type will profit from higher shaking frequencies because larger gas/liquid transfer areas have to be expected.

A scale-down of an established industrial shake flask protocol into MTP was conducted in this work, because, today, in biotechnological industry the implementation of high-throughput screening procedures is a crucial issue. Due to the small well diameters preventing sufficient liquid movement, 96-well MTPs are unsuitable for screening of filamentous fungal strains such as *T. reesei*. As such cultures are highly viscous, and an appropriate volume of supernatant was needed for analytical procedures, 24-well MTPs were chosen in this study. An oxygen-consuming sodium sulfite system was used to generate $OTR_{max}$-datasets for shake flasks and MTPs. These sulfite datasets were then applied to identify those specific operating conditions for MTPs, leading to an $OTR_{max}$ of 20 mmol/L/h – like in shake flasks. The optimal operating conditions for 24-well MTPs are: 200 rpm shaking frequency, 1 mL filling volume, and 50 mm shaking diameter. These operating conditions are crucial to ensure uniform screening conditions among the different screening scales. This was verified by similar $OTR$-curves and enzyme productivities in both screening systems. The proposed approach combining a sodium sulfite system together with RAMOS is practical, easy and accurate. In the future, this approach can be applied to scale-down established screening procedures from shake flasks in order to achieve high-throughput.
REFERENCES


REFERENCES


Figure A.1: Technical drawing of the novel MTP concept in 48-well format.
Figure A.2: Technical drawing of the novel MTP concept in 96-well format.
On the author

Mr. Heiner Giese, born April 18, 1981 in Haan (Rhineland), Germany, studied Mechanical Engineering majoring Process Engineering at Duisburg-Essen University and RWTH Aachen University. He graduated in 2009 and afterwards joined the Bioprocess Engineering group of Prof. Dr.-Ing. Jochen Büchs at the Aachener Verfahrenstechnik, RWTH Aachen, where he wrote the thesis at hand. From 2009 to 2012 he hold a scholarship assigned by the Research-School “Fuels from Renewable Resources”, funded by the government of Northrhine-Westphalia.