

High-Throughput Experimentation for Microscale Cultivations and Recombinant Protein Expression

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Preface

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Abstract

In the postgenomic era, high-throughput processes for cultivating clone libraries become increasingly important, in particular for recombinant protein production. Such processes mostly consist of multiple steps (e.g. colony picking, preculture, main culture, induction) and are mainly conducted in microtiter plates (MTPs). However, with conventional techniques it is very tedious to study microbial growth and product formation in such complex processes. This is mainly due to a lack of appropriate monitoring capabilities and the extensive workload associated with manually conducted high-throughput cultivations.

Hence, the major aim of this thesis was to evaluate the suitability of the online-monitoring technique BioLector to study and optimize high-throughput cultivation processes, with a special focus on recombinant protein expression in *E. coli*. Furthermore, new applications in this area should be elaborated for the BioLector.

The effects of different tools and methods for the replication of cells from one MTP to another MTP on growth kinetics were observed with the BioLector. This resulted in highly variable growth kinetics and as a consequence gave strongly varying product formation upon induction at a defined point of time. To improve the reproducibility and comparability of parallel cultivations, different methods are recommended and elaborated in this thesis, e.g. applying fed-batch operation for equalizing precultures.

In industry and academic research, flexible automated microbioreactor platforms with advanced sensing technology are increasingly needed. To meet this demand, a novel system consisting of a BioLector and a liquid-handling robot (termed Robo-Lector) was successfully built and tested. Three unique exemplary methods were programmed on the Robo-Lector platform that effectively allowed to study in detail high-throughput cultivation processes and an *E. coli* expression system. Additionally, the Robo-Lector platform enabled to extensively study the effects of a phosphate limitation on the same expression system and helped to optimize its specific productivity for the recombinant target protein.

In summary, the results of this thesis demonstrated, that extensive monitoring of key parameters (i.e. biomass and product formation) in small scale cultivations, in particular MTPs, enables a profound understanding and hence optimization of recombinant expression systems and high-throughput cultivation processes. Here, the BioLector proved to be a valuable tool and offers new applications within the design of the RoboLector platform. Ultimately, this platform can contribute to the envisioned paradigm shift in bioprocess development. This entails switching from rather empirical process development (low-throughput experimentation with no or unsophisticated monitoring) to automated high-throughput experimentation with very sophisticated yet simple monitoring to gain deeper knowledge of biological production systems.

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Abbreviations

a.u.	arbitrary units
d_0	shaking diameter [mm]
DOT	dissolved oxygen tension
FDA	food and drug administration
GMP	good manufacturing practice
HEPA	high efficiency particulate air filter
HMI	human machine interface
I	scattered light intensity [a.u.]
I_0	initial scattered light intensity [a.u.]
IPTG	isopropyl- β -D-thiogalactopyranoside
MBCR	miniature bubble column reactor
MBR	microbioreactor
MES	2-morpholinoethanesulfonic acid
MOPS	3-(N-Morpholino)-propanesulfonic acid
MTP	microtiter plate
n	shaking frequency [rpm]
OD	optical density
OD_{t0}	initial optical density
OTR	oxygen transfer rate
PAT	process analytical technology
RAMOS	respiration activity monitoring system
STR	stirred tank reactor
T	temperature [°C]
USB	universal serial bus
V_L	filling volume per well or shake flask [L]

Nomenclature for Chapter 4.3

Feedrate	feeding rate [g/L/h]
K_d	decay term [1/h]
K_S	half-saturation constant [g/L]
rel_{act}	relative activity [-]
S	substrate concentration [g/L]
S_0	initial substrate conc. [g/L]
t	time [h]
t_{acc}	time of acceleration phase [h]
t_{lag}	time of lag phase [h]
V_L	filling volume per well or shake flask [L]
X	biomass concentration [g/L]
X_0	initial biomass concentration [g/L]
$Y_{X/S}$	yield coefficient [g/g]
μ	specific growth rate [1/h]
μ_{max}	maximal sp. growth rate [1/h]

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1. Introduction

1.1. Clone libraries for recombinant protein production

In recent years, many advances have been made in biomolecular disciplines for the genetic manipulation of procaryotes and eucaryotes, such as high-throughput cloning techniques (Alberti et al. 2007; Esposito et al. 2009; Hartley 2006). This leads to an increasing availability and utilization of genetically different clones (clone libraries). Nowadays, one of the most important purposes of clone libraries is to produce recombinant proteins for a huge variety of applications. Here, *Escherichia coli* is still the host of choice for producing proteins in high-throughput applications due to its simplicity, high growth and production rates, cheapness and availability (Hammarström et al. 2002; Hartley 2006). Table 1-1 depicts the most important types of *E. coli* clone libraries, their applications and produced proteins. In subsequent steps, these proteins are normally purified, analyzed and screened for certain parameters, e.g. protein structure or increased expression yield.

In structural genomics/proteomics initiatives, *E. coli* clone libraries (type 1) are used to produce different proteins or protein domains for subsequent structural studies (Berrow et al. 2006; Busso et al. 2008a; Gräslund et al. 2008). Therefore, completely different proteins (designated as A, B, C in Table 1-1) of various origins are expressed, purified

and analyzed. Besides that, clone libraries of type 1 are also used to produce protein biochips/arrays for diagnostic purposes (Kung and Snyder 2006; Lueking et al. 2005; Weiner et al. 2004). The main screening parameters for proteins from type 1 libraries are protein producibility, solubility and structure.

Clone libraries of type 2 produce variants of one specific protein, which is normally an industrially relevant enzyme. These enzyme variants are mainly generated via directed evolution methods (Arnold and Georgiou 2003; Dalby 2007; Leemhuis et al. 2009; Reetz et al. 2008; Wong et al. 2004) and exhibit substitutions of only few amino acids of the target protein (designated as D, D', D'' in Table 1-1). After expression, such variants are screened for improved enzyme activity, affinity, stability or altered specificity (Reymond and Babiak 2007).

Table 1-1: Different types of *E. coli* clone libraries for recombinant protein production.

	type 1	type 2	type 3
applications	structural genomics/ proteomics, protein biochips/arrays	directed evolution	expression optimization
screening for	protein solubility, producibility, structure	enzyme activity, specificity, affinity, stability	increased expression yield
protein variants produced	A  B  C  different proteins or protein domains	D  D'  D''  few amino acid substitutions	E  E  E  identical amino acid sequence

A third type of clone libraries is established by optimizing the gene or the expression of a specific target protein by genetic engineering means, e.g. by changing the codon usage or translation initiation (Burgess-Brown et al. 2008; Hatfield and Roth 2007). The resulting protein variants have an identical amino acid sequence and hence structure (designated as E in Table 1). The clones are mostly screened for an increased expression yield.

1.2. High-throughput cultivation processes

In order to produce a huge number of recombinant proteins, clone libraries are grown in parallel high-throughput cultivation processes. Clone libraries of type 1 (Table 1-1) are the most applied today. Hence, different reports using such clone libraries are reviewed (with a focus on process-related parameters) to evaluate the characteristics of typical high-throughput cultivation processes (refer to Appendix A). Such processes are predominantly performed in microtiter plates (MTPs). These are preferred because of their high parallelism, low costs and easy handling (Duetz 2007; Fernandes and Cabral 2006; Gräslund et al. 2008; Hermann et al. 2003; Kumar et al. 2004; Leemhuis et al. 2009). A typical MTP-based production process for recombinant proteins consists of multiple cultivation steps, applying clone libraries as starting material (Berrow et al. 2006; Busso et al. 2008b; Gräslund et al. 2008). Such clone libraries are established via transformation of competent cells with plasmids harboring different target genes. Then, the transformation mix is normally plated on selective agar plates. In a next step, single colonies from those agar plates are picked and transferred to a preculture MTP. Sometimes the transformation mix is also directly applied to the precultivation step. From the preculture MTP often cryocultures are established, which in turn serve as long-term storage of the clones and also as starting material for further protein expression studies. In summary, there are three possible sources that are used as starting material for inoculation of precultures in high-throughput production processes: cryocultures, freshly transformed cells or single colonies from agar plates (Fig. 1-1).

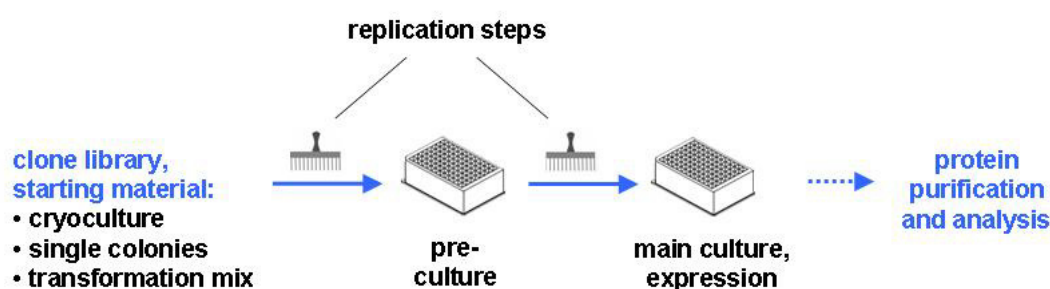


Figure 1-1: Typical layout of a high-throughput production process with clone libraries.

Subsequently to the preculture, the main cultivation is conducted. In such processes, the clones are normally transferred from one plate to another with replicator tools (e.g. 96-pin tools). After protein expression in the main culture, the resulting proteins are purified, analyzed and used for further applications. Although there are new methods

emerging, which try to simplify this complex workflow (Busso et al. 2008b), there are only few exceptions of the above described procedure. Hence, the workflow in Figure 1-1 can be considered as 'state-of-the-art' for producing recombinant proteins in high-throughput.

The protein expression in the main culture of a recombinant *E. coli* strain is dependent on multiple parameters (Baneyx 1999; Donovan et al. 1996; Jana and Deb 2005; Sawers and Jarsch 1996; Schumann and Ferreira 2004; Swartz 2001). On the one hand, the characteristics of the applied strain and expression system are important factors, e.g. host-vector combination, promoter strength, plasmid stability and copy number, translation initiation, codon usage as well as characteristics and localisation of the target protein. On the other hand, the culture conditions play a crucial role for growth and product formation, e.g. pH, temperature, oxygen supply (shaking conditions) and medium composition. Furthermore, other process-related parameters have to be considered, e.g. length of the precultivation, time of harvest of the main culture. Besides that, the time of induction and the inducer concentration need to be optimized, as they are the most critical factors influencing the product yield of an expression system (Donovan et al. 1996). Thus, a tool for convenient testing these parameters for a given host-vector system would be highly beneficial (refer to Chapter 5).

The multiple parameters mentioned above make clear, that the optimization of a protein expression system is very challenging. This is valid already for a single clone. However, if whole clone libraries are applied, there will be some additional challenges arising for parallel cultivations in MTPs.

1.3. Challenges of parallel high-throughput cultivations

The parallelism of cultivations in MTPs leads to the necessity of identical cultivation parameters in each well. This means that different clones in the same MTP are compulsory treated equally, though they might have different growth behavior and production characteristics. These growth differences are caused by variances of individual clones regarding, for example, initial biomass concentrations, lag phases or specific growth rates. Therefore, different physiological states and biomass concentrations of the starting material (e.g. cryocultures, single colonies, transformation mix), represent an inherent source of variance in clone libraries; this aspect will be discussed in Chapter 3. In Figure 1-2, consequences thereof and further possible

influencing factors for the whole high-throughput cultivation process are summarized. Variances of the starting material (e.g. initial biomass) can lead to an often underestimated problem, namely the non parallel and non equal growth of different clones in batch cultures. These variable growth kinetics are illustrated for four different cultures in the lower section of Figure 1-2.

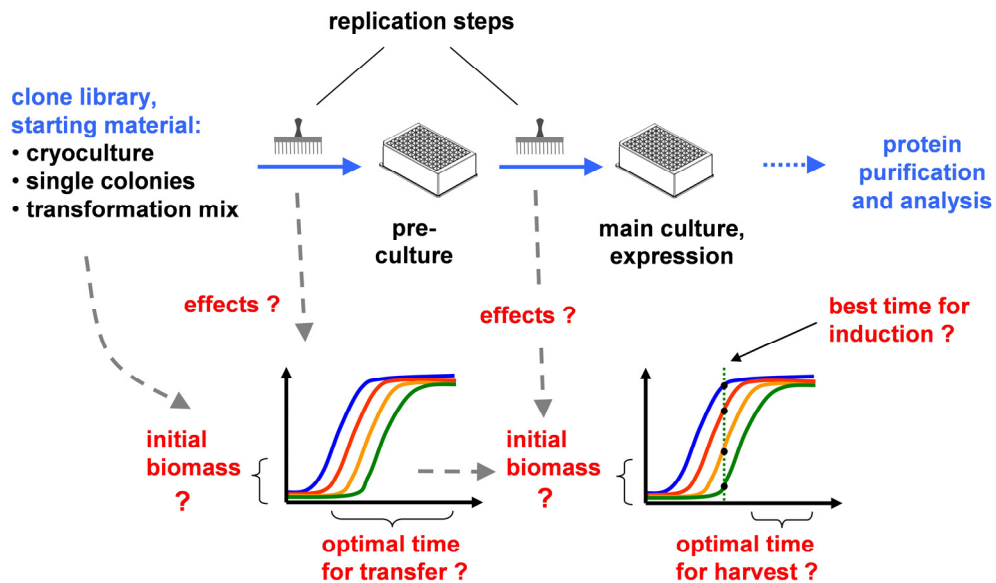


Figure 1-2: Specific challenges in high-throughput cultivation processes.

A question arising for MTP-based cultivation processes is: what effects exhibit the replication steps of the process with respect to the amount of biomass that is transferred? Interestingly, the influence of the replication step on growth and protein expression has not been studied in dedicated investigations so far. Hence, this topic will be addressed in Chapter 3. Another important question for the preculture step is: what is the optimal time for the transfer of cells to the main culture? When all cultures of a preculture MTP are transferred at the same time (regardless of their individual physiological state), then again different initial biomass concentrations and inocula quality might be present in the main culture MTP.

One strategy for achieving uniform starting conditions in the main culture is to cultivate the microorganisms until the stationary growth phase in a preculture MTP (saturation). Though, when using this principle it has to be assured that the final pH of the cultures is not too acidic when they reach saturation (Studier 2005). Moreover, the stationary growth phase in batch mode is characterized by tremendous structural and physiological

effects on bacterial cells (Baev et al. 2006; Hengge-Aronis 1996; Huisman et al. 1996). Some authors have described that precultures remaining unequal time periods in the stationary phase show variations in the lag phase of main cultures (Hornbaek et al. 2004; Pin and Baranyi 2008). It is also known that the inoculum history is very important for the whole cultivation process regarding reproducibility of growth kinetics (Ferenci 1999; Neves et al. 2001; Webb and Kamat 1993). Furthermore, cultivating precultures for an undefined time, e.g. 'overnight', can also contribute to unreproducible results. Nevertheless, this procedure is quite common for high-throughput processes (refer to Appendix A). As a consequence, different growth kinetics might again be observed in the main culture (Fig. 1-2). Then, the question arises: what is the best time for induction of the main culture? The addition of an inducer such as isopropyl- β -D-1-thiogalactopyranoside (IPTG) at a predefined time point to the whole MTP is the most common way to initiate recombinant protein expression in *E. coli* (refer to Appendix A). If different growth kinetics occur in the main culture, the clones of a library will be induced at different metabolic states. It is well known that inducing at different metabolic states or growth phases of a culture is a critical factor regarding protein yield (Donovan et al. 1996; Jenzsch et al. 2006). Therefore, screening for the best producing clones of a library (e.g. for clones of type 3, Table 1-1) might be very difficult or even impossible with such an approach (refer to Chapter 3).

Studier extensively described the problem of simultaneously inducing protein expression of different clones and, thus, developed an autoinduction medium (Studier 2005). With this specially designed medium, there is no need for adding an inducing agent manually, as the culture is induced automatically when a certain amount of glucose is consumed. Hence, this medium is a highly sophisticated way to cope with the problem of different induction points and is, therefore, increasingly used for *lac*- and T7-expression systems; however, it cannot be applied to all microorganisms and host-vector combinations. Furthermore, Studier emphasizes that it is very difficult in high-throughput cultivations to obtain all of the cultures in a comparable state of growth. This aspect will be discussed in Chapter 4, where a new method to equalize different growth kinetics will be presented.

Finally, another question that arises in the main culture is: what is the best time to harvest the cultures? Here, different growth kinetics can lead to various time periods the cultures stay in after induction. Interestingly, typical protein production processes exhibit quite variable harvesting times, although similar *E. coli* expression systems and cultivation conditions are applied (refer to Appendix A). Thus, some cultures might still be in the exponential growth phase, whereas others are already in the stationary phase.

However, the promotion of protease production in stationary phase and the subsequent proteolytic degradation of target proteins may adversely affect product yield and purity (Gräslund et al. 2008). To sum up, the non parallel growth of such cultures can have tremendous effects on the performance of high-throughput production processes.

So far it has been too difficult to study growth kinetics of different cultures in parallel, because of a lack of monitoring tools and sensors for small-scale culture vessels such as MTPs (black-box operation) (Rao et al. 2009; Samorski et al. 2005). Especially for the cell-bank generation stage only little or no process information is available, mainly because only end-point measurements are made (Rao et al. 2009). Nevertheless, monitoring growth kinetics is a prerequisite for understanding the whole production process, for example, for choosing the right time for inoculation from a preculture, for inducing protein expression at an optimal time, and for determining the best harvesting point (e.g. to avoid proteolytic degradation in the stationary phase). Interestingly, only little research is being conducted to investigate processes with multiple cultivation steps systematically and in more detail. This is mainly because conventional approaches (e.g. off-line analysis of bacterial growth in parallel reactors) are too time-consuming and extensive. Therefore, advanced high-throughput cultivation systems are needed (refer to Chapter 5), which preferably provide similar monitoring properties as large scale bioreactors (e.g. sensors for pH, dissolved oxygen tension DOT, biomass). Such systems are termed microbioreactors (MBRs).

1.4. Microbioreactors

Many MBR devices in the milliliter scale evolved in the last years (Betts and Baganz 2006; Betts et al. 2006; Maharbiz et al. 2004; Marques et al. 2009; Puskeiler et al. 2005b). They differ strongly regarding the way of mixing, oxygen transfer, temperature control or analysis of key cultivation parameters, such as biomass or DOT. Table 1-2 gives an overview of the most prominent available MBR systems for culture volumes up to 35 mL (excluding microfluidic devices). This volume range is normally sufficient to produce enough recombinant protein for subsequent utilisation. Some of these systems have already been commercialized and are increasingly applied throughout industry and academia as well as for high-throughput expression of proteins. Further important applications of MBRs include screening of medium compositions as well as bioprocess development, optimization and validation (Betts and Baganz 2006; Duetz 2007; Kumar et al. 2004; Rao et al. 2009).

Table 1-2: Comparison of different available MBR systems; adapted from (Betts and Baganz 2006).

Device/Reference	Based on	Volume (mL)	Agitation/Aeration	pH, DOT and OD instrumentation	Multi-plexing
MicroReactor (μ 24), Applikon (Isett et al. 2007)	MTP & micro-fabrication	3 - 5	Orbital shaker (up to 800 rpm); Gas sparging	pH and DOT via optical probes	24
SimCell, Seahorse Bioscience (Legmann et al. 2009)	Micro Bioreactor Array	0.3 - 0.7	Rotation of MBR chips; Surface via membrane	pH, DOT and OD atline via cell-reading station	1500
Cellstation, Fluorometrix (Harms et al. 2006)	STR	up to 35	Dual paddle impeller (10 - 1000 rpm); Sparger	pH, DOT and OD via optical probes	12
Bioreactor block (Kusterer et al. 2008; Puskeiler et al. 2005a)	STR	8 - 12	Gas-inducing single impeller (up to 4000 rpm)	DOT and pH optically; OD atline via plate reader	48
MBCR (Doig et al. 2005)	Bubble column	2	Gas-sparging	pH and DOT via optical probes	48
Piccolo, The Automation Partnership (Wollerton et al. 2006)	Culture vessel block	10 - 12.5	Stirring rods (up to 3000 rpm)	OD atline via cell-reading station	1152
Bioscreen C, Oy Growth Curves (Weiss et al. 2004)	MTP (honeycomb)	0.4	Linear shaker	OD via plate reader	200
BioLector, m2p-labs (Kensy et al. 2009; Samorski et al. 2005)	MTP	0.1 - 1	Orbital shaker (up to 1800 rpm)	OD via scattered light, pH and DOT via optodes, fluorescence	96

Nomenclature: MTP = microtiter plate, STR = stirred tank reactor, MBCR = miniature bubble column reactor, DOT = dissolved oxygen tension, OD = optical density.

Online monitoring of pH and DOT is realized in many of these MBR systems. However, only few of them are capable to monitor in detail the most dominant indicators characterizing a biological production system, i.e. the formation of biomass and product. Some of these systems measure microbial growth atline (sample is removed from the reactor) via optical density, although this has the disadvantage of only limited data density (Betts and Baganz 2006; Puskeiler et al. 2005b) and sometimes requires interruption of the cultivation process. Furthermore, none of the above described systems has the possibility to conduct fluorescence protein monitoring online in a large number of parallel MBRs.

In contrast, the so-called BioLector technique provides a cultivation system that is able to permanently monitor microbial growth, fluorescence of reporter proteins, pH and DOT under defined conditions in MTPs without interrupting the shaking movement (Kensy et al. 2009; Samorski et al. 2005). This system consists of an orbital shaker, a MTP holder, a x-y-positioning device, an optical fiber, a MTP reader and a computer. With these components it is possible to permanently scan each well of a MTP during the cultivation for different signals. Because the shaking movement is not stopped during the measurement, a permanent oxygen supply can be provided to the cultures. Another advantage of the system is its wide range of biomass quantification, which is realized via scattered light measurements (Kensy et al. 2009).

For operating bioreactors, there is a trade-off between information output and high-throughput capability (Betts and Baganz 2006). As bioreactors increase in scale, more process information is available (due to many available sensors), whereas the experimental throughput is reduced (Fig. 1-3).

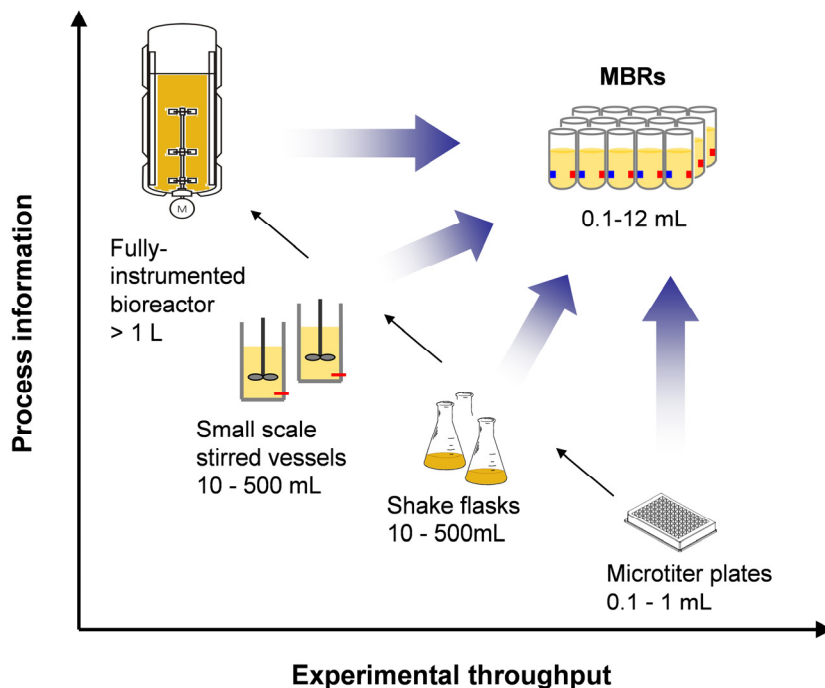


Figure 1-3: Cultivation systems with respect to experimental throughput and available process information; adapted from (Betts and Baganz 2006).

The aim of developing and improving MBRs is to unify high experimental throughput with high process information and, thus, to overcome this trade-off. Therefore, properties of bioreactors of different scales must be combined, e.g. monitoring capabilities of large scale bioreactors with high-throughput of MTPs or shake flasks. These aspects are symbolized as blue arrows in Figure 1-3. Ideally, such MBR systems should also be automatable to further decrease the amount of workload and time needed for high-throughput experiments. Therefore, automated MBRs are promising tools to study parallel processes for protein expression and cultivating clone libraries. Such new tools are urgently required, as reliable and reproducible methods for high-throughput production of proteins become increasingly important (Blommel et al. 2007). A novel approach to establish such a system will be presented in Chapter 5. It entails the combination of a BioLector and a pipetting robot and the elaboration of novel automated cultivation methods. This Chapter is followed by an application example of this technique to study the influence of a phosphate limitation on growth and protein expression of a recombinant *E. coli* strain (Chapter 6).

2. Objectives

As described in Chapter 1, profound knowledge about different growth kinetics in MTPs and their effects on recombinant protein expression is essential for understanding and optimizing MTP-based production or screening processes. In order to study such processes, an online-monitoring device for MTPs – the MBR-system BioLector – is applied throughout this work to

- 1) evaluate its suitability to study and optimize high-throughput cultivation processes and
- 2) to elaborate new applications for the BioLector in this area.

A first aim of this thesis is to systematically investigate different tools and methods for the replication of cells (*E. coli* SCS1) from one MTP to another MTP. Furthermore, the effects of different growth kinetics on protein production are studied with two model expression systems (*E. coli* and *Corynebacterium glutamicum*), which express different fluorescence proteins (EcFbFP and GFP) upon induction (Chapter 3).

Additionally, a new concept to cope with the problems of different growth kinetics in high-throughput cultivation processes is elaborated. Here, one strategy is to use the fed-batch mode instead of the conventional batch mode in the precultivation step. The principle of this strategy is described by a mathematical model and studied experimentally with an *E. coli* and a *Hansenula polymorpha* strain (Chapter 4). Further strategies are elaborated in Chapter 5.

A major aim of this work is to establish a novel automated high-throughput cultivation system based on the BioLector (Chapter 5). This device (termed Robo-Lector) should help to automatically investigate and optimize MTP-based cultivations and protein production, thereby providing a high-throughput experimentation platform with high information content. Three automated methods are programmed which allow to, first, characterize protein expression of a given *E. coli* host-vector system in one automated run (method 'induction profiling'), second, induce protein expression of cultures with different growth kinetics at the same biomass concentration (method 'biomass-specific induction') and third, generate equal initial biomass concentrations in main cultures from precultures with different growth kinetics (method 'biomass-specific replication').

Finally, the tools BioLector, the newly established Robo-Lector platform and the RAMOS device (which measures microbial respiration activity in shake flasks) are applied to demonstrate their usefulness in bioprocess understanding and optimization. Here, the effects of a phosphate limitation on the growth and protein production of a recombinant *E. coli* T7 expression system (producing the fluorescent protein EcFbFP) are investigated (Chapter 6). In the end, the results from the MTP-scale are verified by shake flask experiments.

3. Investigation of replication tools and methods

3.1. Introduction

Typical high-throughput production processes with clone libraries mostly consist of multiple unit operations (refer to Chapter 1.2): transformation, plating, colony picking, preculture, main culture, induction and harvest. To combine the cultivation steps of such a process, a transfer of clones from a source MTP to a target MTP is necessary, which is mainly conducted with replicator tools for liquid transfer. Easy to use tools are for example 96-pin replicators (manufactured as a disposable or as reusable steel variant) (Endo et al. 2008; Scheich et al. 2004). Dependent on the dimensions and characteristics (e. g. coating) of the pins, their transfer volume lies in a range of only few nanoliters to several microliters. Moreover, liquid handling stations with multi-channel pipette heads (automated or manually operated) are applied for replicating clones (Zhu et al. 2006). Interestingly, the influence of the replication step on growth and protein expression in MTP-based high-throughput processes has not been addressed in dedicated investigations so far. A possible reason for that is probably the lack of appropriate monitoring tools for cultivations in MTPs (Rao et al. 2009; Samorski et al. 2005). With the BioLector-technique it is possible to permanently monitor growth and fluorescence of reporter proteins in a MTP without interrupting the shaking motion, thus not interfering with the oxygen supply of the cultures (Kensy et al. 2009; Samorski et al. 2005).

Therefore, the aim of this Chapter was to use the BioLector device to investigate the effects of different replication tools and replication methods on the cultivation of the *E. coli* strain SCS1 pQE-30 pSE111 in MTPs. The influence of these effects on product formation was studied with the strain *E. coli* BL21(DE3) pRhotHi-2-EcFbFP, which expresses a fluorescence protein upon induction. Furthermore, the typical process flow from colony picking, preculture and cryopreservation to protein induction at a defined point of time was studied in respect to growth kinetics and product formation. For that purpose, the strain *Corynebacterium glutamicum* pEKEx2-phoD-GFP was applied.

3.2. Material and methods

Microorganism

The strain *E. coli* SCS1 pQE-30 pSE111 (here referred to as *E. coli* PR02) (Büssow et al. 1998) was used for the replicator tool and replication method experiments. It was kindly provided by Protagen AG, Dortmund, Germany. To prepare stock cultures, a preculture in TB medium was made. After reaching the stationary phase (OD= 22), an equal amount of glycerol solution (500 g/L) was added as a cryoprotectant, resulting in a final glycerol concentration of 250 g/L and a final OD of 11. The cultures were stored at -20°C in 100 µL aliquots in 96-well MTPs, which were closed with an adhesive seal (Cat. AB-0580, Abgene, Epsom, UK). OD was measured at 600 nm with a Uvikon 922 spectrophotometer (Kontron, Milano, Italy).

The strain *E. coli* BL21(DE3) pRhotHi-2-EcFbFP (Katzke et al. 2009) was used to investigate the influence of different growth kinetics on product formation upon induction at a defined point of time. This strain was kindly provided by T. Drepper, Institute of Molecular Enzyme Technology, Heinrich-Heine-University Düsseldorf, Germany. The used expression plasmid harbors the T7 promoter that is under the control of the *lac* operator and a kanamycin resistance gene. The fluorescent protein EcFbFP was used as a model protein. Therefore, the EcFbFP encoding gene was cloned into the pRhotHi-2 vector downstream of the T7 promoter. A His₆-tag was fused to the C-terminus of the EcFbFP resulting in a recombinant fusion protein with a molecular weight of 16.5 kDa. The gene for this FMN-binding fluorescent protein (FbFP) was codon-optimized for expression in *E. coli* (hence, the name EcFbFP) and could even fluoresce in the absence of oxygen (in contrary to GFP and its derivatives) (Drepper et al. 2007). The used FbFP is available under the trademark evoglow (evocatol GmbH, Düsseldorf, Germany). To prepare stock cultures, a preculture in TB medium was made. After reaching the

stationary phase, fresh medium and glycerol solution (500 g/L) was added as a cryo-protectant, resulting in a final glycerol concentration of 150 g/L and a final OD of 4. The cultures were stored at -20°C in 1 mL aliquots in cryo-vials.

The strain *Corynebacterium glutamicum* pEKEx2-phoD-GFP (Meissner et al. 2007) was used for the experiments to simulate a high-throughput cultivation process and was kindly provided by R. Freudl, Institute of Biotechnology 1, Forschungszentrum Jülich, Germany. The transformed colonies were provided on agar plates (modified Eikmanns mineral medium with additional 16 g/L agar). Upon induction with IPTG, this strain produces and secretes the green fluorescence protein (GFP).

Media

Terrific Broth (TB) medium was used for all *E. coli* PR02 cultivations. It consists of 5 g/L glycerol, 12 g/L tryptone, 24 g/L yeast extract, 12.54 g/L K₂HPO₄ and 2.31 g/L KH₂PO₄. The pH was adjusted to 7.2 with NaOH. Additionally, 0.1 g/L ampicillin was added.

E. coli BL21(DE3) pRhotHi-2-EcFbFP was cultivated in MDG medium which was prepared as described by Studier (Studier 2005). It consists of 3.55 g/L Na₂HPO₄, 3.4 g/L KH₂PO₄, 2.675 g/L NH₄Cl, 0.71 g/L Na₂SO₄, 0.493 g/L MgSO₄ x 7H₂O, 0.365 mg/L FeCl₃ x 6H₂O, 0.444 mg/L CaCl₂, 0.396 mg/L MnCl₂ x 4H₂O, 0.575 mg/L ZnSO₄ x 7H₂O, 0.095 mg/L CoCl₂ x 6H₂O, 0.068 mg/L CuCl₂ x 2H₂O, 0.095 mg/L NiCl₂ x 6H₂O, 0.097 mg/L Na₂MoO₄ x 4H₂O, 0.105 mg/L Na₂SeO₃ x 5H₂O, 0.025 mg/L H₃BO₃, 2.5 g/L aspartate and 5 g/L glucose. The pH was adjusted to 6.8 with NaOH. Additionally, 0.05 g/L kanamycin was added to the medium.

A modified Eikmanns mineral medium (Eikmanns et al. 1991) was used for the *C. glutamicum* pEKEx2-phoD-GFP cultivations. It consists of 10 g/L glucose, 10 g/L (NH₄)₂SO₄, 1 g/L KH₂PO₄, 2 g/L K₂HPO₄, 0.25 g/L MgSO₄ x 7H₂O, 21 g/L 3-(N-Morpholino)-propanesulfonic acid (MOPS), 0.2 mg/L biotin, 10 mg/L CaCl₂, 30 mg/L protocatechuic acid, 10 mg/L FeSO₄ x 7H₂O, 0.2 mg/L CuSO₄, 10 mg/L MnSO₄ x H₂O, 0.02 mg/L NiCl₂ x 6H₂O, 1 mg/L ZnSO₄ x 7H₂O. The pH was adjusted to pH 7.2 with NaOH. Additionally, 0.025 g/L kanamycin was added to the medium.

All reagents were purchased from Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany) or Sigma Aldrich (Taufkirchen, Germany).

BioLector

The *E. coli* PR02 and *C. glutamicum* cultivations were conducted in a BioLector prototype, corresponding to the description by Samorski et al. (2005). The experiments with *E. coli* BL21(DE3) pRhotHi-2-EcFbFP were conducted with a commercial BioLector device (m2p-labs GmbH, Aachen, Germany) (Kensy et al. 2009). To detect microbial growth, the scattered light intensity of the cultures in a MTP was measured at 620 nm. The EcFbFP fluorescence of the *E. coli* BL21(DE3) pRhotHi-2-EcFbFP cultivations was measured at an excitation of 460 nm and an emission of 492 nm, the GFP fluorescence of the *C. glutamicum* cultivations was measured at an excitation of 485 nm and an emission of 520 nm. The cultivations were carried out in black 96-well MTPs with a clear bottom (µclear, Greiner Bio-one, Frickenhausen, Germany) which were sealed with adhesive gas permeable seals (Cat. AB-0718, Abgene, Epsom, UK). All cultures were grown at 37°C, a shaking frequency (n) of 950 rpm (*E. coli* PR02 and *E. coli* BL21(DE3) pRhotHi-2-EcFbFP) or 995 rpm (*C. glutamicum* pEKEx2-phoD-GFP), a shaking diameter (d_0) of 3 mm and a filling volume (V_L) of 200 µL per well. The experimental data were standardized to eliminate factors that influence the scattered light intensity (I) such as media background, type of MTP or geometrical positioning (Samorski et al. 2005). Hence, the initial value of scattered light intensity (I_0) was subtracted from every value ($I-I_0$). There is a general linear correlation between scattered light and OD (Kensy et al. 2009). In the current thesis, the scattered light data can be correlated to OD values, which can be determined with a calibration curve as reported by Kensy et al. (2009). Nevertheless, for comparing different cultures in parallel experiments, a calibration to OD values is not essential. Hence, the biomass data are shown throughout this work as scattered light intensity in arbitrary units (a.u.).

Replication tools and experiments

Four replication tools were applied: an 8-channel pipette (Research 5 µL - 100 µL, Eppendorf AG, Hamburg, Germany) to mimic a liquid handling system (e.g. 96-channel pipetting head) and three different pin tools. First, a disposable plastic replicator with fixed pins (QRep 96 pin, Genetix GmbH, München, Germany) made from polypropylene, second, a reusable steel replicator with fixed pins (96-pin replicator, Boekel scientific, Feasterville, USA) and third, a reusable steel replicator with 96 spring-loaded pins that may move vertically, dependent on the surface of the frozen liquid of a cryoculture MTP (Duetz replicator system, EnzyScreen BV, Leiden, Netherlands) (Duetz et al. 2000).

The disposable plastic replicator and the reusable steel replicator with fixed pins were used to inoculate target MTPs filled with 200 µL of TB medium with cells from thawed

and mixed *E. coli* PR02 cryoculture MTPs. As the transfer volume of the pin replicators was lower than 1 μ L compared to the 5 μ L of the 8-channel pipette experiment, a dilution (1:10) with TB medium was made for the 8-channel pipette in order to achieve comparable initial biomass concentrations in the main culture.

In further experiments, the steel replicator with fixed pins was applied to inoculate target MTPs with cells from a frozen cryoculture MTP or from the same cryoculture MTP after thawing without mixing. Replication with the steel replicator with spring-loaded pins was also conducted with a frozen cryoculture MTP. Thus, the pins of the replicator were put into the wells of a cryoculture MTP with light pressure for three seconds so that the pins came into contact with the frozen liquid. Thereafter, the pins were put into the wells of the target MTP filled with 200 μ L TB medium. After these different replications, the corresponding target MTP was cultivated and measured in the BioLector. The time to reach a scattered light intensity of 10000 a.u. was determined for each well and the time difference between the fastest and the slowest growing culture of each plate was determined. These time differences were used to compare the reproducibility of the transferred volume of the different replication tools.

An *E. coli* BL21(DE3) pRHotHi-2-EcFbFP cryoculture was used to establish different initial biomass concentrations for an induction experiment. Therefore, the cryoculture was thawed, centrifuged and resuspended in MDG medium. Different amounts of this suspension were then used to establish different biomass concentrations (OD: 0.0125, 0.025, 0.05, 0.1, 0.2) in a MTP. The cultures were grown and monitored in the BioLector. They were induced by adding IPTG stock solution after 4.9 h of cultivation, resulting in a final IPTG concentration of 0.1 mM. In previous studies this concentration was shown to be optimal for this strain in this specific medium.

Simulation of a typical high-throughput cultivation process

A typical high-throughput cultivation process is depicted in Figure 3-1. To simulate such a process, a MTP filled with 200 μ L modified Eikmanns mineral medium per well was inoculated with 62 genetically identical *C. glutamicum* colonies from an agar plate with sterile toothpicks.

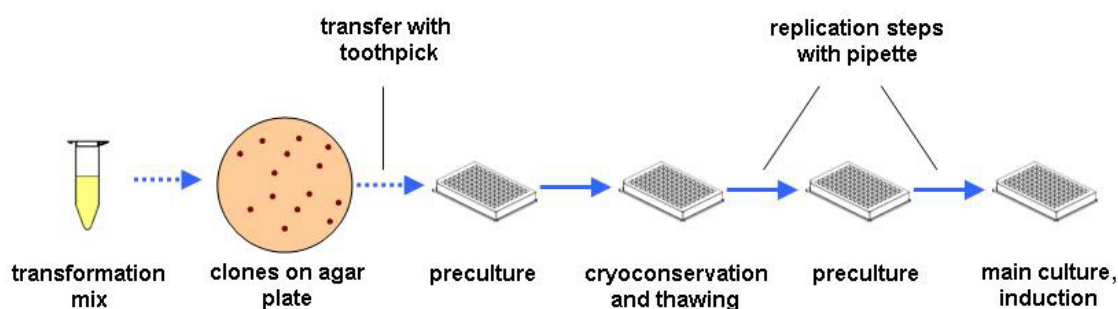


Figure 3-1: Scheme of a typical high-throughput cultivation process. After transformation, the clones are plated and grown on agar. Colonies are transferred with sterile toothpicks to a preculture MTP and cultivated. Glycerol is added and the MTP is frozen (cryoculture). After thawing and mixing, liquid is transferred from the cryoculture MTP to a new preculture MTP. This MTP is incubated and subsequently a new main culture MTP is inoculated with liquid from the preculture MTP. At a defined point of time, inducer is added to the main culture to induce protein expression.

Then, the MTP was cultivated overnight (14-16 h) at 37°C, a shaking frequency of 995 rpm and a shaking diameter of 3 mm. Subsequently, 20 μ L glycerol stock solution (500 g/L) was added to each well of the MTP as a cryoprotectant, resulting in a final glycerol concentration of 45 g/L. The MTP was then frozen at -20°C. For the experiment, the cryoculture MTP was thawed and mixed before 10 μ L of each well were transferred to 190 μ L modified Eikmanns mineral medium in a preculture MTP with an 8-channel pipette. Subsequently, a precultivation was conducted overnight (14-16 h). 10 μ L of each well of this preculture MTP were then transferred to 190 μ L modified Eikmanns mineral medium in a main culture MTP with an 8-channel pipette. After this, the corresponding MTP was cultivated and measured in the BioLector. The cultures were induced by adding IPTG stock solution after 5.25 h of cultivation, resulting in a final IPTG concentration of 0.5 mM.

3.3. Results and discussion

A set of experiments was conducted to evaluate the influence of the replication step on growth kinetics of a single *E. coli* strain (PR02). Cultures were transferred from a source cryoculture MTP with 96 homogeneous cultures with identical biomass concentrations to a target MTP with different replication tools and replication methods. Figure 3-2A-F shows the growth curves of these cultures in TB medium. A diauxic growth can be observed. The first growth phase on the carbon source glycerol ends with its depletion at

a scattered light intensity of about 20000 a.u.. A second growth phase on complex compounds of the medium follows, resulting in a stationary phase at about 40000 a.u.. This growth pattern is consistent with the growth curves of *E. coli* BL21 in TB medium, as described previously (Kensy et al. 2009). The time difference between the fastest and the slowest culture for the particular replicator system was determined at 10000 a.u. as an indicator for the accuracy of the applied replication tools. While the curve progression of all six experiments (Fig. 3-2A-F) is similar, their corresponding time difference of the fastest and slowest culture varies dramatically. Fig. 3-2A-C depicts the time difference of *E. coli* PR02 cultures that were inoculated with different replication tools. All cryocultures were thawed and mixed before replication. The application of an 8-channel pipette leads to the lowest time difference (0.3 h). For the disposable replicator and the steel replicator with fixed pins, it is 1.6 h and 3.1 h, respectively (Fig. 3-2B and C).

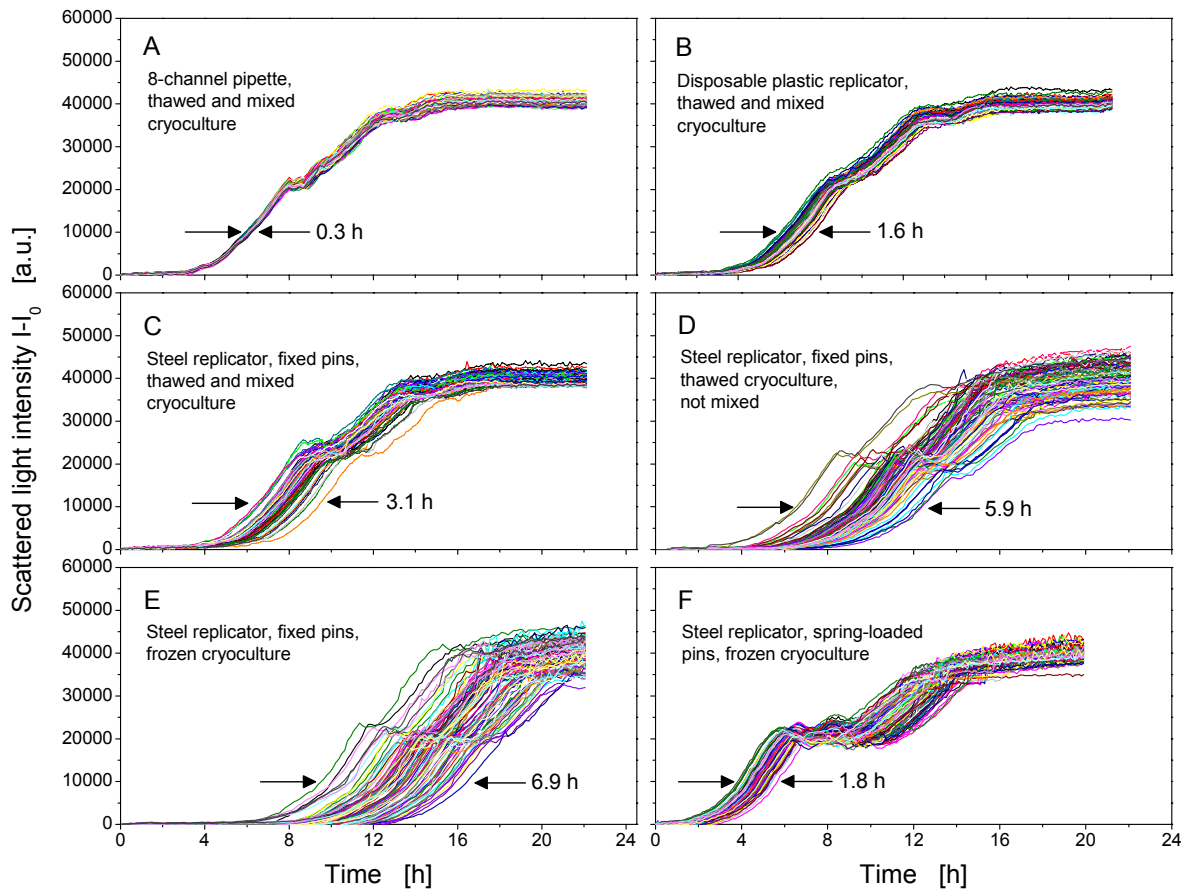


Figure 3-2: Influence of different replication tools on growth of 96 cultures of *E. coli* PR02. Replication tools: (A) 8-channel pipette, thawed and mixed cryoculture. (B) Disposable plastic replicator, thawed and mixed cryoculture. (C) Steel replicator, fixed pins, thawed and mixed cryoculture. (D) Steel replicator, fixed pins, thawed cryoculture, not mixed. (E) Steel replicator, fixed pins, frozen cryoculture. (F) Steel replicator, spring-loaded pins, frozen cryoculture. Conditions: TB medium with 5 g/L glycerol, $T = 37^\circ\text{C}$, $V_L = 200 \mu\text{L}$, $n = 950 \text{ rpm}$, $d_0 = 3 \text{ mm}$.

According to the manufacturer, the precision of the 8-channel pipette for a transfer volume of 5 μL is high with a coefficient of variance of only $\leq 2\%$. Higher transfer volumes even increase this precision. In contrast to the 8-channel pipette, the transfer volume of the applied replicators is below 1 μL . In this low range, the volume is strongly affected by the manufacturing accuracy of the pins, leading to a lower precision of the replicator tools. This results in variances in transferred biomass and thus variable growth kinetics.

As depicted in Figure 3-2C-E, the time difference also varies strongly depending on the way the cryoculture MTP is treated. Whereas thawing and mixing of the cryoculture MTP results in a time difference of 3.1 h with the steel replicator (Fig. 3-2C), merely thawing without mixing leads to a nearly doubled time difference of 5.9 h (Fig. 3-2D). This might be explained by concentration gradients in the wells, caused by cell sedimentation during freezing of the cryoculture. When such a cryoculture is not mixed after thawing, the transferred amount of inoculum is obviously highly variable, leading to different growth kinetics. The cultures that were inoculated without thawing the cryocultures have the longest time difference of 6.9 h (Fig. 3-2E). As the pins of the applied steel replicator are fixed, their contact to the frozen liquid in the wells of the cryoculture MTP varies, caused by slight differences of the height of the frozen liquid in each well. These differences can occur because of variable freezing patterns in the wells (Duetz et al. 2000), resulting in varying filling heights. Thus, different amounts of liquid are thawed by the heat introduced by the pins and transferred to the target MTP, resulting in differing initial biomass concentrations. In contrast to these fixed pins, the pins of the other steel replicator are spring-loaded to equalize even small differences in the filling height of the wells. This grants a homogeneous contact of the pins to the surface of the frozen liquid in the wells of the cryoculture MTP (Duetz et al. 2000). Thus, a reduced variance in transferred biomass in comparison to replicators with fixed pins was observed. This led to a time difference of only 1.8 h (Fig. 3-2F) in comparison to 6.9 h for the steel replicator with fixed pins (Fig. 3-2E). In summary, the variance in transfer volume is affected not only by the replication tool itself, but also by the replication method (e. g. mixing of thawed cryocultures). Furthermore, it is influenced by several other factors. According to a manufacturer of replicator tools, the transfer volume of pin replicators is, for example, affected by the speed of withdrawal of the replicator from the liquid as well as the source plate volume. The sum of all mentioned influences can, hence, lead to unpredictable and irreproducible replication steps in high-throughput cultivation processes.

The effects of the different growth kinetics caused by the applied replication tools on product formation were studied with the strain *E. coli* BL21(DE3) pRhotHi-2-EcFbFP. This strain produces a model fluorescent protein upon induction with IPTG and thus allows the monitoring of the product formation with the BioLector. Figure 3-3 shows the growth and protein expression of *E. coli* BL21(DE3) pRhotHi-2-EcFbFP cultures with five different initial biomass concentrations, simulating the possible effects from replication steps. Because of the varying initial biomass concentration, different growth kinetics can be observed up to 4.5 h (Fig. 3-3A). At that time, all cultures of the MTP are induced simultaneously with IPTG, which is the most common procedure in high-throughput production processes (refer to Appendix A). Here, this induction at a defined point of time means that the cultures are induced at different physiological conditions. The cultures with an initial OD of 0.2, for example, are in the late exponential growth phase at the time of induction, whereas the cultures with an initial OD of 0.0125 are in the early exponential growth phase. With a final EcFbFP fluorescence intensity of about 80 a.u., the cultures with an initial OD of 0.2 have the lowest amount of product, whereas the highest amount of about 160 a.u. is reached by the cultures with an initial OD of 0.05 (Fig. 3-3B). Apart from the different amount of synthesized product, the rate of product formation also varies. The cultures with an initial OD of 0.1 and 0.2 have the fastest rate of product formation, those with an initial OD of 0.0125 the slowest.

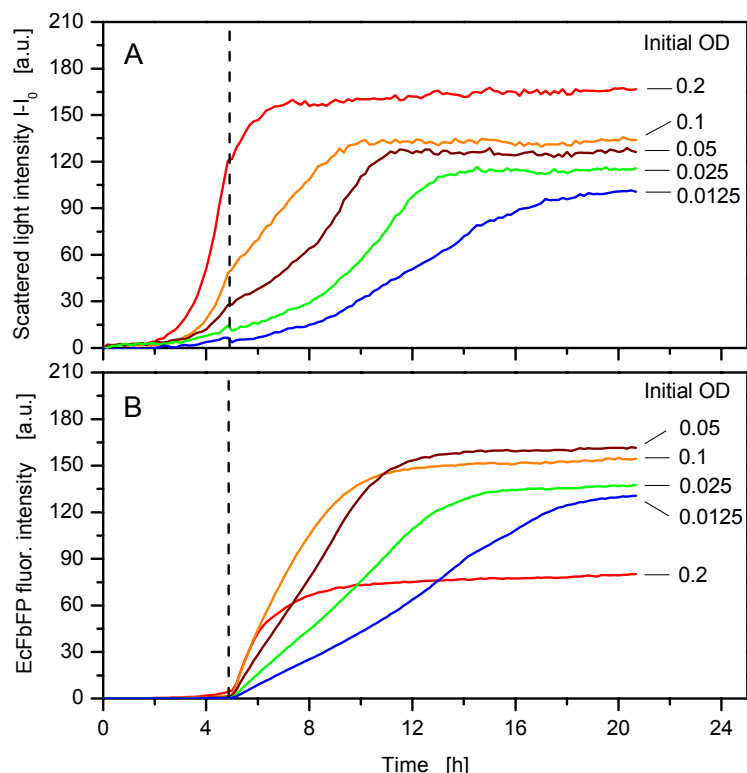


Figure 3-3: Influence of different initial biomass concentrations on growth and product formation of *E. coli* BL21(DE3) pRhotHi-2-EcFbFP. (A) Scattered light intensity (mean of eight parallel wells per condition). (B) EcFbFP fluorescence intensity (mean of eight parallel wells per condition). Vertical dashed line: time of induction with 0.1 mM IPTG after 4.9 h. Conditions: MDG medium, $T = 37^\circ\text{C}$, $V_L = 200\ \mu\text{L}$, $n = 950\ \text{rpm}$, $d_0 = 3\ \text{mm}$.

The reason for the different growth after induction is the metabolic burden the cultures are exposed to (Bonomo and Gill 2005; Glick 1995). When the cells are induced, cellular resources are redirected to product formation, thus reducing the growth of the cultures. According to Donovan et al. (1996), this burden influences the growth rate, cell yield and product expression of the culture. Furthermore, the amount of produced protein is dependent on the growth phase in which the cultures are induced. Therefore, if all cultures of a MTP are induced at the same time, differences in the initial biomass concentration lead to variances in product formation. Taking these variances into account, it seems impractical to screen and compare different clones regarding their product formation. This is especially valid when different clones of a library of type 3 (refer to Chapter 1, Table 1-1) have to be compared in respect to an optimized expression yield.

Differences in initial biomass concentration could be equalized by a robotic liquid handling system in combination with a BioLector. Such a platform (Robo-Lector, refer to

Chapter 5) could permanently monitor the preculture MTP and calculate the amount of liquid that has to be transferred from each well of the preculture MTP to the target MTP to reach homogeneous initial biomass concentrations.

A further possibility to equalize different biomass concentrations could be to utilize silicone elastomer depots at the bottom of a MTP that release glucose during cultivation to establish fed-batch conditions. In contrast to the aforementioned method that acts at the replication step from preculture to main culture, this fed-batch technique for MTPs could be applied at the precultivation step. Due to the fed-batch cultivation, all wells of a MTP would then be in the same growth phase and have equal biomass concentrations after a certain time of cultivation. Transferring an inoculum from such an equalized preculture to the main culture could thus minimize the differences in initial biomass concentrations. This concept will be described in detail in Chapter 4.

Another way to deal with different initial biomass concentrations is not to equalize, but to compensate variable growth kinetics during the main culture. The aforesaid Robo-Lector platform could be used to induce single wells automatically when a defined biomass concentration is reached. Thus, all screened cultures of a MTP would be in the same growth phase and metabolic state when induced and this could result in an improved comparability between them (refer to Chapter 5). Nevertheless, such a system can only be applied for medium-throughput cultivation processes, as the BioLector can currently monitor only one MTP.

For high-throughput processes, which cultivate many MTPs in parallel, the autoinduction principle can be applied, which uses a specially designed medium (Studier 2005). Here, the induction is triggered automatically for T7 *E. coli* expression systems by lactose when another carbon source (glucose) in the medium is exhausted. Nevertheless, the simultaneous induction by manually adding the inducer to cultures of a MTP is still widespread.

Although new techniques as described above are being developed and other methods are emerging, which try to simplify the complex workflow (Busso et al. 2008b), the typical high-throughput cultivation process (as shown in Fig. 3-1) with its multiple steps is still a common procedure. In order to simulate such a high-throughput cultivation process, typical steps (clone picking, cryoculture, preculture, main culture and induction at a defined point of time) were conducted with *C. glutamicum*, another frequently applied and industrially important microorganism. Clones of *C. glutamicum* were

transferred with sterile toothpicks from an agar plate to a preculture MTP. After cultivation overnight glycerol was added and the culture was frozen. After thawing and mixing the cryoculture, another MTP was inoculated and a second preculture was conducted overnight. Subsequently, a main culture MTP was inoculated which was induced after 5.25 h for protein production. This last and most important step – the protein expression step – was monitored with the BioLector. For the replication step, an 8-channel pipette was applied which caused the lowest time difference in the first experiment (Fig. 3-2A). Figure 3-4A shows the growth curves of 62 *C. glutamicum* pEKEx2-phoD-GFP cultures in a MTP. A strong variance can be seen between the different cultures. Whereas the fastest culture has a scattered light intensity of about 5500 a.u. after 5 h, the slowest culture reaches only about 1000 a.u..

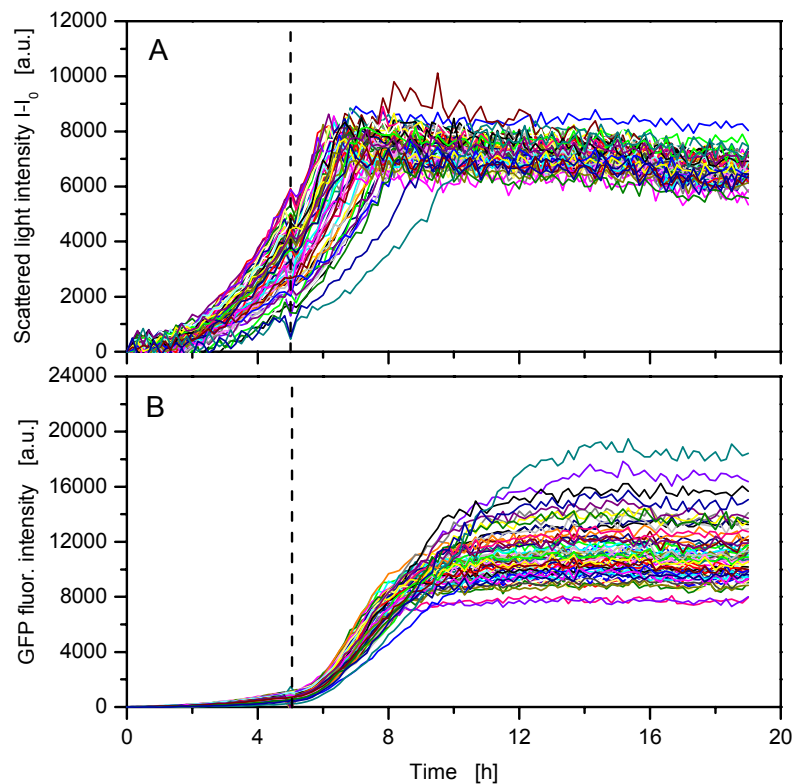


Figure 3-4: Simulating a typical high-throughput cultivation process: effect on growth and product formation of *Corynebacterium glutamicum* pEKEx2-phoD-GFP. (A) Scattered light intensity. (B) GFP fluorescence intensity. Transformed genetically identical *C. glutamicum* clones were grown on an agar plate. Sixty-two colonies from this plate were transferred to a MTP with toothpicks and cultivated overnight. Glycerine was added to the MTP which was then frozen at -20°C . The cryoculture MTP was thawed and mixed. Subsequently, 10 μL of each well were transferred to 190 μL medium in a preculture MTP. After cultivation overnight 10 μL of each well were transferred to 190 μL medium in a main culture MTP. The following cultivation with induction was monitored with the BioLector and is shown above. Vertical dashed line: time of induction with 0.5 mM IPTG after 5.25 h. Conditions: Eikmanns mineral medium, $T = 37^{\circ}\text{C}$, $V_L = 200 \mu\text{L}$, $n = 995 \text{ rpm}$, $d_0 = 3 \text{ mm}$.

When all cultures of the MTP are induced at a defined point of time (after 5.25 h in this study) variable product formation kinetics become visible (Fig. 3-4B). The GFP fluorescence deviates by a factor of more than two, with a minimal GFP fluorescence signal of 8000 a.u. and a maximal GFP fluorescence signal of about 18000 a.u. at the end of the cultivation. The varying growth curves are presumably caused by varying initial biomass concentrations that lead, as a consequence, to strongly varying product formation upon induction.

As the replications were conducted with the highly accurate 8-channel pipette, the occurring strong variances can not be caused by the replication tool. It should be noted that cells were manually transferred with sterile toothpicks from an agar plate to the first preculture MTP. The amount of inoculum transferred probably varied strongly on each toothpick, leading to varying initial biomass concentrations. In addition, because of the manual picking, the inoculum was extracted from different regions of the single colonies (cell cluster) on the agar plate. Cells inside the cell cluster were probably in a different metabolic state, as the oxygen supply can be limited (Stewart and Franklin 2008) and key nutrients can locally be depleted (Borriello et al. 2004). Hence, the transferred cells might not only have varied in their amount, but also in their metabolic state. Instead of manual tooth picking, more sophisticated techniques such as automated clone picking systems may be used (Lane et al. 2008). Although these picking systems reduce time and workload for colony picking, the problem of varying cell concentrations and metabolic states is an inherent one. Cultures with a higher initial biomass concentration reach their stationary phase earlier and are thus longer in this phase. As the entrance into the stationary phase means morphological and physiological changes (Huisman et al. 1996; Nyström 2004), this different length of the stationary phase might result in different physiological states of the cultures. These can be carried over from the first preculture over the cryoculture and second preculture to the main culture. The initial biomass concentration in some wells might even have been too low to reach the stationary phase during precultivation. In that case, the cultures that were applied for inoculation might not only have differed in their physiological state, but also in their biomass concentration. Thus, although two precultures were conducted (one before and one after the cryoconservation), the variances in biomass concentrations caused by tooth picking were probably carried over to the main culture. Further studies should, therefore, monitor the whole process, beginning with the first preculture to investigate the influencing factors for such high-throughput cultivation processes.

3.4. Conclusions

This study showed that the conventional method of using 96-pin replicators to transfer *E. coli* PR02-cells from one MTP to another MTP can result in highly variable growth kinetics of the applied cultures. To our knowledge, this problem could be demonstrated and quantified for the first time (with the help of the BioLector). The degree of variance of the growth kinetics depends on the applied replicator and replication method. It is caused by variances of the transferred liquid volume, resulting in varying initial biomass concentrations. In contrast to the pin replicators, the 8-channel pipette showed nearly identical growth kinetics. Since there is a need for reliable and reproducible methods for the production of proteins in MTP-based high-throughput processes (Blommel et al. 2007), applying liquid handling systems is strongly advised to reduce variances in transferred liquid volume. Furthermore, growth and product formation of a simulated high-throughput process with *C. glutamicum* was monitored online with the BioLector technique. It showed that the typical steps of a high-throughput production process (colony picking, preculture, main culture and induction) can lead to strongly varying growth kinetics even if a defined single strain is investigated. These growth differences resulted in a strongly varying product formation upon induction of the main culture at a defined time. Additionally, this study showed that the initial biomass concentration strongly affects the product formation if all cultures of *E. coli* BL21(DE3) pRhotHi-2-EcFbFP in a single MTP are induced simultaneously. To sum up: high-throughput cultivation processes with multiple steps are prone to irreproducible protein expression results.

New methods are required to address the above problems. The combination of online monitoring (BioLector) with an automated liquid handling system could be a promising tool to equalize varying growth kinetics by establishing homogeneous initial biomass concentrations in MTPs (refer to Chapter 5). This combination could also compensate varying growth kinetics and hence product formation, because it could automatically induce different cultures at a defined biomass concentration, in contrast to the manual induction at a defined time. Such compensation can also be achieved by the utilization of autoinduction medium or possibly fed-batch precultures in MTPs (refer to Chapter 4). The presented results and the awareness that varying initial biomass concentrations can occur during replication may minimize this problem and, therefore, might help to increase the robustness and reproducibility of high-throughput cultivation processes.

4. Equalizing growth kinetics in fed-batch cultivations

4.1. Introduction

The screening of different clones in shake flasks and MTPs is mainly conducted in batch mode. As described in Chapter 1.3, the growth of precultures to stationary phase in the batch mode can have negative effects on the following main cultivation and hence protein expression. Nevertheless, batch cultivations are predominately applied for small-scale cultures because of their easy use, flexibility, low cost and lack of alternative methods. However, fed-batch mode would often be superior for producing biomass and product. Furthermore, the fed-batch mode provides more defined physiological conditions and is more often applied in industrial scale than the batch mode. Jenzsch et al. (2006) presented the concept of greatly improving the reproducibility of main cultivations in stirred tank reactors via initiating a fed-batch mode already very early in the fermentation. Reproducibility is of utmost importance especially for good manufacturing practice (GMP), as recommended in the PAT initiative from the FDA (Jenzsch et al. 2006). The authors showed that using fed-batch mode starting in the early biomass formation phase can lead to identical growth profiles of differently inoculated cultures because of the fixed feed profile (Jenzsch et al. 2007). This concept which Jenzsch et al. proposed for the early phase of a main cultivation served as a basis for equalizing growth in small-scale precultivations with fed-batch mode. The fed-batch mode can be used for different precultures with diverse growth parameters. Therefore, a fed-batch preculture could

improve the comparability and the selection of clones in screening and high-throughput applications.

Fed-batch systems on a microliter to milliliter scale for screening and bioprocess development are increasingly being developed. These techniques comprise automated stirrer-driven microbioreactors (Puskeiler et al. 2005b), microfluidic devices (Buchenauer et al. 2009; Leeuwen van 2008) or fed-batch in shake flasks (Ruottinen et al. 2008; Weuster-Botz et al. 2001). As these kinds of devices require pumps and additional equipment, they are somewhat impractical for fed-batch in real high-throughput. Panula-Perälä et al. recently published an enzyme controlled glucose auto-delivery system for MTPs and shake flasks (Panula-Perälä et al. 2008). Jeude et al. first have developed a slow-release system to feed substrates such as glucose to shake flasks (Jeude et al. 2006) and MTPs in a easier way without the need for additional equipment or enzymes. In these MTPs, silicone elastomer depots are immobilized at the bottom of each well, thereby allowing the controlled release of glucose to the culture broth. This Chapter presents a novel method for equalizing growth kinetics in high-throughput precultures in shake-flasks and MTPs applying fed-batch mode. *Escherichia coli* and *Hansenula polymorpha* are used as model microorganisms.

4.2. Material and methods

Microorganisms

E. coli BL21 pRSET eYFP-IL6 was maintained in glycerol stocks at -80°C in LB medium with 100 µg/mL ampicillin. This strain was described previously with an additional plasmid pLysS (Samorski et al. 2005). *Hansenula polymorpha* RB11 pC10-FMD (P_{FMD}-GFP) (Amuel et al. 2000; Gellissen 2000) was maintained in glycerol stocks at -80°C in YNB medium and was kindly provided by Dr. C. Amuel (Heinrich-Heine University, Department of Microbiology, Düsseldorf, Germany).

Media and Solutions

Modified Wilms & Reuss synthetic medium was used for the *E. coli* cultivations (Scheidle et al. 2007; Wilms et al. 2001). The medium consists of 20 g/L glucose; 5 g/L (NH₄)₂SO₄; 0.5 g/L NH₄Cl; 3 g/L K₂HPO₄; 2 g/L Na₂SO₄; 0.5 g/L MgSO₄ x 7H₂O; 41.85 g/L MOPS; 0.1 g/L ampicillin; 0.01 g/L thiamine hydrochloride; 1 mL/L trace element solution [0.54 g/L ZnSO₄ x 7H₂O; 0.48 g/L CuSO₄ x 5H₂O; 0.3 g/L MnSO₄ x H₂O; 0.54 g/L CoCl₂ x 6H₂O;

41.76 g/L $\text{FeCl}_3 \times 6\text{H}_2\text{O}$; 1.98 g/L $\text{CaCl}_2 \times 2\text{H}_2\text{O}$; 33.39 g/L Na_2EDTA (Titriplex III)]. The pH was adjusted to 7.5 with NaOH.

Hansenula polymorpha was cultivated in Syn6-MES medium. The Syn6-MES mineral medium consisted of 1.0 g/L KH_2PO_4 , 7.66 g/L $(\text{NH}_4)_2\text{SO}_4$, 3.3 g/L KCl, 3.0 g/L $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.3 g/L NaCl, 27.3 g/L 2-morpholinoethanesulfonic acid (MES). This aqueous basic solution was adjusted to pH 6.4. Then the following substances were added (per L basic solution): 6.67 mL calcium chloride solution (150 g/L $\text{CaCl}_2 \times 2\text{H}_2\text{O}$), 6.67 mL microelement solution (10.0 g/L $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \times 6\text{H}_2\text{O}$, 0.8 g/L $\text{CuSO}_4 \times 5\text{H}_2\text{O}$, 3.0 g/L $\text{ZnSO}_4 \times 7\text{H}_2\text{O}$, 4.0 g/L $\text{MnSO}_4 \times \text{H}_2\text{O}$, 10.0 g/L EDTA (Titriplex III)), 6.67 mL of vitamin solution (0.06 g/L D-biotin, 20.0 g/L thiamine hydrochloride), 3.33 mL of trace element solution (0.2 g/L $\text{NiSO}_4 \times 6\text{H}_2\text{O}$, 0.2 g/L $\text{CoCl}_2 \times 6\text{H}_2\text{O}$, 0.2 g/L boric acid, 0.2 g/L KI and 0.2 g/L $\text{Na}_2\text{MoO}_4 \times 2\text{H}_2\text{O}$). The medium resulted in a final volume of 1023.33 mL and no final pH adjustment was necessary (Gellissen 2004; Jeude et al. 2006).

The medium used for fed-batch cultivations had no initial glucose because of the immediate release of glucose from the slow-release system (Jeude et al. 2006). All chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany), Carl Roth (Crailsheim, Germany) or Sigma (Taufkirchen, Germany).

Manufacture of MTPs with slow-release system

To manufacture MTPs with slow-release system, denominated as 'FeedPlates', solvent-free two-component silicone Sylgard™184 was used. The ratio between the two components was 10:1 as recommended by the manufacturer. Anhydrous glucose was supplied by Sigma Aldrich (Crailsheim, Germany) with the highest degree of purity. The glucose was milled with a vibration micromill (Spartan™, Fritsch, Idar-Oberstein, Germany) in a high-grade steel mortar and then sieved with test sieves (Fritsch, Idar-Oberstein, Germany). The fraction with particle sizes ranging from 20 to 50 μm was used. First, the two silicone components of the Sylgard™184 and glucose were mixed. Second, chloroform (1-5 mL chloroform per 10 g silicone-glucose mixture) was added to decrease the viscosity of the mixture allowing it to flow more easily. Thereafter, 100 μL of the compounds were filled at the bottom of each cavity of a 2.2 mL polypropylene MTP (HJ Bioanalytik, Mönchengladbach, Germany) using a multipipette (Eppendorf, Wesseling-Berzdorf, Germany). The plate was stored at 50°C for 12 h to aid cross-linking.

Cultivation

For reproducible inoculation of the example precultures in this paper, additional pre-cultures were performed in Wilms & Reuss synthetic medium and Syn6-MES medium for *E. coli* and *H. polymorpha*, respectively. The following cultivation parameters were applied: 350 rpm shaking frequency, 50 mm shaking diameter, 10 mL filling volume in 250 mL shake flasks. These pre-cultures were centrifuged and washed two times in 5 mL fresh medium and optical densities were measured. These OD values were used for the calculation of the required inoculation volume for each described experiment. Cultures in shake flasks were conducted in an in-house made Respiration Activity Monitoring System (RAMOS) for online-monitoring of oxygen transfer rates (OTR), as previously described by Anderlei et al. (Anderlei and Büchs 2001; Anderlei et al. 2004). The following cultivation parameters were applied: 350 rpm shaking frequency, 50 mm shaking diameter, 10 mL filling volume in 250 mL RAMOS flasks. The applied fed-batch mode in shake flasks was realized by using three slow-release discs per flask. These discs are denominated as 'FeedBeads' and are available from Adolf Kühner AG, Birsfelden, Switzerland. In general, the discs with silicon elastomer did not adversely affect microbial growth (Jeude et al. 2006).

For fed-batch cultivations, deepwell plates with immobilized silicon elastomer depots at the bottom (FeedPlates) were used under the following conditions: 700 μ L filling volume, 25 mm shaking diameter and 400 rpm shaking frequency. The MTPs were sealed with an airpore-sheet (nonwoven sealing foil, HJ Bioanalytik, Mönchengladbach, Germany) and cultivated under a humified aerated hood to minimize evaporation. The substrate released up to the time t can be described by the following equations (Eq.):

$$\text{glucose-release} = 2 \cdot t^{0.69} \quad [\text{mg/disc}] \quad \text{for FeedBeads and} \quad \text{Eq. 1}$$

$$\text{glucose-release} = 0.8 \cdot t^{0.72} \quad [\text{mg/well}] \quad \text{for FeedPlates.} \quad \text{Eq. 2}$$

The glucose release kinetic of Eq. 2 is shown in Figure 4-1D. For subsequent fed-batch cultivations, precultures were centrifuged and resuspended in glucose-free medium to prevent possible residues of glucose from the preculture. All cultures were incubated at 37°C in shakers of type LS-W or ISF-4-W from Adolf Kühner AG (Birsfelden, Switzerland).

Analytical methods

Optical density of MTP experiments was measured at 600 nm with the MTP reader Powerwave X340 (Bio-Tek Instruments GmbH, Bad Friedrichstal, Germany) and for shake flask experiments a Uvikon 922 spectrophotometer (Kontron, Milano, Italy) was used. Samples were measured in the linear range of OD measurements after dilution in fresh medium. Sampling and OD measurement were each conducted twice or thrice. Samples from the fed-batch MTPs were withdrawn from different wells. Otherwise, the lower volume and the continuous release of substrate would have led to glucose concentrations that are higher than those in the unsampled wells.

4.3. Theoretical background

A simple model for batch and fed-batch cultivations in MTPs was applied to demonstrate the concept of equalizing the growth in precultures. The variation of initial biomass concentrations (inocula), lag phase and specific growth rates was chosen to visualize growth and substrate kinetics of different cultures. These cultures may represent various clones of a clone library.

Modeling

A simple model for fed-batch cultivations in MTPs with standard bioreaction equations based on Monod kinetics was applied (Eq. 3 to 7; for a nomenclature of the symbols refer to Chapter Abbreviations).

$$\frac{dX}{dt} = (\mu - K_d) \cdot X \quad [g/L/h] \quad \text{Eq. 3}$$

$$\frac{dS}{dt} = -\frac{1}{Y_{X/S}} \cdot \mu_{\max} \cdot X + \text{Feedrate} \quad [g/L/h] \quad \text{Eq. 4}$$

$$\mu = \frac{\mu_{\max} \cdot S}{(S + K_S)} \cdot \text{rel}_{\text{act}} \quad [1/h] \quad \text{Eq. 5}$$

$$\text{Feedrate} = \frac{0.8 \cdot 0.72 \cdot t^{(-0.28)}}{V_L} \quad [g/L/h] \quad \text{Eq. 6}$$

$$\text{rel}_{\text{act}} = \frac{1}{1 + e^{(2+4 \cdot \frac{t-t_{\text{lag}}}{t_{\text{acc}}})}} \quad [-] \quad \text{Eq. 7}$$

Eq. 6 was obtained by differentiating Eq. 2 and referring it to the utilized filling volume in a well. Eq. 7 is an equation to represent the lag and acceleration phase of a culture. The term rel_{act} (relative activity) in Eq. 5 and 7 accounts for the lag phase and acceleration phase and varies from 0 (start of cultivation) to 1 (cells adapted to the new medium). The advantage over existing equations is that the two parameters t_{lag} and t_{acc} can directly be interpreted as the time constant for the lag and acceleration phase, respectively. A decay term was introduced to simulate the decrease in biomass due to the lack of a carbon source and the accumulation of end products in the stationary phase of batch cultivations. As there is no general consensus for mathematical modeling of a decay term (K_d) or death rate (Toal et al. 2000), a constant decay term was assumed in the model (Moser and Steiner 1975). Simulations were conducted with Modelmaker (Cherwell Scientific, Oxford, UK).

Typical values for cultivation parameters of *E. coli* were introduced into the model ($K_S = 0.2$ g/L, $t_{acc} = 0.5$ h, $K_d = 0.01$ 1/h, $Y_{XS} = 0.5$ g/g, $S_0 = 15$ g/L for batch, $S_0 = 0$ g/L for fed-batch). All the other model parameters were selected according to the caption of Figure 4-1. The volume of the cultivation was set at 700 μ L as a typical value for small-scale cultivations in deepwell MTPs. Within the simulation time of 34 h, the initial glucose concentration (substrate S_0) in the batch model was comparable to the total amount of glucose fed in the fed-batch mode. The term *Feedrate* in Eq. 4 was omitted for the batch simulations.

Batch cultivation

After the lag phase, the different batch cultivations grow exponentially (Fig. 4-1A) until the substrate is exhausted (Fig. 4-1B). Then, the stationary phase begins and the decay of viable biomass becomes apparent. This applies for all four simulations which results in different growth kinetics and physiological states at any time in batch mode. Moreover, non-optimal conditions for inoculation of the main culture are realized in the stationary phase (see arrow in Fig. 4-1A), because the clones are nutrient depleted in the stationary phase for different times. When these cells are used as a preculture for screening experiments, differences in growth kinetics in the main cultivation are most probable. This might also be the reason for the different growth kinetics that were observed for a simulated high-throughput cultivation process in Chapter 3.3, Fig. 3-4.

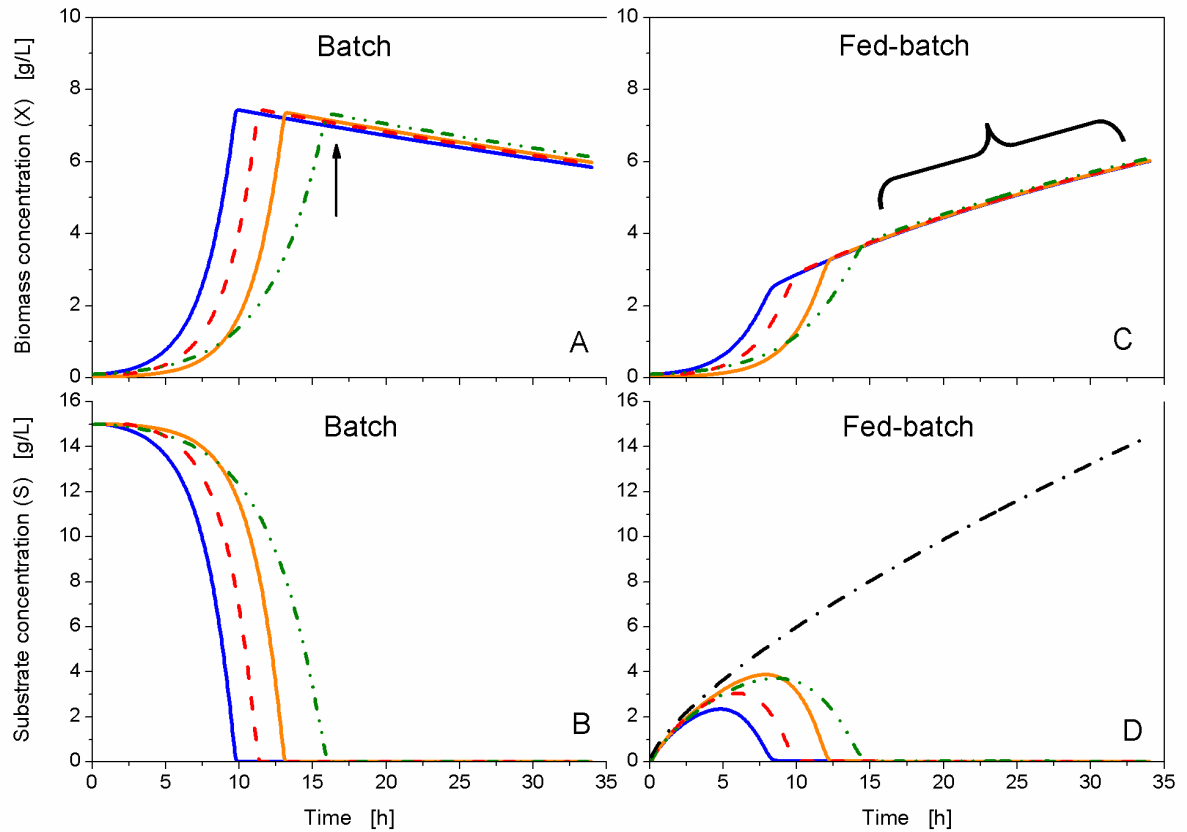


Figure 4-1: Comparison of batch and fed-batch modes for cultivating precultures. Variations in lag phase, maximum specific growth rate or initial biomass concentration were simulated. (A) growth kinetics in batch mode; (B) substrate kinetics in batch mode; (C) growth kinetics in fed-batch mode; (D) substrate kinetics in fed-batch mode. (blue —) reference growth parameters ($X_0 = 0.1$ g/L; $t_{lag} = 0.5$ h; $\mu_{max} = 0.5$ 1/h); (---) decreased initial biomass concentration ($X_0 = 0.02$ g/L; $t_{lag} = 0.5$ h; $\mu_{max} = 0.5$ 1/h); (orange —) increased lag time ($X_0 = 0.1$ g/L; $t_{lag} = 2.0$ h; $\mu_{max} = 0.5$ 1/h); (-•-) decreased μ_{max} ($X_0 = 0.1$ g/L; $t_{lag} = 0.5$ h; $\mu_{max} = 0.3$ 1/h); (-•-) total release of substrate from slow-release system in fed-batch mode (calculated using Eq. 2). The arrow and the bracket indicate the time for inoculation of the main culture from batch and fed-batch preculture, respectively.

Fed-batch cultivation

The fed-batch mode allows predefined growth behavior of the culture via feeding and, thus, offers the advantage of a more controlled process than the batch mode. In fed-batch simulations, shown in Figure 4-1C and D, the addition of glucose to the applied medium was unnecessary, because of the immediate release of glucose from the release system. This is also apparent in Figure 4-1D, which shows the total release of glucose throughout the cultivation. The substrate accumulates at the beginning of the fermentation when the cell concentration is still too low to consume the released glucose. After 5 to 10 h the biomass of the simulated cultures consume more glucose than provided by the slow-

release system and, consequently, the substrate concentration decreases (Fig. 4-1D). The culture turns from a batch to a fed-batch phase after 8 to 15 h (Fig. 4-1C). In this phase the microorganisms are growing in a substrate-limited fashion and show a growth rate predefined by the feed rate of the slow-release system (Eq. 6). It is obvious that the fed-batch mode equalizes the different growth behavior of all cultures. The cells are constantly supplied with substrate, thereby resulting in constant growth and a defined metabolic state from 15 h onwards (Fig. 4-1C). In this way, the adverse effects of nutrient starvation or accumulation of overflow metabolites are minimized.

This system is self regulating concerning the consumption of glucose of each culture. After the different bacterial clones switched over to glucose-limited growth, they all consume equal amounts of glucose and, hence, produce equal cell densities. The simulation demonstrates that fed-batch fermentations with defined feeding rates can equalize cultures that have different inocula, specific growth rates and lag phases. Moreover, the time for inoculation of main cultures is, in contrast to batch-precultures, no longer important. Even if the transfer of inocula is postponed relative to a fixed schedule, e.g. due to some practical reasons, the precultures do not suffer from carbon source depletion (see bracket in Figure 4-1C) and preserve their metabolic activity.

4.4. Results and discussion

4.4.1. RAMOS cultivations

Batch mode

Batch cultures of the recombinant strain *E. coli* BL21 pRSET eYFP-IL6 were cultivated as reference at three different initial optical densities (OD_{t_0}) from 0.1 to 0.5. These represent a range commonly applied when inoculating cultures. The cultures were inoculated from the same preculture. The oxygen transfer rates are depicted in Figure 4-2 against the fermentation time. The oxygen transfer rate (OTR) signal shows typically exponential growth and oxygen is limited only for a short period (plateau) upon attaining an OTR of approximately 60 mmol/L/h. When glucose is exhausted, the OTR decreases sharply (e.g. at 12 h for the culture with $OD_{t_0} = 0.1$). Due to overflow metabolism, acetate is formed during the exponential growth phase; its assimilation marks the second peak in the OTR curve of each culture. These phenomena have been previously described for this strain (Scheidle et al. 2007).

The different inocula resulted in a time variation of approximately 4 h in growth. The results demonstrate clearly the significant difference in growth kinetics of the microorganisms caused only by different initial biomass concentrations.

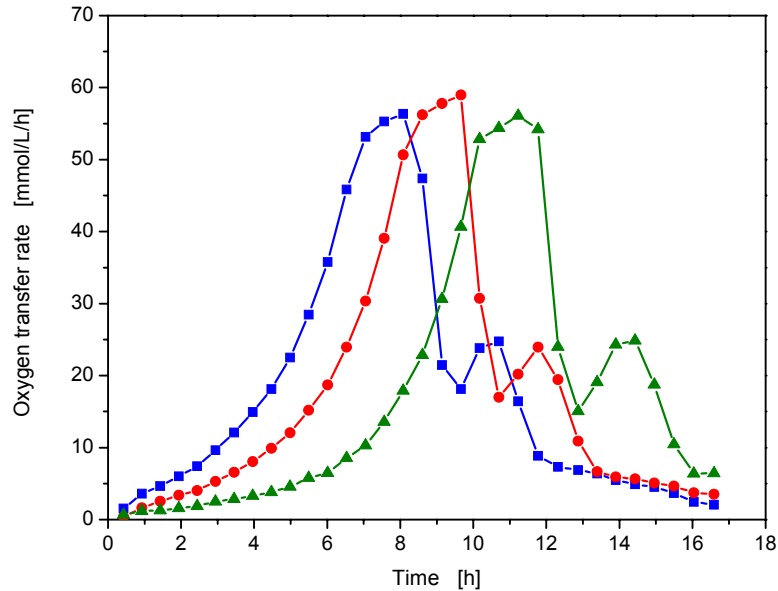


Figure 4-2: Batch cultivation of *E. coli* BL21 pRSET eYFP-IL6 in RAMOS flasks with various initial biomass concentrations: $OD_{t0} = 0.5$ (■), $OD_{t0} = 0.3$ (●), $OD_{t0} = 0.1$ (▲). Conditions: Wilms-MOPS medium with 15 g/L glucose, $T = 37^{\circ}\text{C}$, $V_L = 10$ mL, $n = 350$ rpm, $d_0 = 50$ mm.

Fed-batch mode

For fed-batch cultures in shake flasks, FeedBeads were applied, and the fermentation was monitored online with the RAMOS device (Fig. 4-3A). The exponential growth of the batch phase in the beginning is followed by substrate limitation and a sharp OTR decrease. It is noteworthy that this decrease does not fall to zero but rather to a defined value of approximately 3 mmol/L/h. This value reflects the constant release of the substrate from the FeedBeads during the fed-batch phase. This growth pattern is obvious for all of the three differently inoculated cultures. The OTR peak of the batch phase increases for cultures with less inoculum (from 10 mmol/L/h for $OD_{t0} = 0.5$ to 17 mmol/L/h for $OD_{t0} = 0.1$; Fig. 4-3A). This increase is caused by the fact that more glucose accumulates in the flasks with lower inoculum before the fed-batch phase starts (refer to Fig. 4-1D). Nevertheless, as soon as all cultures have reached the fed-batch mode (11 h), they all have consumed the same total amount of glucose. In fed-batch mode neither acetate formation nor oxygen limitation can be observed due to the lower initial glucose concentration and subsequent glucose-limited growth as compared with the batch mode (compare Fig. 4-3A and Fig. 4-2). All the cultures are found to be in a defined and similar metabolic state beginning at 11 h and are actively growing. At the end of the

experiment, the OD of the cultures with an initial biomass of OD_{t_0} of 0.1, 0.3 and 0.5 were 4.4, 4.5 and 4.2, respectively. This demonstrates equalized growth.

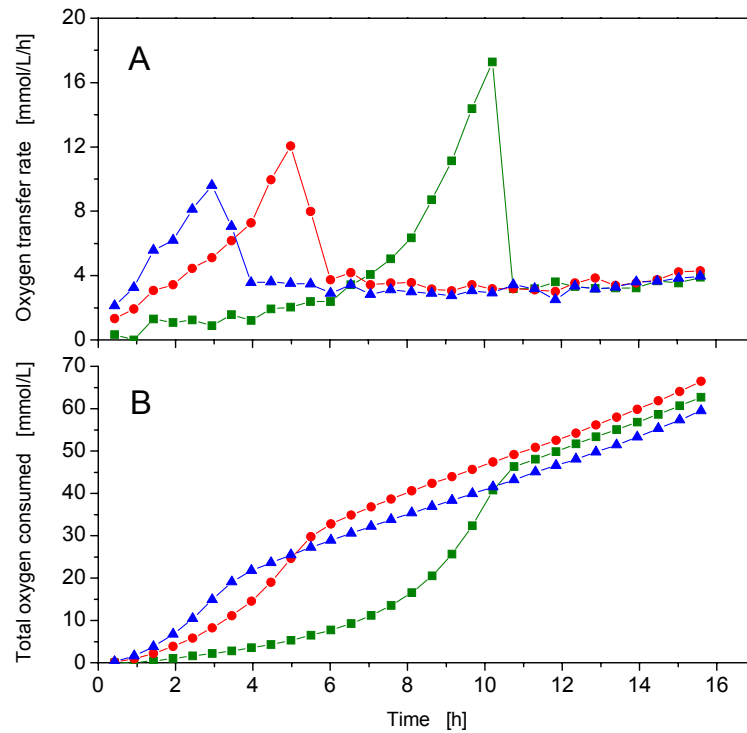


Figure 4-3: Fed-batch cultivation of *E. coli* BL21 pRSET eYFP-IL6 in RAMOS flasks with various initial biomass concentrations: $OD_{t_0} = 0.5$ ($\blacktriangle$), $OD_{t_0} = 0.3$ ($\bullet$), $OD_{t_0} = 0.1$ ($\blacksquare$). (A) oxygen transfer rate and (B) calculated total oxygen consumption. Conditions: Wilms-MOPS medium with no additional glucose, 3 FeedBeads per flask, $T = 37^{\circ}\text{C}$, $V_L = 10\text{ mL}$, $n = 350\text{ rpm}$, $d_0 = 50\text{ mm}$.

Figure 4-3B shows the total oxygen consumed by the cells at any given time obtained by the integration of the OTR. By assuming a constant yield coefficient of biomass to oxygen during fermentation, the total oxygen consumption reflects the increase in biomass and is in good agreement with the simulated data (Fig. 4-1C). Variations in the total oxygen consumption during the fed-batch phase (Fig. 4-3B) result from errors in integrating curves with only few data points.

4.4.2. Fed-batch in microtiter plates

The newly developed fed-batch system for MTPs was used. Two microorganisms were tested to equalize the various cultures. Initial optical densities of 0.05, 0.1 and 0.3 were applied to simulate variable growth kinetics. The different cultures were inoculated from the same pre-preculture. For *E. coli*, varying the inoculum from the highest to the lowest initial biomass concentration yielded a ca. 10 h delay in growth (Fig. 4-4). In fed-batch mode the cultures turned one after another from an exponential growth to a controlled nearly linear increase in biomass concentration. After approximately 15 h, all cultures were equalized and attained the same biomass concentration of OD 6.5 at the end of the experiment. As previously mentioned, this effect was caused by the same total amount of glucose being released per well.

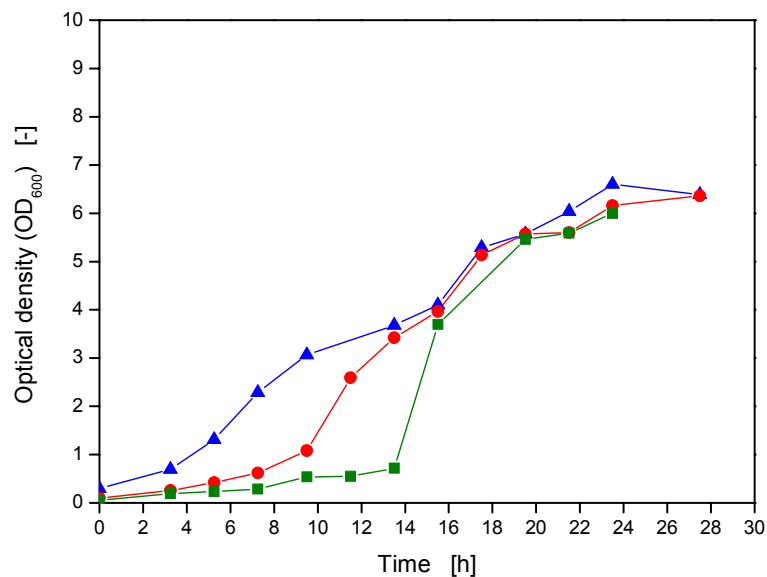


Figure 4-4: Fed-batch precultivation of *E. coli* BL21 pRSET eYFP-IL6 in a fed-batch deepwell plate with various initial biomass concentrations: OD_{t0} = 0.5 (▲), OD_{t0} = 0.3 (●), OD_{t0} = 0.1 (■). Wilms-MOPS medium with no additional glucose, T = 37°C, V_L = 700 µL, n = 400 rpm, d₀ = 25 mm.

The same behavior as for *E. coli* can be observed for *Hansenula polymorpha* cultures. Here, a 10 h delay in growth is observed for cultures with high and low inoculum concentration (Fig. 4-5). The applied fed-batch mode enables equalization of all cultures after 24 h. At 50 h the different cultures still have the same biomass concentration and are actively growing.

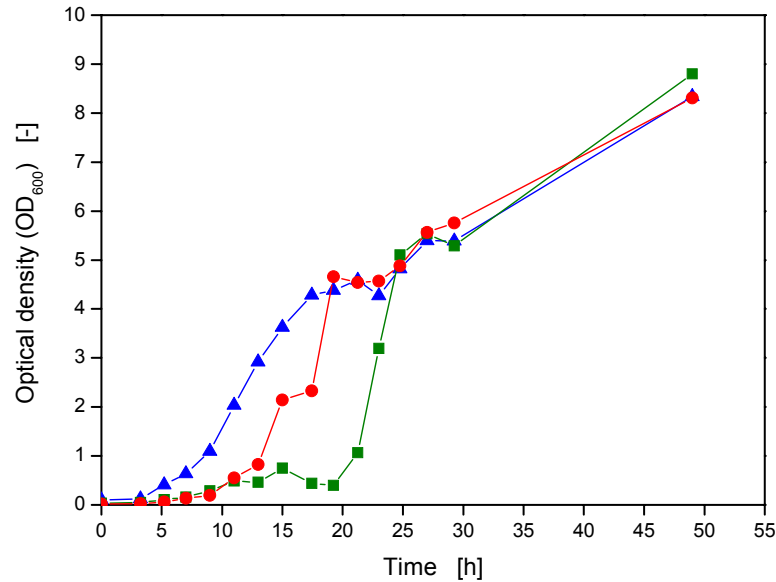


Figure 4-5: Fed-batch precultivation of *Hansenula polymorpha* in a fed-batch deepwell plate with various initial biomass concentrations: $OD_{t_0} = 0.5$ (▲), $OD_{t_0} = 0.3$ (●), $OD_{t_0} = 0.1$ (■). Conditions: Wilms-MOPS medium with no additional glucose, $T = 37^{\circ}\text{C}$, $V_L = 700\ \mu\text{L}$, $n = 400\ \text{rpm}$, $d_0 = 25\ \text{mm}$.

4.5. Conclusions

A new technique for growth equalization in high-throughput cultivations by applying fed-batch mode in the precultures was simulated and verified experimentally. Growth of differently inoculated cultures in shake flasks and MTPs could be equalized and, therefore, the feasibility of this concept could be demonstrated. The concept worked for procaryotic and eucaryotic microorganisms. Consequently, this technique seems to be of general applicability.

The inherent advantages of this method are that it is easy to use as it requires no additional equipment for fed-batch cultivations on a small-scale. Furthermore, there is no need for permanent and laborious offline-monitoring of precultures to determine the right time of transfer to a main cultivation. Moreover, the exact time for inoculation of

main cultures is, in contrast to batch precultures, no longer important. The system is self-regulating; the cells are continuously supplied with substrate and are in a defined metabolic state. This represents another strategy for achieving uniform conditions in the growth of different clones as recommended by Studier (Studier 2005), with the exception that the microorganisms are not in the stationary growth phase. Possible oxygen limitations and adverse effects of the batch mode can also be avoided in the fed-batch mode.

This technique might be especially useful for microorganisms exhibiting decreasing viability in the stationary phase and in which synchronous growth of distinct cultures is very important. Furthermore, precultures in fed-batch mode MTPs can generate more relevant data in screening processes (Jeude et al. 2006), because the starting conditions for all strains under study are equal. A possible disadvantage of the introduced equalization technique may be the expression of toxic products in hosts which are de-repressed by low glucose concentrations during the fed-batch phase. Further investigations have to be performed to prove this method with different clone libraries and to study the impact of equalized precultures on product formation (e.g. recombinant proteins or amino acids) in subsequent main cultivations.

5. Robo-Lector – a novel automated microbioreactor system for high-throughput experimentation

5.1. Introduction

The induction point for protein expression in *E. coli* as well as the inducer concentration are the most critical factors which influence product yield (Donovan et al. 1996; Miao and Kompala 1992). Interestingly, only few studies have systematically investigated this aspect (Donovan et al. 1996). In small-scale cultivations, e.g. in shake flasks or MTPs, it is very tedious to investigate in detail recombinant protein production with conventional means. These studies entail exhaustive work (e.g. induction at different times, drawing samples, optical density measurement with dilution of samples, protein analytics via SDS-PAGE or other assays). This might be the reason, why in many laboratories a generic procedure is applied for routine protein expression, namely to induce cells in the early to mid-log growth phase with an IPTG ‘standard concentration’ of 1 mM (Berrow et al. 2006; Donovan et al. 1996). However, protein expression is also influenced by many other factors (refer to Chapter 1.2), such as the host-vector system, promoter strength, characteristics of the target protein, media composition and culture conditions (Donovan et al. 1996; Jana and Deb 2005). Therefore, a generic approach may often lead to suboptimal induction conditions and is normally unsuitable for taking into account multiple parameters to understand and optimize specific expression systems. A MBR

system that automatically checks at least the influence of the two main parameters - induction time and inducer concentration - would help to systematically investigate and understand a given host-vector system and could contribute to better expression results than the generic procedure.

Induction at different growth phases can lead to great variations in product formation (Chapter 3.3, Fig. 3-2 and 3-4). Therefore, when different clones of a clone library have to be compared regarding recombinant protein yield (e.g. clones of type 3, Table 1-1), it is necessary to induce the clones at comparable biomass concentrations. The conventional clone screening approach would be to monitor the growth of a limited number of clones off-line (e.g. via OD measurement) and then induce them at an appropriate time. This, however, is tedious and not feasible for a large number of clones. Studier considers it even impossible to monitor growth and induce parallel cultures at the same growth phase in high-throughput cultivations (Studier 2005). Consequently, the autoinduction medium has been developed. Being a highly sophisticated method, it requires no manual addition of IPTG. It automatically induces the cultures with lactose after the cells have consumed a certain amount of glucose (Studier 2005). Nonetheless, this principle cannot be applied for all host-vector systems. Therefore, it would be useful to have a system that permanently monitors growth of different clones in parallel MBRs and uses this information to automatically add inducer at the same physiological state of the cultures.

MBRs have also become increasingly important for industrial production processes. Here, many microbial processes are still poorly understood, especially in the area of recombinant protein expression (Clementsich and Bayer 2006). Hence, process analytical technology (PAT)-driven initiatives tend to use scale-down systems such as (disposable) MBRs in process characterization and validation (Chen et al. 2009; Rao et al. 2009). In these areas, there is also a strong need to combine different unit operations (upstream, downstream and product analysis) in small-scale automated routines (Betts and Baganz 2006; Lye et al. 2003), which demand automation, high-throughput sampling and, ideally, direct product analysis from MBRs (Rao et al. 2009). Quantitative kinetic data for protein production are also needed in systems biology to validate dynamic mathematical models of the regulatory mechanisms and networks of expression systems. Therefore, the use of parallel MBRs with integrated online-monitoring of product formation would be highly beneficial in systems biology applications and, in general, for industries producing recombinant proteins.

To summarize, the development and application of more advanced MBRs, sampling and measurement devices is necessary for better understanding biological systems and whole bioprocesses (Känsäkoski et al. 2006). Consequently, as demonstrated above, many disciplines of biotechnological research and development that use MBRs require:

- 1) flexible, automated cultivation systems to reduce human manual error and workload, increase throughput and enable the investigation of complex workflows (e.g. multiple cultivation steps);
- 2) MBRs with advanced sensing technologies capable of providing high-content data of key cultivation parameters (pH, DOT, biomass) and especially protein production; and
- 3) modular platforms for allowing the combination of easy to use, disposable MBRs with downstream processes and analytical assays.

Various systems that partially fulfill these requirements will be discussed as follows (also refer to Chapter 1.4, Table 1-2). Only systems which use MBRs with volumes of approximately 0.1 to 35 mL (excluding microfluidic devices) are presented.

MBR automation has been addressed in some systems. There are two main concepts to implement automated cultivation systems. The first one is to integrate MBRs in existing liquid-handling workstations or to combine them with standard robotic devices. For instance, Puskeiler et al. have developed such a system, consisting of a MBR device (called bioreactor block) integrated in a liquid-handling robot (Puskeiler et al. 2005b). Zimmermann and Rieth established a system that utilizes MTPs and standard robotic equipment in a climate chamber to screen mutant libraries (Zimmermann and Rieth 2007). Another MBR device with 24 individual minireactors (μ 24, Applikon) can be combined with a plate crane and a single-channel pipettor for sampling and feeding (Chen et al. 2009).

The second concept to automate the operation of MBRs is to design and build completely new combinations of devices for a particular task. Such systems are highly sophisticated and consist of multiple devices (MBR, robotic arm, sampling module, sensing module), which are linked to each other via complex control software. In order to provide a fully automated option for protein expression and purification in high-throughput, a special cultivation system with discontinuous optical density measurement (PiccoloTM) has been developed for *E. coli* or insect cell expression systems (Wollerton et al. 2006). Another automated system (SimCellTM) uses cell culture MBR arrays that are handled in a cluster design around a central robotic arm (Legmann et al. 2009). Because such systems are

highly complex and need special equipment and periphery, they tend to have high investment costs, prohibiting the widespread use in industry and even more in academia.

Online monitoring of pH and DOT is realized in many of these MBR systems (refer to Chapter 1.4, Table 1-2). Additionally, some of these systems measure microbial growth via optical density atline (sample is removed from the reactor), although this has the disadvantage of only limited data density (Betts and Baganz 2006; Puskeiler et al. 2005b) and sometimes requires interruption of the cultivation process. Furthermore, none of the above described systems has the possibility to conduct fluorescence protein monitoring online in a large number of parallel MBRs.

Unlike most MBR systems, the BioLector technique provides a cultivation system that is able to permanently monitor microbial growth, fluorescence of reporter proteins, pH and DOT under defined conditions in MTPs without interrupting the shaking movement (Kensy et al. 2009; Samorski et al. 2005). Using MTPs offers the advantage of high-throughput, low costs, standardization and thoroughly studied engineering parameters for cultivation of microorganisms (Duetz 2007; Fernandes and Cabral 2006; Hermann et al. 2003; Islam et al. 2008; Kensy et al. 2005a; Kensy et al. 2005b). Furthermore, MTPs – being the international standard for laboratory automation – offer widespread and easy automation possibilities and allow high-throughput bioprocess development (Samorski et al. 2005; Zhang et al. 2008).

In this Chapter, the development and first results of a new concept for an automated microfermentation platform (for shortness here called 'Robo-Lector') is presented. This platform combines a liquid-handling workstation with a BioLector, resulting in a flexible system to study small-scale cultivation processes in detail. Here, the following three automated methods using the Robo-Lector are described:

- 1) 'induction profiling' comprises a method to investigate the influence of induction time and inducer concentration on protein expression;
- 2) 'biomass-specific induction' is a method that enables one to induce cultures with different growth kinetics at a similar physiological state (meaning that the different wells are induced at different specific time points); and
- 3) 'biomass-specific replication' is a method that equalizes the biomass concentration of a preculture MTP for further experiments.

In these methods, a common expression system (*E. coli* BL21(DE3) with a plasmid harboring a fluorescent reporter protein under the control of the T7 promoter) was used to study in detail recombinant protein expression of this host-vector system and to validate the established platform.

5.2. Material and methods

Organism, medium and solutions

For all experiments the strain *E. coli* BL21(DE3) pRhotHi-2-EcFbFP was used. For a detailed description of this expression system refer to Chapter 3.2. To induce protein expression, sterile filtered IPTG stock solutions of different concentrations (0.2 mM to 30 mM) were applied. For all cultivation experiments, MDG mineral medium with glucose as a carbon source was used (Studier 2005). It was prepared according to Chapter 3.2. All chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany), Carl Roth (Crailsheim, Germany) or Sigma (Taufkirchen, Germany).

Cultivation

All the cultivations were carried out in sterile black 96 well MTPs (μ Clear, Greiner Bio-One, Frickenhausen, Germany) in the MBR system BioLector (m2p-labs, Aachen, Germany) (Kensy et al. 2009). The MTPs were sealed with sterile pierceable, resealable tape (X-Pierce, Excel Scientific, Victorville, USA), allowing ventilation of the wells at reduced evaporation rates. The following conditions were applied for all cultivations in the BioLector: temperature 37°C, shaking diameter 3 mm, shaking frequency 950 rpm. The EcFbFP fluorescence was monitored at an excitation of 460 nm and an emission of 492 nm. The biomass concentration was measured via scattered light intensity (I) (Kensy et al. 2009; Samorski et al. 2005) and was detected at an excitation of 620 nm. The initial scattered light intensity (I_0) was mainly attributed to such factors as the media background or the type of the MTP and was subtracted from the residual scattered light data ($I-I_0$) (Samorski et al. 2005). There is a general linear correlation between scattered light and OD (Kensy et al. 2009). In the current Chapter, the scattered light data can be correlated to OD values according to the equation $OD = \text{scattered light} / 22.4$, which was determined with a calibration curve (similar to (Kensy et al. 2009)). Nevertheless, for comparing different cultures in parallel experiments, a calibration to OD values is not essential. Hence, the biomass data are shown as scattered light intensity in arbitrary units and only at important points OD values are given. The measurement cycle for EcFbFP and scattered light monitoring was 5 min for the method ‘induction profiling’.

This means that the EcFbFP and scattered light signals were measured every 5 min in every single well of a MTP. For the method ‘biomass-specific induction’ this cycle time was 4 min and for the ‘biomass-specific replication’ method the scattered light was monitored every 10 min. The total filling volume per well at the beginning of the cultivation was 190 μ L or 200 μ L. Precultures were made in a 250 mL shake flask under the following conditions unless otherwise stated: inoculation with a cryoculture to yield an OD at the start of 0.1, temperature 37°C, total filling volume 10 mL of MDG mineral medium, shaking diameter 50 mm, shaking frequency 350 rpm, duration 16 to 24 h. OD was measured at 600 nm with a Uvikon 922 spectrophotometer (Kontron, Milano, Italy).

Design of the automated microfermentation platform

The Robo-Lector system presented in this study was made up of the MBR system BioLector and a liquid-handling workstation (Microlab STAR, Hamilton Robotics, Martinsried, Germany) (Fig. 5-1). Here, a custom-built BioLector was placed down into the free space under the deck of the pipetting robot. The height of the BioLector setting was adjusted so that the traverse height of the pipetting arm did not interfere with the BioLector. Moreover, the liquid-handling workstation was equipped with a HEPA (High Efficiency Particulate Air Filter) hood providing an air stream with a very low particle fraction for transferring and storing liquids (e.g. medium, inducer stock solutions) under sterile conditions. The pipetting robot was connected to a computer (via USB connection) and actuated via a control software (Vector software, Hamilton Robotics). In addition, the BioLector was connected to the same computer (via a TCP/IP-network) and controlled with the BioLector HMI (Human Machine Interface). Different command codes for controlling the BioLector (e.g. open lid, pause measurement) were implemented in the Vector control software of the pipetting robot, thereby allowing the integration of the BioLector in complex workflows including liquid-handling processes and MTP movements. Different methods were programmed, tested and optimized regarding time schedules and ease of use. For a more detailed description of the system setup refer to Appendix B.

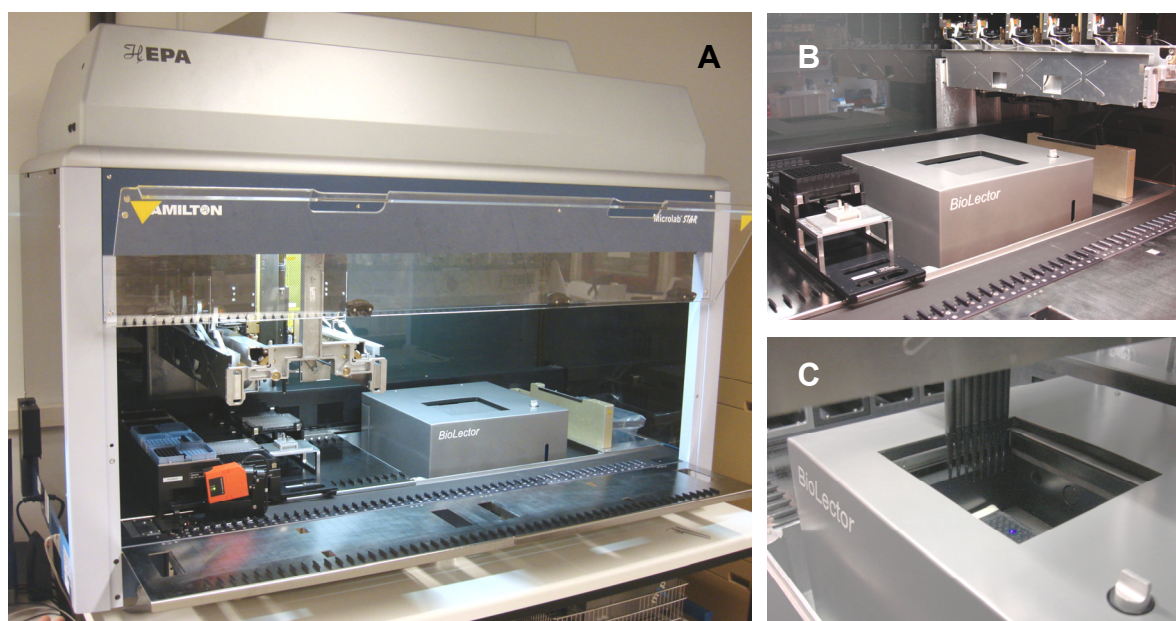


Figure 5-1: The Robo-Lector automated microfermentation system. (A) BioLector (m2p-labs) integrated into a liquid-handling workstation (Microlab STAR, Hamilton Robotics). (B) detail view of the integrated BioLector. (C) 8 channel pipetting head adding liquid to a MTP in the BioLector.

Induction profiling

The induction profiling method was established to study recombinant protein expression in host cells that require inducing agents (e.g. IPTG, arabinose) to start protein production. A method was programmed for the liquid-handling platform that enables the automatic induction of up to 96 cultures of a recombinant strain in a MTP with varying inducer concentrations at different times. The principle of this so-called induction profiling method is shown as a simplified flowchart in Figure 5-2. To start the method, the user first has to define the induction parameters in the program, i.e. the first point of induction (t_0), the time interval between two induction points (Δt) and the desired volume of inducer to be added to each well (v) (Fig. 5-2A, step 1.). Besides that, the cultivation parameters for the BioLector have to be defined (step 1.), e.g. the desired temperature (T) or the shaking frequency (n) (see also *Cultivation* section).

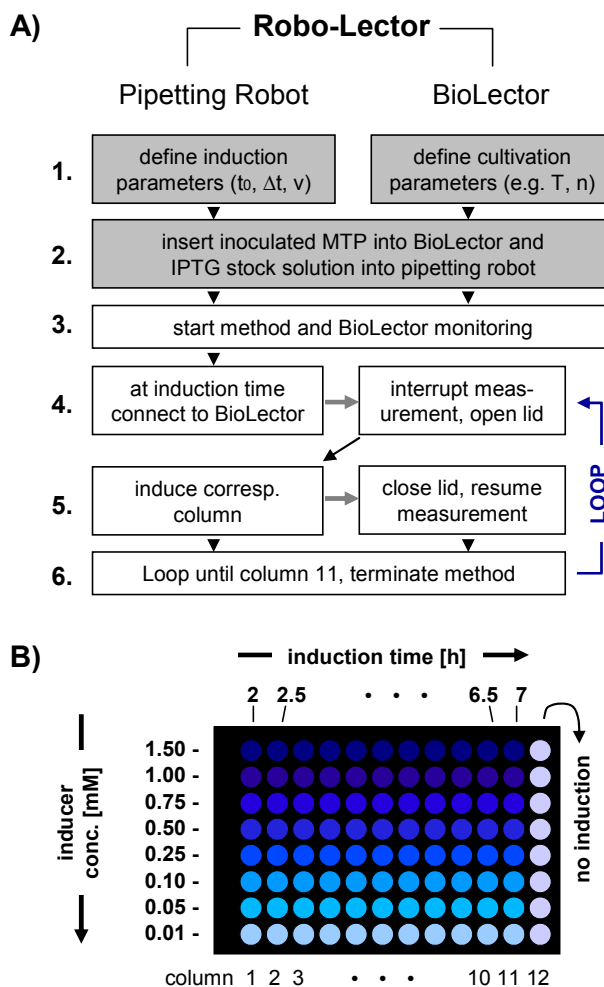


Figure 5-2: Principle of method *induction profiling*. (A) Simplified process flowchart; shaded boxes indicate necessary user input; gray arrows indicate interaction of the pipetting robot and the BioLector; parameters t_0 : time of first induction, Δt : time interval between two induction points, v : volume of inducer added per well, T : cultivation temperature, n : shaking frequency; refer to *Cultivation* section for more details. (B) Established experimental procedure for induction profiling used in this work.

Then, a MTP with medium is inoculated with a preculture of *E. coli* BL21(DE3) pRhoHi-2-EcFbFP (to give 190 μ L of culture with an OD of 0.1) and placed inside the BioLector (step 2.). In parallel, a MTP with 8 different IPTG stock solutions ranging from 0.2 mM to 30 mM is placed inside the pipetting robot. At the different times of induction, 10 μ L of the 8 stock solutions are transferred to the corresponding wells of the culture plate in the BioLector, thereby yielding IPTG concentrations of 0.01 to 1.5 mM (Fig. 5-2B). The aforementioned steps are manually conducted by the user, whereas after the start of the method (step 3.) the whole process (monitoring of the cultivation in the BioLector, addition of inducer) is run completely automated.

Once the cultivation reaches the predefined first induction point (2 h in this study), the liquid-handling robot picks up 8 sterile filtered tips and finally aspirates 10 μL of each IPTG stock solution. Thereafter, the pipetting channels move over the BioLector lid. Then the pipetting robot software links up to that of the BioLector, interrupts the measurement and opens the BioLector lid (step 4.). When the lid opens, the shaking movement in the BioLector stops, and the IPTG solutions are dispensed into the first column of the culture plate (taking less than 20 seconds). After the induction with IPTG, the lid is closed and the cultivation and measurements resume (step 5.). This induction process is repeated in a loop (step 6.) with the predefined time interval Δt (0.5 h in this study) until column 11 of the culture plate is attained (induction after 7.5 h). Here, column 12 is not induced and serves as a reference. The resulting induction conditions for every culture in a well is shown in Figure 5-2B.

Biomass-specific induction

With this method, the biomass in a main culture is permanently monitored using the BioLector and the cultures are induced at a specific, predefined biomass concentration. The principle of this so-called biomass-specific induction method is shown as a simplified flowchart in Figure 5-3A. Again, the user first has to specify the cultivation conditions of the BioLector. Moreover, the parameters for the induction step are defined (step 1.). These included:

- 1) the target biomass concentration, at which all the cultures of a MTP are induced (scattered light at induction sc_{ind} , also called induction criterion);
- 2) the inducer volume added per well v (10 μL); and
- 3) the induction phase ip (4 min). This specific time interval is defined to avoid that the BioLector is interrupted too often for induction, as this would eventually have negative effects on the oxygen supply and as a consequence the performance of the cells.

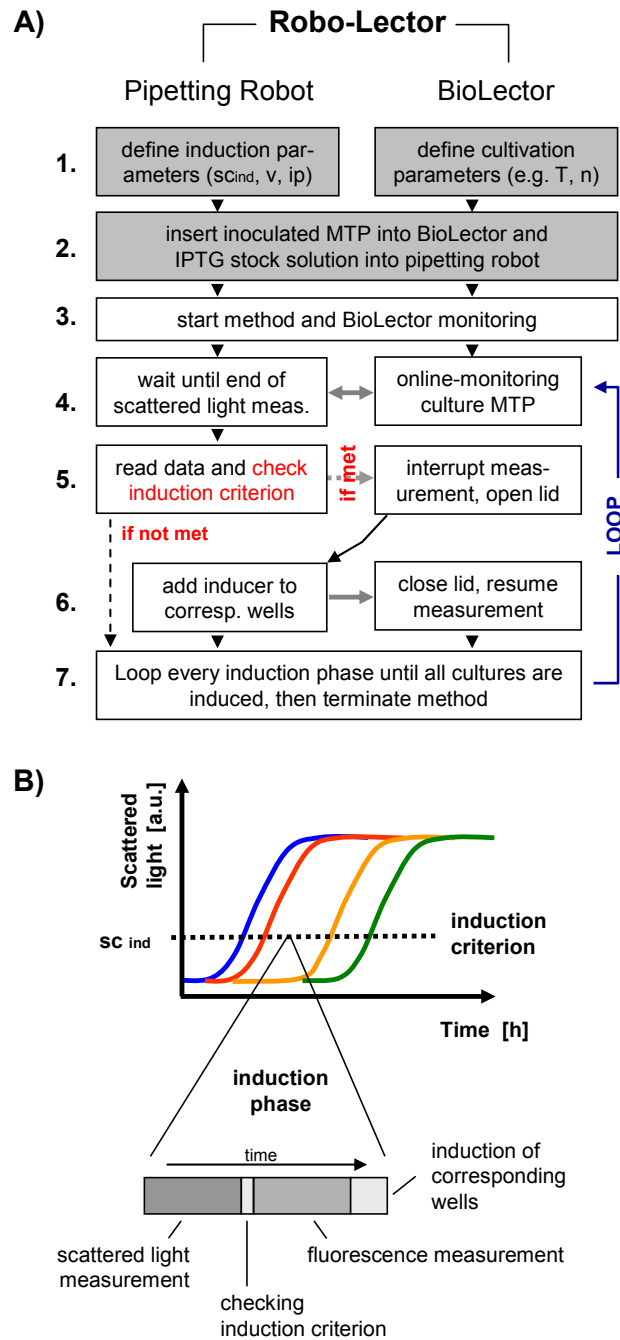


Figure 5-3: Principle of method *biomass-specific induction*. (A) Simplified process flow chart; shaded boxes indicate necessary user input; gray arrows indicate interaction of pipetting robot and BioLector; parameters sc_{ind} : scattered light at induction (induction criterion), v : volume of inducer added per well, ip : induction phase, T : cultivation temperature, n : shaking frequency. (B) Schematic principle of the established procedure for biomass-specific induction; throughout the complete cultivation, subsequent induction phases are conducted for the whole MTP; refer to *Cultivation* section for more details.

The whole cultivation process is divided into subsequent induction phases, which are conducted for the whole MTP. During the induction phase, the scattered light and EcFbFP fluorescence are measured whereby the induction criterion is also checked and induction takes place in the corresponding wells which reached the induction criterion (Fig. 5-3B).

After the above parameters for the BioLector and the induction step were set, an inoculated MTP is placed in the BioLector (step 2.). In this model experiment, the wells of the MTP were inoculated to give initial biomass concentrations of OD 0.16, 0.11, 0.05 and 0.03. Respectively, six wells were inoculated with each initial OD concentration (resulting in 24 cultures) to simulate different growth kinetics and to test the feasibility of the method. In addition, a sterile plate with an IPTG stock solution (2 mM) was placed inside the pipetting robot (step 2.). These steps are conducted manually. Afterwards, the method and the BioLector monitoring is initiated (step 3.).

The BioLector monitors the culture MTP and waits until the scattered light measurement is finished for the whole plate (Fig. 5-3A, step 4.; Fig. 5-3B). These data are then read from the BioLector and checked to see if the induction criterion is met (Fig. 5-3A, step 5.; Fig. 5-3B). Meanwhile, the BioLector continues with the monitoring of the EcFbFP fluorescence in each well (Fig. 5-3B). The following program is then run accordingly. If no culture meets the induction criterion, the program waits until the next induction phase (Fig. 5-3A, 'if not met', go from step 5. to step 7.). The other condition is that at least one well reaches a scattered light intensity greater than the induction criterion sc_{ind} (50 a.u., corresponds to an OD of approximately 2.2) (Fig. 5-3A, 'if met'). Then, the program calculates how much inducer is needed in every one of the 8 rows of the plate (e.g. 3 wells in row 5 have to be induced; this requires 30 μ L of IPTG to be aspirated by one pipetting channel). Afterwards, the liquid-handling robot aspirates the appropriate volume from the plate with the IPTG solution with 8 sterile tips and moves to the BioLector lid. The program waits until the product fluorescence measurement is completed (Fig. 5-3B). Subsequently, the robot software then links to the BioLector software, interrupts the measurement and opens the BioLector lid (Fig. 5-3A, step 5.). Once this lid opens, the shaking movement in the BioLector is interrupted for 20 to 30 seconds (depending on the number of wells that have to be induced) and the IPTG solutions are dispensed into the corresponding wells that reached the induction criterion (10 μ L per well). After the induction, the lid is closed and the BioLector cultivation and measurement resumes (Fig. 5-3A, step 6.). This workflow of measuring, checking and

induction is repeated in a loop at the predefined time of the induction phase (step 7. to 4.). This loop is repeated until every culture of the MTP has been induced.

Biomass-specific replication

The principle of this method is to transfer (replicate) cultures from one MTP (preculture) to another one (main culture) taking into account their specific biomass concentrations. This is achieved by permanently monitoring the biomass of the preculture plate with the BioLector and subsequent mixing of fresh medium in a main culture plate with an appropriate amount of inoculum from this preculture. This concept is shown as a simplified flowchart in Figure 5-4.

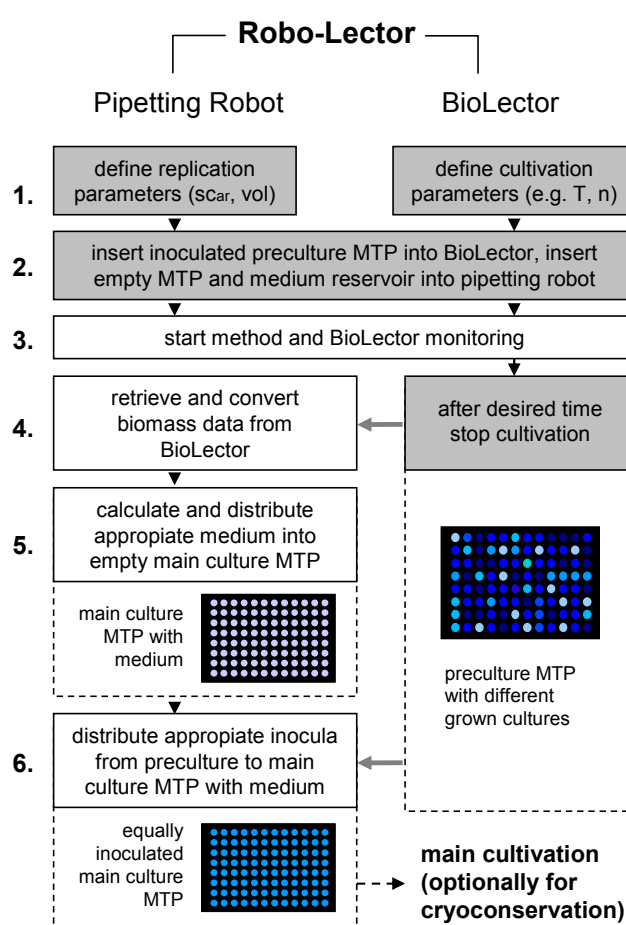


Figure 5-4: Principle of method *biomass-specific replication*. Simplified process flow chart; shaded boxes indicate necessary user input; gray arrows indicate interaction of pipetting robot and BioLector; parameters sc_{ar} : scattered light after replication, vol: total volume of wells after replication, T: cultivation temperature, n: shaking frequency; refer to *Cultivation* section for more details.

First, again the user has to specify the cultivation conditions of the BioLector (step 1.). Besides that the parameters for the replication step are defined. This is the target scattered light intensity (sc_{ar}) at which all the cultures of a 96 well MTP (main culture) are equally inoculated after the replication step. Another parameter is the total liquid volume of the wells (vol), in which the equally inoculated main cultures are prepared (200 μ L in this study). After setting above parameters for the BioLector and the replication step, an inoculated preculture MTP is placed in the BioLector (step 2.). This plate is prepared as follows. To simulate different growth kinetics during a cultivation, a cryoculture plate (stored at -80°C, containing 150 g/L glycerol) with different biomass concentrations (six different concentrations in triplicate) is thawed, mixed and 10 μ L from each well are transferred to the corresponding wells of the preculture plate with 190 μ L of MDG medium (giving initial optical densities of 0.2, 0.1, 0.05, 0.025, 0.0125, 0.005). Besides this preculture plate a sterile empty MTP and a reservoir (Nerbe Plus, Winsen, Germany) with MDG-medium is placed inside the pipetting robot. Subsequently the method and the BioLector monitoring is started (step 3.). After a desired time (8.2 h in this study) the cultivation is stopped manually in this study (although this step can also be automatized) and the replication is started (step 4.). The pipetting robot connects to the BioLector and retrieves the scattered light data from the cultures of the monitored plate. These data are converted into a specific file for further processing in the method program. Based on the biomass concentration of all cultures it is calculated how much medium and how much inoculum from the preculture is needed to achieve a uniformly inoculated main culture MTP (step 5.). Then, the liquid handling robot pipets the appropriate volume from the medium reservoir into each well of the empty main culture plate. Afterwards the appropriate inoculum volume from the preculture plate is distributed to the main culture plate (step 6.), resulting in an equally inoculated culture plate with the predefined sc_{ar} (20 a.u., corresponds to an OD of approximately 0.9). This MTP is then used for the main cultivation, which is again monitored with the BioLector.

Another possibility would be to use the equally inoculated culture plate for cryoconservation. Therefore, a portion of a sterile glycerol stock solution could be added to each well by the pipetting robot and afterwards the plate would be frozen.

5.3. Results and discussion

5.3.1. Induction profiling

In this study the new Robo-Lector platform combining automated liquid-handling and cultivation in monitored MTPs (Fig. 5-1) was used to study the expression of a fluorescent model protein from the strain *E. coli* BL21(DE3) pRhotHi-2-EcFbFP. The automated method designated ‘induction profiling’ was used as shown in Figure 5-2 and resulted in 96 growth and product formation kinetics (Fig. 5-5).

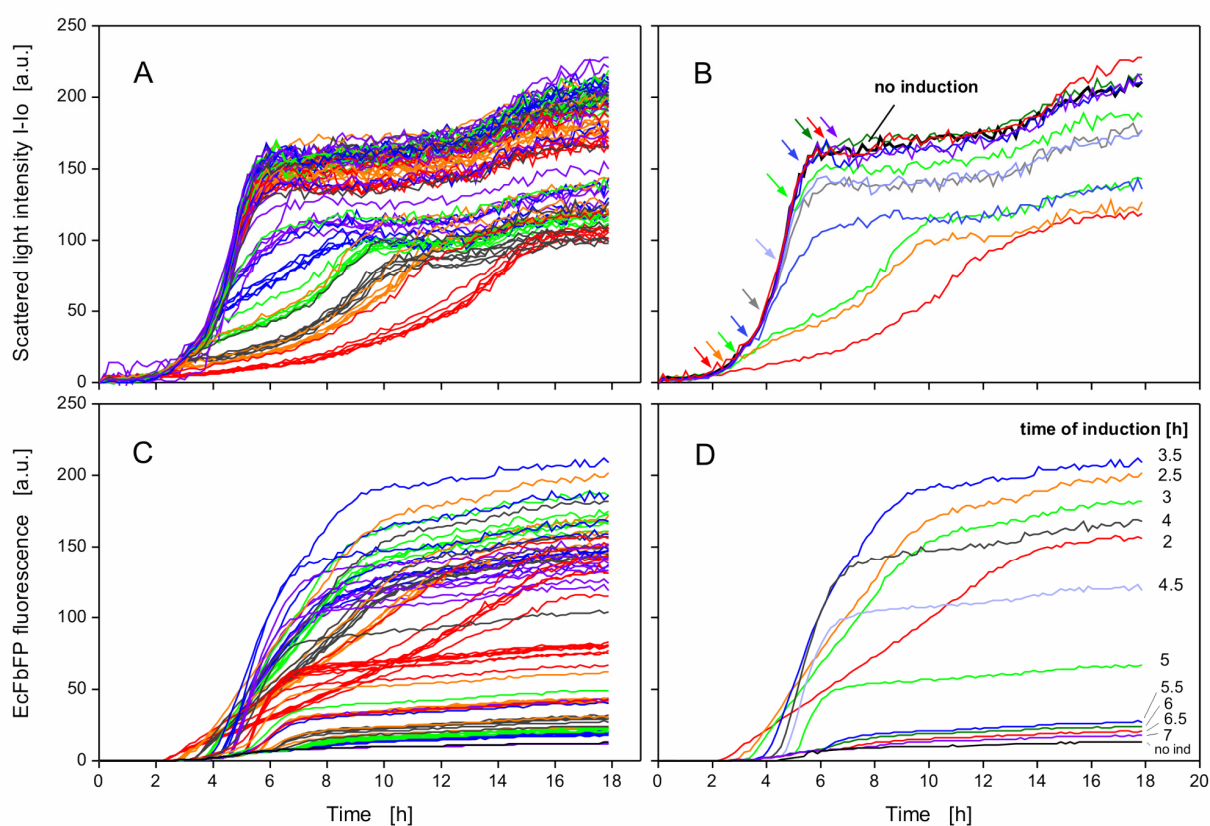


Figure 5-5: Influence of induction time and inducer concentration on EcFbFP production of *E. coli* BL21(DE3) pRhotHi-2-EcFbFP. 96 Induction experiments were conducted according to the profile shown in Figure 5-2B. Scattered light intensities of all cultures (A) and cultures induced with 0.05 mM IPTG at various points of time, as indicated by arrows (B). EcFbFP production of all cultures (C) and cultures induced with 0.05 mM IPTG (D). In Figure 5-5B and D also a reference cultivation (no induction) is shown; Conditions: MDG mineral medium, $T = 37^{\circ}\text{C}$, $V_L = 190\ \mu\text{L}$, $n = 950\ \text{rpm}$, $d_0 = 3\ \text{mm}$.

The growth of all cultures, being induced at various times with various IPTG concentrations during the batch cultivation, varied as well as the product formation (Fig. 5-5A and C). To simplify the evaluation of data shown in Figure 5-5A and C, only the curves of the 0.05 mM IPTG induction experiments are depicted in Figure 5-5B and D. The uninduced culture showed a typical sigmoid batch cultivation curve (Fig. 5-5B, black line) with a stationary phase starting at 6 h at a scattered light of 160 a.u. (corresponds to an OD of approximately 7.1) and no significant expression of the fluorescence protein (20 a.u., Fig. 5-5D). When protein expression was induced at the very beginning of the batch culture (2 h, red line), the growth rate decreased markedly, whereas the product signal rose. The EcFbFP expression continuously increased with a constant rate until the cells entered the stationary phase after 14 h. A later induction of the cells (2.5 to 4 h) led to a sharper increase and greater values of the EcFbFP expression due to the higher biomass at the point of induction. When induction occurred after 4 h, the EcFbFP expression sharply decreased (Fig. 5-5D), resulting from the transition from the late exponential to the stationary phase and hence a lack of nutrients and metabolic activity of the cells. Therefore, the EcFbFP and also the growth kinetics of these late-induced cultures showed a similar curve shape as the uninduced cultures (Fig. 5-5B and D).

Unlike the non-induced cultures, the early induced cultures grew significantly slower. This phenomenon can be attributed to the so-called 'metabolic burden' through recombinant protein expression (Bentley et al. 1990; Glick 1995). Metabolic burden is still poorly understood (Bonomo and Gill 2005), because there are no appropriate methods to monitor it online (Reischer et al. 2004). Recent research focuses on in-depth analysis of the molecular physiological reactions in host cells during protein expression, e.g. through the use of methods such as DNA microarrays or 2D-electrophoresis (Nemecek et al. 2008). Unfortunately, such methods are complex and quite time-consuming. The approach to fuse green fluorescent protein (GFP) with stress-sensitive promoters and to measure fluorescence is an easier and promising way to achieve online-monitoring of the metabolic burden during protein expression in bioreactors (Nemecek et al. 2008). Until now such studies have been mainly conducted with larger bioreactor configurations that can be more easily equipped with fluorescence probes than most MBRs. However, with the Robo-Lector platform presented here, it is now possible to provide online fluorescence measurements in parallel experiments and to automate high-throughput cultivations. This platform thus provides an easy and efficient tool to systematically study and possibly quantify metabolic burden.

By systematically varying the induction point and inducer concentration over a wide range in parallel experiments, it is possible to obtain a so-called ‘induction profile’ of the specific strain tested. Such a profile is presented in Figure 5-6, where data from the experiment of Figure 5-5 (at end-point of cultivation) were plotted as contour plots. These color-coded profiles can also be calculated at any time of the process because of the extensive data provided by the BioLector. For example, in this study the biomass and fluorescence in each of the 96 wells has been detected every 5 min, resulting in over 20000 data points.

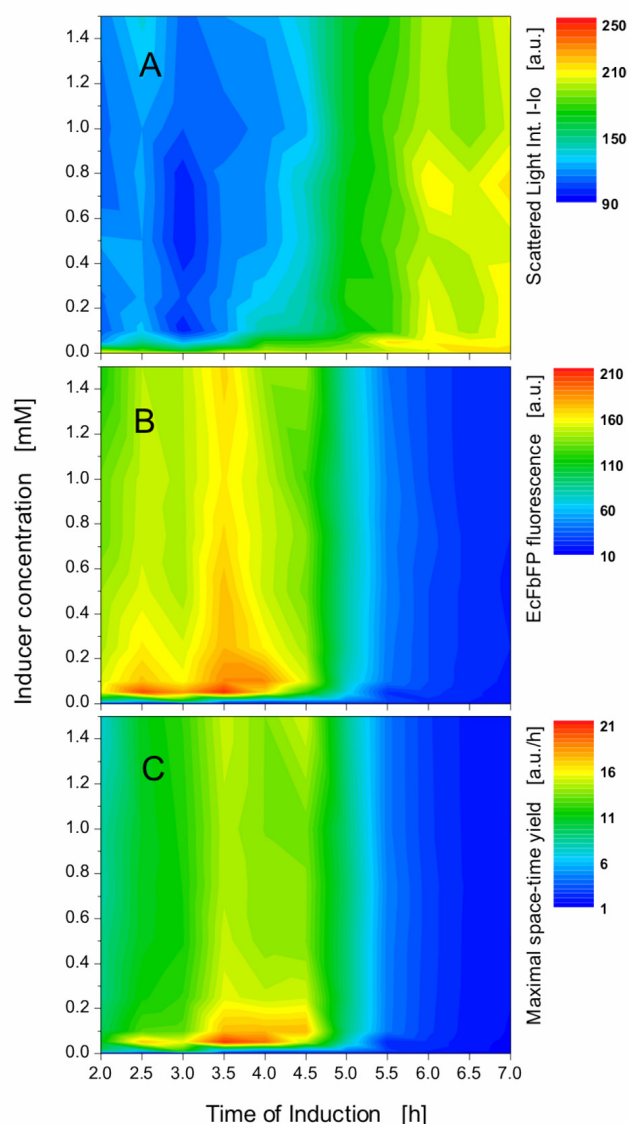


Figure 5-6: Induction profiles of *E. coli* BL21(DE3) pRhotHi-2-EcFbFP. (A) Scattered light intensity and (B) EcFbFP fluorescence; data taken from 96 expression experiments of Figure 5-5A and C at the end of the cultivation (18 h). (C) Maximal space-time yield at various inducer concentrations and induction points; the maximal space-time yield was calculated from the curves of Figure 5-5C by dividing the EcFbFP fluorescence by the corresponding time.

The contour plot of the scattered light signal at the end of the experiment reveals that biomass concentration peaks when induction occurs after 4.5 h, independent of the inducer concentration (Fig. 5-6A; green to yellow region). Only the uninduced cultures (inducer 0.0 mM) also show high biomass concentrations. Even though this can also be seen in Figure 5-5A, the contour plot as shown in Figure 5-6A depicts this bundle of information much easier and in a concise way.

The fluorescence of the EcFbFP is greatest (> 200 a.u.; red region) at an inducer concentration of only 0.05 to 0.1 mM and an induction time at 2.5 to 3.5 h (Fig. 5-6B). High to moderate EcFbFP production (160 to 200 a.u.; yellow to red region) is also reached at an induction time of 3 to 4 h. Interestingly, at this time the protein expression is much less dependent on the inducer concentration above 0.2 mM IPTG. Miao and Kompala, using a similar host-vector system, have also found that protein expression stagnates at inducer concentrations greater than 0.2 mM (Miao and Kompala 1992). However, this observation results from different possible factors that are not the focus of this Chapter. When induction takes place after 4 h, there is a sharp decrease in EcFbFP production (green to blue region), as already discussed for Figure 5-5D. Upon comparing Figure 5-6A with 5-6B, it becomes obvious, the more protein expressed, the lower the biomass growth is. This effect can also be explained by the metabolic burden which the recombinant protein expression exhibits on the host bacteria. Figure 5-5D shows that when induction occurs after 2 h, one obtains the same product quantity as after induction at 4 h. Only the product formation rate is different. Since the culture induced after 2 h reaches its maximum EcFbFP fluorescence approximately 6 h later than the 4 h-induced culture, it makes sense to basically induce cultures at 4 h to save time. This aspect is not visible in Figure 5-6B. Hence, the maximum EcFbFP produced per time (maximum space-time yield) would be a better indicator to distinguish between different induction experiments. Furthermore, maximizing the space-time yield is the principal goal of biological production processes (Clementsich and Bayer 2006). Thus, the space-time yield has been calculated from the data of Figure 5-5C by dividing the EcFbFP fluorescence (a.u.) by the corresponding time (h). The maxima of the resulting space-time yield curves are depicted in Figure 5-6C. This space-time yield calculation results in a distinct region with the highest EcFbFP production per time (yellow to red) at inducer concentrations of 0.05 to 0.2 mM added to the culture after 3.5 to 4.5 h. Comparing this with Figure 5-6B clearly shows that inducing these specific cultures earlier than 3.5 h or at higher IPTG concentrations makes no sense in respect to fast and high product formation. It should be emphasized that different recombinant strains may behave completely different. The induction profiling method presented here allows to

automatically conduct and monitor up to 96 induction experiments in just one run (e.g. overnight), thereby allowing the researcher to attain a fast, easy and profound understanding of the given expression system, especially when fluorescent marker proteins are used.

5.3.2. Biomass-specific induction

As demonstrated in Figure 5-5 and Chapter 3.3 (Fig. 3-3), induction at different growth phases leads to great variations in product formation. Thus, it is a large problem to simultaneously induce cultures which show different growth kinetics with the conventional method (induce all cultures of a MTP at the same time). Since the BioLector permanently monitors growth in MTPs, it is possible to trigger actions of the liquid-handling robot in response to the scattered light data. This allows one to conduct a biomass-specific induction of different cultures (that means at different appropriate times according to the requirement of the individual culture) in a completely automated way. Results of this method (Fig. 5-3) are depicted in Figure 5-7.

To simulate different growth kinetics, some wells of the MTP were inoculated with different amounts of preculture. These growth differences can be seen in Figure 5-7A. When the first cultures reached the induction criterion of 50 a.u. for the scattered light signal (corresponds to an OD of approximately 2.2), the Robo-Lector automatically induced the corresponding wells of the MTP with IPTG. In Figure 5-7A, the scattered light curves show a short drop in the signal intensity after induction, caused by dilution of the culture through the added inducer solution. After the automated induction, the growth is immediately slowed down (Fig. 5-7A, also visible in Fig. 5-5A) because of the starting EcFbFP production, thereby leading to an increase in the fluorescence signal (Fig. 5-7C). As the growth curves in Figure 5-7A and the product curves in Figure 5-7C of the wells treated in the same way (curves with same colors) are similar, this demonstrates that the automated induction took place at a comparable growth phase. This fact is also supported by normalizing all culture curves to have the same induction time as the first induced culture and by calculating the mean and standard deviation of these curves (Fig. 5-7B and D).

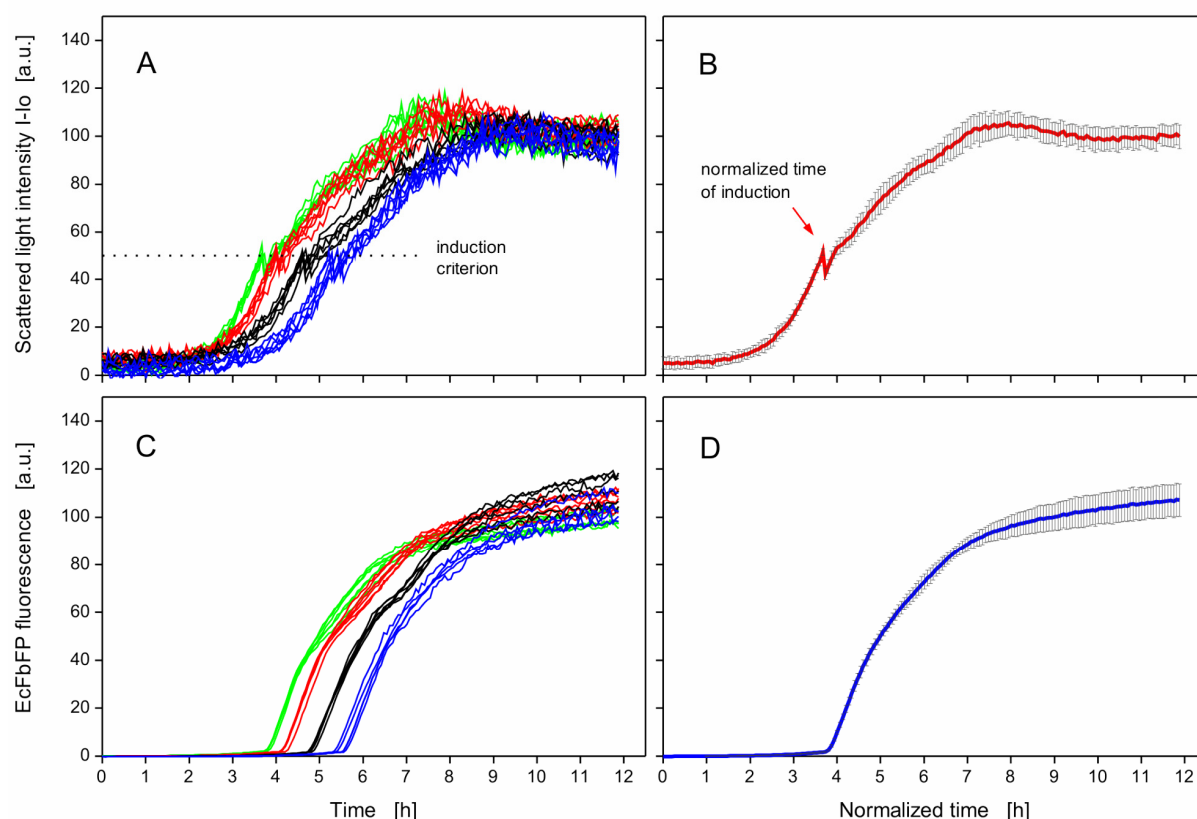


Figure 5-7: Effects of *biomass-specific induction* on *E. coli* BL21(DE3) pRhotHi-2-EcFbFP. (A) Growth of different inoculated cultures, induced at an induction criterion of 50 a.u. with 0.1 mM IPTG; cultures from left to right (in sixfold): optical density at start $OD_{t_0} = 0.16$ (green curves), 0.11 (red curves), 0.05 (black curves), 0.03 (blue curves). (B) Mean and standard deviation of normalized growth of 24 cultures from Figure 5-7A; the growth kinetics were normalized to have the same induction time as the first induced culture. (C) EcFbFP production of all induced cultures. (D) Mean and standard deviation of normalized product formation of 24 cultures from Figure 5-7C. Conditions: MDG mineral medium, $T = 37^{\circ}\text{C}$, $V_L = 190\ \mu\text{L}$, $n = 950\ \text{rpm}$, $d_0 = 3\ \text{mm}$.

The EcFbFP fluorescence after 12 h ranges from 100 to 120 a.u. (Fig. 5-7C) with a mean fluorescence of 107 ± 7 a.u. (Fig. 5-7D), representing a relative standard deviation of only $\pm 7\%$ (the accuracy of the measurement system is $\pm 5\%$, according to the manufacturer of the BioLector). This variance is quite low in comparison with the conventional approach, namely to induce all cultures of a MTP at the same time. In Figure 3-3 (Chapter 3.3), such a simultaneous induction was conducted after 4.9 h with cultures of similar initial OD values of 0.01 to 0.2 in comparison with the experiment of Fig. 5-7 (initial OD values of 0.03 to 0.16). As a consequence, the cultures in Fig. 3-3 were induced at scattered light intensities of 5 to 120 a.u. (Fig. 5-7 had an induction criterion of 50 a.u.) and therefore exhibited a high variance of product formation from approximately 80 to 160 a.u. (mean of 133 ± 32 a.u.; relative standard deviation of

± 24 %). However, an exact quantitative comparison between both methods is difficult, as the induction point plays the major role in the overall yield of protein expression.

Upon comparing Figure 5-7C with Figure 5-5C, the differences in product yield is reasonably smaller because of the induction at a similar growth phase. The demonstrated data here show the feasibility of this concept and can be important for applications where an action (e.g. addition of liquids, drawing samples) has to be triggered depending on the growth phase of different cultures.

5.3.3. Biomass-specific replication

To simulate different growth kinetics in high-throughput cultivations, a preculture MTP with different initial biomass concentrations was used. The resulting growth differences can be seen in Figure 5-8A. After 8.2 h this cultivation was stopped. At that moment, the different cultures had scattered light intensities of 240 to 300 a.u. and were at different phases of the batch cultivation. Some of the cultures were already in the stationary phase, whereas others were still in the late-exponential growth phase (Fig. 5-8A). At this time, the biomass-specific replication was conducted (Fig. 5-4). Fresh medium was distributed to a new MTP and appropriate amounts of the cultures from the preculture plate (13 to 17 μ L) were transferred to the corresponding wells of the main culture plate to give a final volume of 200 μ L per well with a scattered light intensity of approximately 20 a.u. (sc_{ar}). The subsequent main cultivation showed that all 18 cultures had very similar growth kinetics after the biomass-specific replication (Fig. 5-8B) with a relative standard deviation of ± 4 % regarding scattered light (after 7.8 h), demonstrating that the method can provide equal starting conditions and hence growth kinetics in the main culture.

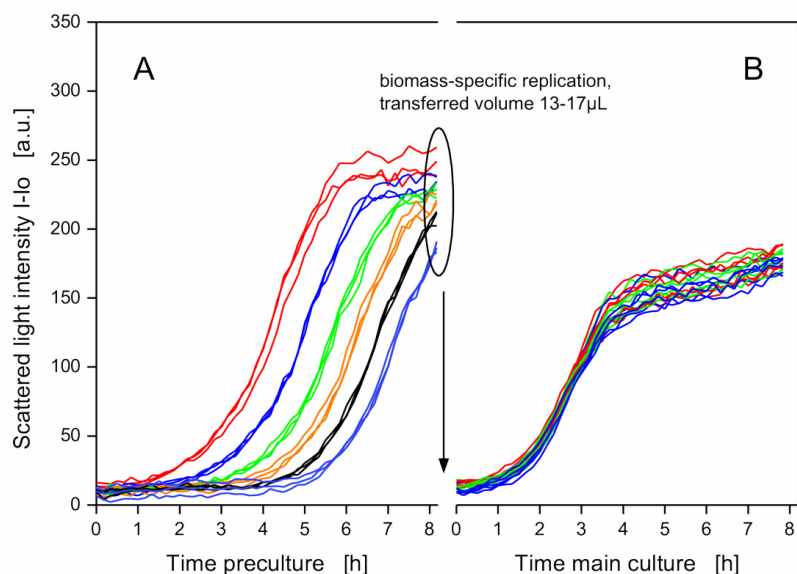


Figure 5-8: Results of the method *biomass-specific replication*. (A) Growth of precultures with different initial biomass concentrations, from left to right (in triplicate): optical density at start $OD_{t_0} = 0.2, 0.1, 0.05, 0.025, 0.0125, 0.005$; after 8.2 h the first cultivation was stopped and the automated method for replication (see also Fig. 5-4) started. (B) Main culture: growth of 18 replicated cultures; the relative standard deviation of the scattered light after 7.8 h is $\pm 4\%$. Conditions: strain *E. coli* BL21(DE3) pRhotHi-2-EcFbFP, MDG mineral medium, $T = 37^\circ\text{C}$, $V_L = 200\ \mu\text{L}$, $n = 950\ \text{rpm}$, $d_0 = 3\ \text{mm}$.

This method can be used to systematically study multiple cultivation steps, such as preculture-main culture cascades (e.g. as shown in Fig. 1-1) and influencing factors of the inoculum on a main culture (e.g. lag-time, volume ratio of the preculture to fresh medium). If the differences in biomass concentration in different preculture wells are too large to be equalized in one single replication step, it is also possible to include another preculture before the main culture and to conduct the biomass-specific replication twice.

5.4. Conclusions

In industry and academic research, there is an increasing demand for flexible automated microfermentation platforms with advanced sensing technologies. The Robo-Lector, a new platform consisting of a BioLector and a liquid-handling robot, has been successfully built and tested. Unlike other MBR systems, the BioLector generates extensive kinetic data in high-throughput cultivations concerning biomass and fluorescence protein formation. Based on the non-invasive online-monitoring signal for microbial growth, actions of a liquid-handling robot can easily be triggered and controlled. This interaction

between the robot and the BioLector combines high-content data generation with systematic high-throughput experimentation (refer to Chapter 1.4, Fig. 1-3) in an automated fashion, offering new possibilities to study biological production systems.

The ‘induction profiling’ and ‘biomass-specific induction’ methods presented here allow one to study recombinant protein expression in detail and optimize expression. This, in turn, leads to a fast and profound comprehension of host-vector systems and the metabolic burden phenomenon. The method ‘biomass-specific replication’ enables to generate main cultures with equal biomass concentrations from different growing precultures. Additionally, the presented method could be useful for establishing standardized cryocultures and to study the optimal time for freezing the cells in respect to the lag-time or the viability of the cryocultures. This can provide a more defined starting material for clone libraries, thus avoiding negative effects on the whole production process (refer to Chapter 1.3).

As the Robo-Lector has a simple but efficient design (MTP format; use of standard liquid-handling robot and hence widespread automation possibilities), it can be easily combined with established techniques for small scale downstream processing (Bensch et al. 2007; Bensch et al. 2005; Titchener-Hooker et al. 2008; Wiendahl et al. 2008) and analytical assays. This fits the current trend for small-scale process integration in just one automated platform (Rao et al. 2009). Liquid-handling workstations permit high-throughput sampling and addition of liquids to MTPs so that for example intermittent fed-batch processes with the Robo-Lector can be realized. Furthermore, as liquid-handling workstations allow fast and accurate pipetting, many different media compositions can be generated in the Robo-Lector. This enables one to combine media preparation with subsequent parallel cultivations to optimize media in an automated fashion, e.g. with the help of DoE (design of experiments) (Mandenius and Brundin 2008) or genetic algorithms (Etschmann et al. 2003; Weuster-Botz 2000). Methods implementing fed-batch and automated media preparation have already been established for the presented platform (data not shown).

The novel automated platform and methods presented here can aid in modelling recombinant protein expression, systems biology research and in particular bioprocess development and optimization. In these fields, the Robo-Lector can accelerate and intensify research and development.

6. Studying phosphate limitation with high-throughput experimentation

6.1. Introduction

One of the major goals in biological process development is to increase the productivity of fermentation processes, e.g. in particular to increase the amount of protein produced per cell (specific productivity). Advantages thereof are usually less effort for product purification, lower fermentation volumes and thus smaller bioreactors that are needed to produce a certain amount of product. This can lead to significant cost reductions. The specific productivity can be influenced by genetic engineering of strains, choice of the host-vector system (e.g. plasmid copy number and promoter), process optimization (e.g. optimizing induction time and inducer concentration; reducing acetate formation by using fed-batch mode) or media optimization (Markland 2006).

Another widespread concept to increase specific productivity is to uncouple biomass buildup from product formation (Flickinger and Rouse 1993; Matin 1991; Tunner et al. 1992). When recombinant proteins are produced in growing cells, energy and nutritional resources are often directed toward biomass production rather than target protein formation (Rowe and Summers 1999). To circumvent this, the idea is to use cells that are non-growing (dormant), but metabolically active and capable to produce protein at higher

levels. Rowe and Summers designed such a system (termed Quiescent Cells) using a genetically modified *E. coli* strain that provides a higher specific productivity by decreasing chromosomal gene expression (and hence growth) in favor of plasmid-encoded gene expression (Mukherjee et al. 2004; Rowe and Summers 1999; Summers and Chant 2009). Another concept is to induce protein expression in the stationary phase of a batch culture (Matin 1991; Rowe and Summers 1999; Tunner et al. 1992). Here, the gene of interest is placed under the control of a starvation-inducible promoter that is turned on when glucose becomes exhausted (Sawers and Jarsch 1996). Compared with exponentially growing cells, stationary phase cells are far more resistant to stress (Han and Lee 2006; Peterson et al. 2005) and might therefore be suited to produce recombinant proteins (Vila et al. 1997), as this can place a huge metabolic burden on the cells (Bentley et al. 1990; Glick 1995). Another starvation-inducible promoter is based on the alkaline phosphatase (*phoA*) gene. When phosphate is depleted in the medium, the target genes under the control of the *phoA* promoter are induced (Champion et al. 2001; Wanner 1996). This system is described for the expression of a number of recombinant proteins (Champion et al. 2001; Lübke et al. 1995; Sawers and Jarsch 1996; Shin and Seo 1990). A further possibility to limit growth and hereby enhance product formation is to limit secondary substrates like magnesium, potassium or especially phosphate in the cultivation medium (Kottmeier et al. 2009).

Phosphate - being one of the most abundant elements in *E. coli* in terms of the cellular content - plays a crucial role in the cell. Wanner summarized many aspects of the role of phosphate on cell's structure and function (Wanner 1996). This includes the incorporation of inorganic phosphate in membrane lipids, nucleic acids, carbohydrates and other cellular metabolites. Here, phosphate also acts as an effector of enzymatic reactions (Ryan et al. 1989) and is thus involved in many metabolic pathways, especially in the energy metabolism of the cell (e.g. as high-energy phosphoanhydride bond in ATP). Furthermore, polyphosphate acts as an energy and phosphate storage polymer that is used during phosphate limitation (Van Dien and Keasling 1999; Wanner 1996). Because of this universal role of phosphate, a limitation of this nutrient has tremendous effects on the cell growth and physiology. Therefore, many genes are found to be regulated by the level of inorganic phosphate in the medium (Torriani 1990). A phosphate exhaustion in an *E. coli* cultivation leads to activation of the Pho regulon (Wanner 1996). Furthermore, the RpoS response is triggered (Schurdell et al. 2007) and cells enter the stationary phase (Peterson et al. 2005). Due to phosphate limitation the expression of hundreds of proteins is induced and at the same time the levels of more than two hundred proteins are reduced (Han and Lee 2006; VanBogelen et al. 1996). In summary, a phosphate

limitation leads to quite complex regulatory mechanisms in the cell and is still not yet fully understood on the molecular level.

Nevertheless, phosphate exhaustion was shown to enable enhanced product formation. This was recently proved in batch cultures for a catabolite-repressed *Hansensula polymorpha* strain with glucose or glycerol as carbon source (Kottmeier et al. 2009). Jensen and Carlsen cultivated an *E. coli* strain with an constitutive promoter for a recombinant protein in fed-batch mode. By limiting phosphate in the medium they were able to stop the biomass buildup and enhance specific productivity (Jensen and Carlsen 1990). A similar effect could be achieved for producing shikimic acid in *E. coli* in a phosphate-limited chemostat culture (Johansson et al. 2005) and for lysine production with *Corynebacterium glutamicum* (De Hollander et al. 1998). However, besides using the *phoA* promoter, there are only few reports on phosphate-limited batch cultivations of *E. coli* (Johansson et al. 2005). There are no dedicated reports on using phosphate limitation with the most common inducible promoter system, namely the T7 or *lac* promoter, to enhance specific productivity of recombinant protein expression. The reason for this might be the difficulty to establish optimal conditions for inducing protein expression in phosphate-limited cultures. For this purpose, many experiments have to be conducted in parallel batch cultures to determine the best phosphate content of the medium and the optimal time of induction. So far, such studies are very tedious with conventional means (e.g. shake flask or MTPs experiments with offline-measurement of growth and manual induction of protein expression at an appropriate time).

Hence, the aim of this work was to use advanced online-monitoring tools for small scale cultivations – RAMOS (Anderlei and Büchs 2001; Anderlei et al. 2004) and BioLector (Kensy et al. 2009; Samorski et al. 2005) – to investigate the influence of phosphate limitation on growth and recombinant protein expression in batch cultures. Furthermore, the novel cultivation platform Robo-Lector was applied (refer to Chapter 5). Consequently, these tools were used to determine the possibility to increase specific productivity in a T7 expression system via phosphate limitation.

6.2. Material and methods

Organism

For all experiments the strain *E. coli* BL21(DE3) pRhotHi-2-EcFbFP was used. For a detailed description of this expression system refer to Chapter 3.2. To induce protein

expression, a sterile-filtered IPTG stock solution (100 mM) was used to give a final IPTG concentration of 0.1 mM in the cultures. This concentration was identified in previous experiments to be suitable for efficient recombinant protein expression (refer to Chapter 5.3.1). All chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany), Carl Roth (Crailsheim, Germany) or Sigma (Taufkirchen, Germany).

Media

For all precultures TB-Medium was used, consisting of 24 g/L yeast extract, 12 g/L tryptone, 12.54 g/L K_2HPO_4 , 2.31 g/L KH_2PO_4 and 5 g/L glycerol. The medium was supplemented with kanamycin (50 μ g/L) and the pH was adjusted to 7.2 with NaOH.

All main cultivations were performed in a modified form of Wilms & Reuss synthetic medium (Scheidle et al. 2007; Wilms et al. 2001). The medium consists of 7.5 g/L glucose, 5 g/L $(NH_4)_2SO_4$, 0.5 g/L NH_4Cl , 3 g/L K_2HPO_4 (for phosphate unlimited conditions), 2 g/L Na_2SO_4 , 0.5 g/L $MgSO_4 \times 7H_2O$, 41.85 g/L MOPS, 0.01 g/L thiamine hydrochloride, 1 mL/L trace element solutions. For the determination of the phosphate demand of the used strain, different media with the following phosphate concentrations (as K_2HPO_4) were prepared (mg/L): 3000, 750, 300, 225, 195, 180, 150, 105, 90, 75, 60, 45, 30. All media preparations were supplemented with kanamycin (50 μ g/L) and the pH was adjusted to 7.5 with NaOH. All chemicals were of analytical grade and supplied by Merck (Darmstadt, Germany), Carl Roth (Crailsheim, Germany) or Sigma (Taufkirchen, Germany).

Cultivation

Preculture

The precultures for all experiments were inoculated from a cryoculture of *E. coli* BL21(DE3) pRhotHi-2-EcFbFP and cultivated in a 250 mL Erlenmeyer shake flask with 10 mL TB-medium. The flask was placed on a rotary shaker at a shaking frequency of 300 rpm, a shaking diameter of 50 mm and at a temperature of 37°C for 16 h. To make sure that no disturbing media components from the preculture medium – especially phosphate – influence the limitation experiments, the harvested cells were washed twice. Therefore, cells of the preculture were pelleted by centrifugation at 4000 rpm and resuspended in phosphate-free modified Wilms & Reuss medium. After two washing steps, the subsequent main cultures were inoculated to give an initial OD of 0.4. OD was measured at 600 nm with a Uvikon 922 spectrophotometer (Kontron, Milano, Italy).

Microtiter plate cultivations

Experiments to determine the phosphate demand and the influence of phosphate limitation on protein production were conducted in a BioLector prototype (Samorski et al. 2005). The screening for the optimal induction time was carried out with the Robo-Lector platform (refer to Chapter 5). Here, the method ‘induction profiling’ was used, which performs an automated induction of parallel cultures by pipetting IPTG-solutions in the respective wells of a main culture MTP at different times. For a detailed description of the method refer to Chapter 5.2. The method was applied with the following modifications: the induction time was varied from 1 to 10 h and the inducer concentration was kept constant at 0.1 mM. The following conditions were applied for all cultivations in the BioLector prototype and the Robo-Lector: 37°C, shaking diameter 3 mm, shaking frequency 950 rpm. All cultivations were performed in sterile black 96 well MTP (μ Clear, Cat. 655087, Greiner Bio-One, Frickenhausen, Germany) with an initial filling volume of 200 μ L (BioLector prototype) or 190 μ L (Robo-Lector). To avoid evaporation the used MTP was sealed with a gas-permeable adhesive seal (Cat. AB-0718, Abgene, Epsom, UK) for the cultivations in the BioLector prototype. For the Robo-Lector experiments the MTP was sealed with sterile pierceable, resealable tape (X-Pierce, Excel Scientific, Victorville, USA), allowing ventilation of the wells at reduced evaporation rates. The EcFbFP fluorescence was monitored at an excitation of 460 nm and an emission of 492 nm. The biomass concentration was measured via scattered light intensity (I) and was detected at an excitation of 620 nm. The initial scattered light intensity (I_0) was mainly attributed to factors such as the media background or the type of the MTP and thus was subtracted from the residual scattered light data ($I-I_0$) (Samorski et al. 2005). For the experiments different signal gain factors for scattered light and fluorescence were applied, as stated in the Figure captions.

RAMOS cultivations

The RAMOS device provides analysis of microbial growth in shake flasks by measuring the respiratory activity of the microorganisms (Anderlei and Büchs 2001; Anderlei et al. 2004; Seletzky et al. 2007). The respiration activity (Oxygen Transfer Rate, OTR) during phosphate-limited cultivations was measured with the RAMOS device in modified 250 mL Erlenmeyer shake flasks with a filling volume of 10 mL. All cultivations were carried out with a shaking frequency of 350 rpm at a shaking diameter of 50 mm on a Lab-Shaker LS-K (Kühner, Birsfelden, Switzerland) at $T = 37^\circ\text{C}$.

Shake flask cultivations

Shake flask experiments were conducted to verify results from the MTP scale. These cultures were grown in 250 mL Erlenmeyer shake flasks which were filled with 10 mL modified Wilms & Reuss medium with different phosphate concentrations (3000, 225, 150, 75 mg/L K_2HPO_4) at a shaking frequency of 350 rpm, a shaking diameter of 50 mm and 37°C. The cultures were induced with IPTG (final concentration of 0.1 mM) after 6h of growth. After 26 h the OD of the cultures was measured and cells were harvested to perform product analysis via SDS-PAGE. Furthermore, scattered light and fluorescence intensities of culture samples (200 μ L) were measured in the BioLector prototype.

SDS-PAGE and densitometry

SDS-PAGE was performed to correlate the specific productivities of the shake flask cultivations with different phosphate concentrations. The SDS-PAGE was conducted with the XCell SureLock MiniCell (Invitrogen, Carlsbad, USA) and a Bis-tris-gel (NuPAGE 4-12 %, Invitrogen) according to the manufacturer's instructions. Normalized samples were prepared by suspending equal amounts of cells from the shake flask experiments in SDS-buffer and loading them on the gel. Additionally, a molecular weight standard (RotiMark, Roth, Crailsheim, Germany) was loaded on the gel. After electrophoresis, the gel was stained and destained according to the manufacturer's instructions (SimplyBlue SafeStain, Invitrogen). The destained gel was photographed with the documentation system ChemiDoc (Biorad, Hercules, USA) and analyzed with the densitometry computer program Total Lab TL100 (Nonlinear Dynamics, Newcastle upon Tyne, UK) to determine the specific productivity of the different samples. The specific productivity was defined as the ratio of the density of the target protein band (EcFbFP) to the total density of all bands of the corresponding sample (band %).

6.3. Results and discussion

6.3.1. Effects of phosphate limitation on growth

The aim of this study was to examine the effects of a phosphate limitation on the growth of the *E. coli* strain BL21(DE3) pRhotHi-2-EcFbFP and its recombinant product formation. First of all, the growth behavior at different phosphate concentrations was investigated in the BioLector device. A graduated growth pattern resulted from this experiment (Fig. 6-1). When no phosphate was added to the medium (0 mg/L) there was nevertheless some growth visible. This might be explained by some residual phosphate from the preculture (though these cells were washed two times with phosphate-free

medium) and most likely because of intracellular polyphosphate stores which were mobilized during phosphate starvation (Peterson et al. 2005). The phosphate content of the original medium was 3000 mg/L and this culture showed a typical batch culture curve with exponential growth. The stationary phase began at approximately 13 h with a biomass concentration of 30000 a.u. (Fig. 6-1). This culture was phosphate unlimited, although the cultures with 300 and 750 mg/L reached a slightly higher scattered light intensity. This could be explained by the fact that excess phosphate in the medium (3000 mg/L) could complex trace elements (unpublished results) and this, in turn, led to slightly lower biomass yield compared to 300 or 750 mg/L phosphate. The cultures with less than 300 mg/L phosphate showed lower biomass yields as phosphate became more and more limited. The curves of these cultures deviated from the unlimited cultures as soon as phosphate became limited and then continued with slow growth until they reached a plateau. This slight increase in biomass concentration had been shown elsewhere (Peterson et al. 2005; Shin and Seo 1990; Wang et al. 2005) and seemed typical because of the metabolism of intracellular phosphate reserves. With the BioLector the demand of the examined *E. coli* strain for phosphate could be investigated in detail (Fig. 6-1). Furthermore, the time when phosphate became limited was derived from the online-monitored growth curves.

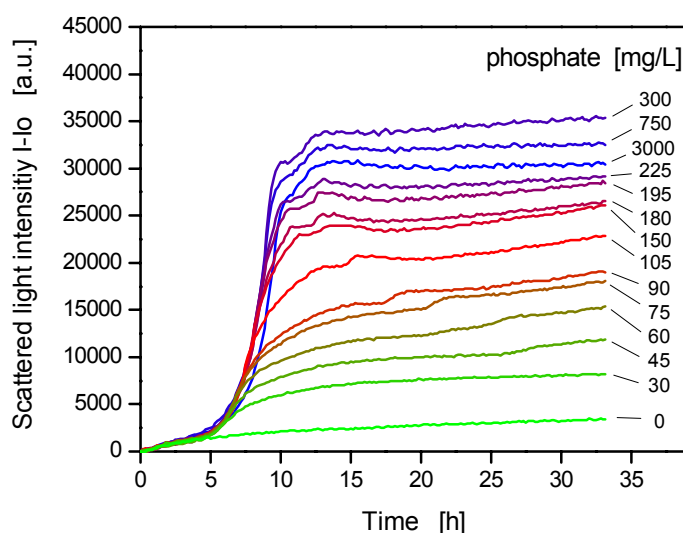


Figure 6-1: Determination of phosphate demand of *E. coli* BL21(DE3) pRhotHi-2-EcFbFP by performing batch experiments in different media (modified Wilms & Reuss media with 7.5 g/L Glucose) with varied phosphate concentrations. Conditions: $T = 37^{\circ}\text{C}$, $V_L = 200\ \mu\text{L}$, $n = 950\ \text{rpm}$, $d_0 = 3\ \text{mm}$, no induction. Measurement with BioLector prototype, signal gain factor 30. Six cultures of every medium composition were conducted, the mean value is depicted above, the relative standard deviation of all cultures did not exceed $\pm 10\ \%$.

In order to verify these results a cultivation in the RAMOS system with three selected phosphate concentrations was conducted (Fig. 6-2). Derived from the results of Figure 6-1, a culture with 3000 mg/L phosphate was chosen as reference for phosphate unlimited conditions. Besides that, cultivations with 150 mg/L and 75 mg/L phosphate were conducted to represent intermediate and strong phosphate limitations, respectively. The unlimited culture showed a typical exponential growth reaching a maximal OTR of 55 mmol/L/h after 11 h. At this time, the OTR decreased sharply, showing a second small peak at 13 h. This behavior is typical for an *E. coli* batch cultivation where the primary carbon source, namely glucose, became exhausted (drop in OTR at 11 h) and the overflow metabolite acetate was consumed subsequently (second peak in OTR until 13 h). The OTR of the limited culture with 150 mg/L phosphate started to stagnate at an OTR of 30 mmol/L/h after 8 to 9 h when phosphate limitation began. After 13 h, the OTR dropped again because glucose was exhausted. This culture showed a smaller second peak, because less acetate is normally produced during slower growth. The 75 mg/L-culture showed a similar pattern, although the OTR reached only 18 mmol/L/h at its maximum and slowly decreased until glucose was exhausted after 20 h.

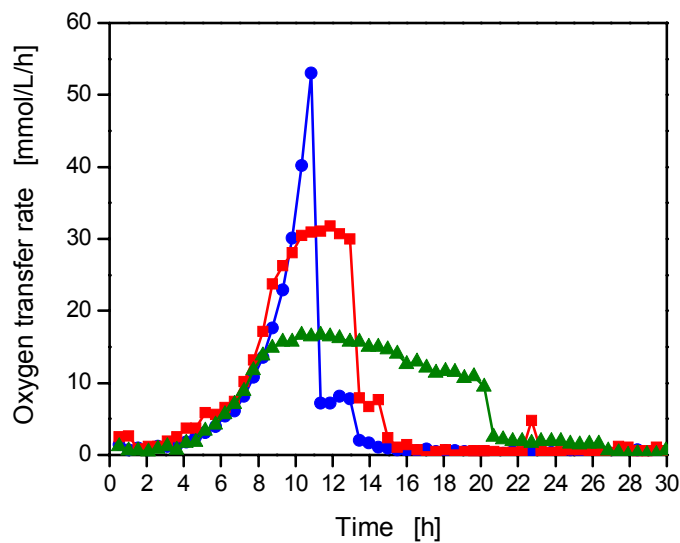


Figure 6-2: Influence of phosphate limitation on respiration activity during cultivation. Conditions: $T = 37^{\circ}\text{C}$, $V_L = 10 \text{ mL}$, $n = 350 \text{ rpm}$, $d_0 = 50 \text{ mm}$, no induction; modified Wilms & Reuss medium (7.5 g/L Glucose) with 3000 mg/L K_2HPO_4 (●), 150 mg/L K_2HPO_4 (■), 75 mg/L K_2HPO_4 (▲). Measurement with the RAMOS device.

This behavior is typical for a limitation of secondary substrates such as phosphate (Anderlei and Büchs 2001; Kottmeier et al. 2009). The culture started phosphate-limited growth at 7 to 8 h and showed no second OTR peak, because obviously only little acetate was produced or consumed. These findings, especially the times when phosphate limitation started and glucose was exhausted, are in good agreement with the growth data from the BioLector (Fig. 6-1).

The above results clearly showed that after a limitation of phosphate, the cells grew much slower and exhibited a prolonged respiration activity due to the ongoing consumption of the primary substrate (glucose). Similar results have been found with extensive offline analysis of growth, phosphate and glucose in a bioreactor (Shin and Seo 1990; Wang et al. 2005). Although in this study glucose and phosphate was not measured directly, the measurement of the respiration activity provided valuable insight into the metabolism of the cells and confirmed the proposed events during phosphate limitation. The period of a stagnating OTR depicted in Figure 6-2 (phosphate-limited cultures) represented a time of metabolic activity, but reduced growth of the cells and might be used to uncouple recombinant protein expression from biomass growth.

6.3.2. Effects of phosphate limitation on protein expression

A BioLector experiment was conducted to study in detail the general effects of a phosphate limitation on the expression of the fluorescence protein EcFbFP. Therefore, an intermediate phosphate concentration (150 mg/L) was chosen. Besides this, also the unlimited medium was used as a reference. From the results of Figure 6-1 and 6-2 an induction time of 9.5 h was chosen because at that time the phosphate became limited (for the 150 mg/L-culture). The unlimited 3000 mg/L-culture grew to a scattered light intensity of 20000 a.u., whereas the 150 mg/L-culture reached only 12000 a.u. (Fig. 6-3A). The unlimited culture immediately started to express the fluorescent protein EcFbFP upon induction and reached a plateau at a fluorescence of 2500 a.u. (Fig. 6-3B).

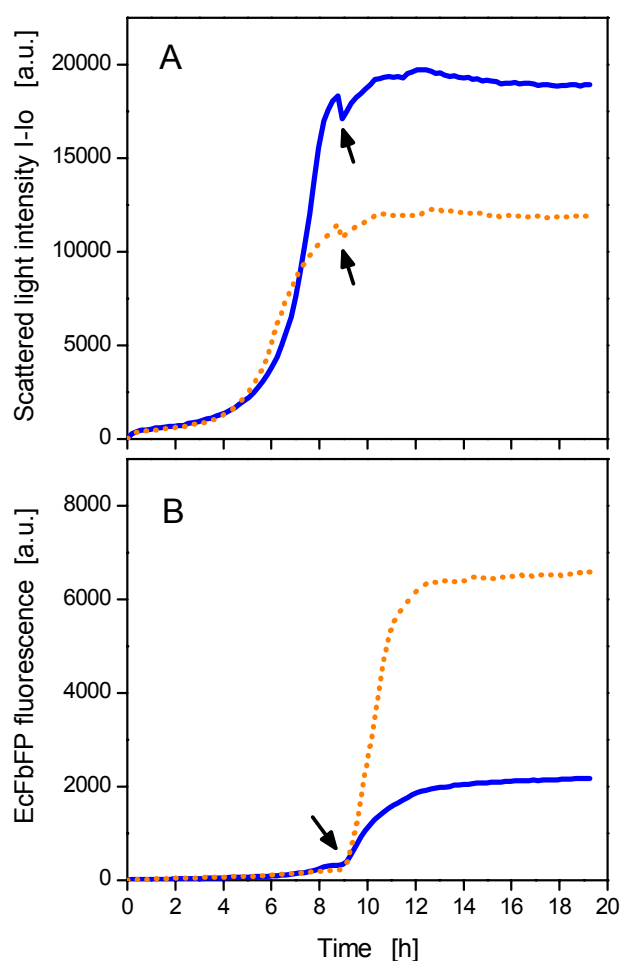


Figure 6-3: Influence of phosphate limitation on growth (A) and recombinant protein production (B). Conditions: $T = 37^{\circ}\text{C}$, $V_L = 200\ \mu\text{L}$, $n = 950\ \text{rpm}$, $d_0 = 3\ \text{mm}$, modified Wilms & Reuss medium (7.5 g/L Glucose) with two different phosphate concentrations: 3000 mg/L K_2HPO_4 (unlimited conditions, blue solid lines), 150 mg/L K_2HPO_4 (phosphate-limited conditions, orange dotted lines), induction with IPTG (final concentration 0.1 mM) after 9.5 h (indicated by arrows). Measurement with BioLector prototype, signal gain factor 25. Twelve cultures of each medium composition were conducted, the mean value is depicted above, the relative standard deviation of all cultures did not exceed $\pm 10\ \%$.

After this culture consumed all glucose, it reached stationary phase (13 h) and the cells had no longer the resources (substrate, energy) to express the target protein. On the other hand, the limited culture exhibited a much greater EcFbFP fluorescence upon induction, even when the important nutrient phosphate was limited (Fig. 6-3B). The production of EcFbFP continued until 14 h (probably because glucose was exhausted as shown in Figure 6-2 for an uninduced culture), although the cells did not grow anymore. Although a lower biomass was available in the phosphate-limited culture, even more EcFbFP was produced as in the unlimited culture. This provided evidence that phosphate-limited cells are still capable to produce recombinant proteins. The phosphate

unlimited culture in this experiment was induced almost in the stationary phase and hence had only little resources left for efficient EcFbFP expression. Furthermore, the time of induction is probably the most crucial factor for recombinant protein expression (Donovan et al. 1996). This was verified for the applied strain in mineral medium in Chapter 5 (Fig. 5-5 and 5-6). Therefore, it is important to screen different induction times for phosphate-limited and unlimited cultures to evaluate if a phosphate limitation is generally suitable to enhance specific productivity.

For such a screening, the Robo-Lector platform for automated high-throughput experimentation was used. With this platform it is possible to automatically induce up to 96 parallel cultures in a MTP at different times. This method is called 'induction profiling' and was modified here to test 9 different induction times (at a constant IPTG concentration of 0.1 mM) for phosphate-limited (150 mg/L) or unlimited cultures (3000 mg/L). Figure 6-4A depicts the EcFbFP expression in phosphate unlimited cultures at different induction times. As a reference, cells without induction were cultivated and showed only small background fluorescence. At every induction point (from 1 to 9 h after inoculation), an immediate increase of the EcFbFP fluorescence became visible for the different cultures. Noteworthy, the slope of this increase was steeper the later the cells were induced. This could be explained by the increased biomass at the later times of induction. The maximum EcFbFP production was achieved with an induction after 6 h. After this time the protein expression decreased dramatically probably because the medium became exhausted for glucose as already discussed for Figure 6-3. Similar effects resulted from inducing phosphate-limited cultures (Fig. 6-4B), although here in most cases a higher EcFbFP fluorescence was observed than in unlimited cultures.

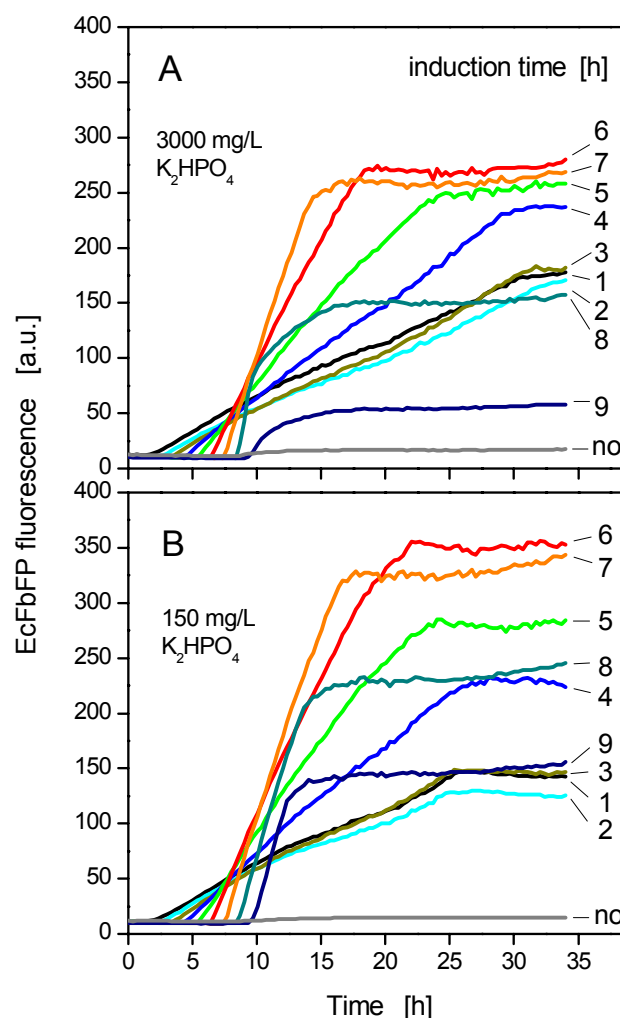


Figure 6-4: Influence of induction time on formation of EcFbFP under (A) unlimited (3000 mg/L K_2HPO_4) and (B) limited (150 mg/L K_2HPO_4) conditions. Each curve represents the EcFbFP production for every different time of induction. Conditions: $T = 37^\circ C$, $V_L = 200 \mu L$, $n = 950 \text{ rpm}$, $d_0 = 3 \text{ mm}$, modified Wilms & Reuss medium (7.5 g/L Glucose), induction with IPTG (final concentration 0.1 mM) at 1 to 9 h. Measurement with the Robo-Lector, signal gain factor 70. Duplicate cultures for each induction time were conducted, the mean value is depicted above, the relative standard deviation of all cultures did not exceed $\pm 10 \%$.

To compare phosphate unlimited and limited conditions, the maximum values for EcFbFP fluorescence at the different induction points were plotted in Figure 6-5. The phosphate-limited and unlimited cultures displayed a nearly constant EcFbFP production until 3 h of induction time. After that, both phosphate conditions showed a peak in EcFbFP fluorescence at 6 h, whereas the phosphate-limited cultures reached higher values than the unlimited cultures. When cells were induced earlier than 4 h after inoculation, then the phosphate-limited cultures showed a lower EcFbFP fluorescence. From this experiments it became obvious that phosphate limitation can only enhance product

formation when an appropriate induction time was chosen. This effect and an optimal time of induction could easily be determined with the Robo-Lector platform.

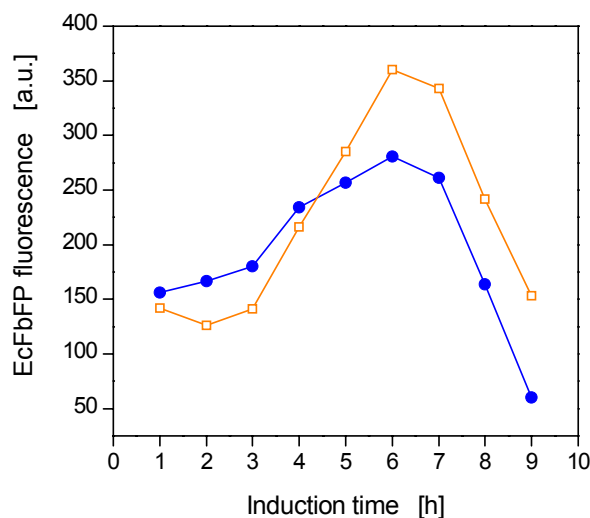


Figure 6-5: Influence of induction time on formation of EcFbFP under unlimited (3000 g/L K_2HPO_4 , ●) and phosphate-limited conditions (150 mg/L K_2HPO_4 , □). For growth conditions refer to Figure 6-4, data taken from experiment of Figure 6-4 at 34 h of cultivation.

6.3.3. Verification of results in shake flasks

To verify the obtained results from the previous experiments, a cultivation in shake flasks was conducted under different phosphate-limited (225 mg/L, 150 mg/L, 75 mg/L) and unlimited conditions (3000 mg/L). The induction conditions were the same as in the Robo-Lector test with induction after 6 h, as this was the optimal time identified previously (Fig. 6-5). After 26 h, samples from the different flasks were taken and the OD, scattered light and EcFbFP fluorescence was measured off-line. Furthermore, samples were taken for SDS-PAGE and subsequent densitometric analysis. The results of these experiments were summarized in Figure 6-6.

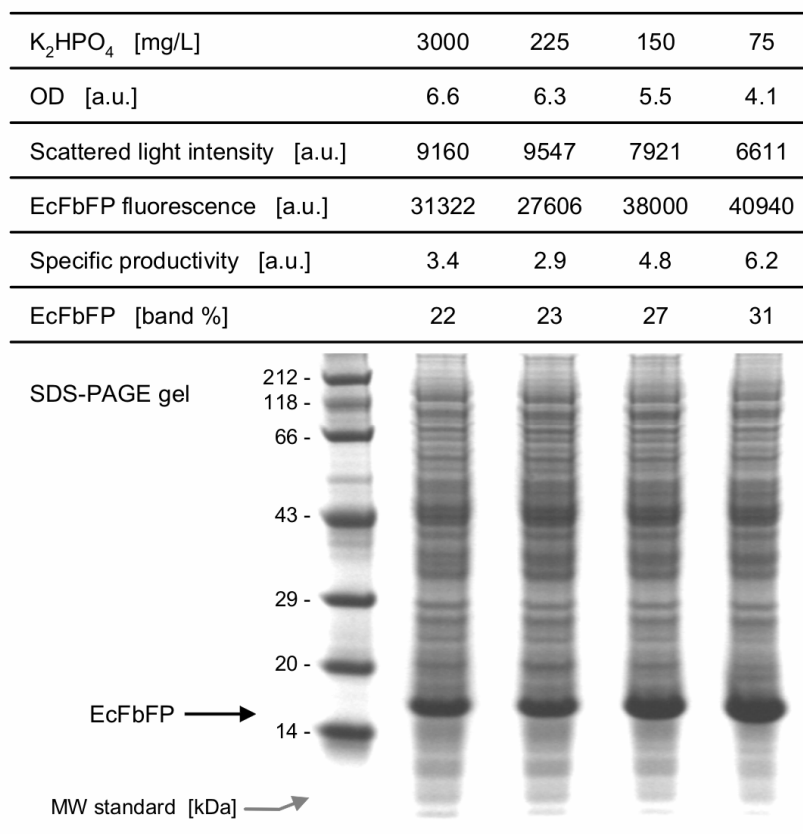


Figure 6-6: Comparison of phosphate-limited and unlimited conditions in shake flasks concerning biomass (OD and scattered light), product formation (EcFbFP fluorescence and SDS-PAGE gel) and specific productivity (fluorescence per scattered light and percent of EcFbFP band). Data from shake flask experiments. Conditions: harvest time 26 h, $T = 37^\circ\text{C}$, $V_L = 10$ mL, $n = 350$ rpm, $d_0 = 50$ mm, modified Wilms & Reuss medium (7.5 g/L Glucose) with four different phosphate concentrations, induction with IPTG after 6 h (final concentration 0.1 mM), samples for SDS-PAGE were normalized to a comparable protein content and EcFbFP bands were quantified with densitometry, MW: molecular weight standard. Measurement of scattered light (signal gain factor 20) and EcFbFP fluorescence (signal gain factor 45) with BioLector prototype.

The OD and scattered light intensity decreased with decreasing phosphate content of the medium (as could be expected from results of Figure 6-1). On the other hand, the EcFbFP fluorescence increased with decreasing phosphate, confirming the concept of increased product formation via a phosphate limitation in batch cultures (Fig. 6-4). This was also indicated by the calculated specific productivity (EcFbFP fluorescence per scattered light), as this rose from 3.4 to 6.2 a.u. under phosphate-limited conditions. The percentage of the EcFbFP band per total protein content of the *E. coli* cells (band %), as determined with SDS-PAGE and densitometry, confirmed this finding (increase from 22 to 31 %; see also SDS-PAGE gel).

6.4. Conclusions

In this Chapter, different online-monitoring tools, such as RAMOS and BioLector, were used to study, characterize and optimize the given T7 expression system *E. coli* BL21(DE3) pRhotHi-2-EcFbFP. With these tools, the demand of the examined strain for phosphate as well as the time of the limitation onset could easily be determined. Furthermore, the effect of phosphate limitation on target protein production and the optimal time for induction was efficiently investigated by the automated high-throughput cultivation platform Robo-Lector. In the end, the results were successfully verified by shake flask experiments.

These results revealed, that the phosphate limitation was suitable to redirect the available resources during cultivation (after 'enough' biomass has formed) to protein expression rather than in biomass production. This effect finally led to an increase in specific productivity of the target protein EcFbFP from 21 % to 31 % of total protein. To our knowledge, such an effect was for the first time shown for an IPTG-inducible expression system. Possible reasons for that result are that cells can be metabolically active for a long time even under a phosphate limitation and the resulting stationary phase (Moreau et al. 2001). This principle was verified with RAMOS cultivations shown in Figure 6-2. Furthermore, as demonstrated for Figure 6-2, phosphate-limited cultures seemed to produce lower amounts of overflow metabolites such as acetate. Although not directly measured here, the lower acetate formation was probably additionally favoring a higher specific productivity, since acetate is known to be detrimental to protein expression (Akeson et al. 2001; van de Walle and Shiloach 1998). Han and Lee also showed by proteome analysis that a subset of proteins involved in protein synthesis in *E. coli* was greatly enlarged during a phosphate limitation (Han and Lee 2006). This, in turn, could increase protein synthesis capacity. Additionally, a higher plasmid copy number was observed in a non-induced fed-batch culture because of a phosphate limitation (Horn et al. 1990). This might lead to higher amounts of mRNA for the target protein and consequently to a higher product yield in induced cultures. Furthermore, the aspects described here are most probably also the main reason why host-vector systems using the phosphate starvation-inducible promoter *phoA* show high productivities, as they tend to be very efficient expression systems (Lübke et al. 1995; Sawers and Jarsch 1996).

Increasing the specific productivity by phosphate limitation can be especially important in high-throughput cultivations such as MTP-based processes. Since in MTPs the cultivation volume is mostly fixed and the biomass often can not be increased due to oxygen limitations, the only way to increase the total productivity is to raise the specific productivity (Markland 2006). A promising idea would also be to combine the presented online-monitoring tools with 'omics'-techniques to further investigate the described effects of phosphate limitation, as the exact molecular reasons for the increased specific productivity remain unclear. This could help to find new targets to further enhance the production capacity of recombinant *E. coli* strains.

7. Conclusions and outlook

In the postgenomic era, there is an increasing availability and utilization of clone libraries, in particular for recombinant protein production. Therefore, high-throughput processes for cultivating different clones, which are mainly conducted in MTPs, gain in importance. However, with conventional techniques it is very tedious to study microbial growth and product formation in such complex processes, which normally consist of multiple steps (e.g. colony picking, preculture, main culture, induction). This is mainly due to a lack of appropriate monitoring capabilities and the extensive workload associated with manually conducted high-throughput cultivations.

Hence, the major aim of this thesis was to evaluate the suitability of the BioLector technique to study and optimize high-throughput cultivation processes, with a special focus on recombinant protein expression in *E. coli*. Furthermore, new applications in this area should be elaborated for the BioLector.

In Chapter 3, the effects of different tools (e.g. 8-channel pipette, 96-pin replicators) and methods for the replication of cells from one MTP to another MTP on growth kinetics of *E. coli* SCS1 pQE-30 pSE111 were observed with the BioLector. This resulted in highly variable growth kinetics, caused by different amounts of inoculum that are transferred by 96-pin replicators in conventional high-throughput cultivation processes. Furthermore, the influence of different growth kinetics on product formation of *E. coli* BL21(DE3)

pRhotHi-2-EcFbFP was investigated and showed strongly varying product formation (approximately twofold) upon induction at a defined point of time. Finally, a typical high-throughput cultivation process was simulated with *Corynebacterium glutamicum* pEKEx2-phoD-GFP, beginning at the colony picking step. Again, the online monitoring with the BioLector revealed strong variances in growth kinetics and a more than twofold difference in product formation. To sum up: high-throughput cultivation processes with multiple steps proved to be prone to irreproducible protein expression results. Hence, to improve the reproducibility and the comparability between different applied cultures, it is strongly recommended to use automated or manual liquid handling stations or, alternatively, multi-channel pipettes to improve the precision of the transferred inoculum volume. With respect to the results obtained, other methods to increase the comparability of parallel cultivations by compensating differences in initial biomass concentrations are required, such as using autoinduction media, fed-batch operation of precultures (Chapter 4) or online monitoring in MTPs combined with automated liquid handling (Robo-Lector, Chapter 5).

As demonstrated in Chapter 3, different growth kinetics are likely to occur in high-throughput batch cultivation processes. Problems arising from unequal growth kinetics are different induction points in expression studies or uneven cultivation periods at the time of harvest. Screening for the best producing clones of a library under comparable conditions is thus often impractical or even impossible. A new approach to circumvent this problem is to apply fed-batch mode for precultures, which was described in Chapter 4. This concept for equalizing growth in high-throughput cultivations was simulated and verified experimentally. For realizing fed-batch, a slow-release system for glucose was applied in special MTPs and disks for shake flasks. The growth of distinct inoculated cultures in such MTPs and shake flasks could be equalized for different microorganisms such as *Escherichia coli* and *Hansenula polymorpha*. This technique might be especially useful for microorganisms exhibiting decreasing viability in the stationary phase and in which synchronous growth of distinct cultures is very important. Inherent advantages of this method are that it is easy to use, requires no additional equipment like pumps and needs no permanent and laborious offline-monitoring of precultures to determine the right time of transfer to a main cultivation. Nevertheless, further investigations are needed to proof the general applicability of this method with different clone libraries and to evaluate its robustness.

In industry and academic research, there is an increasing demand for flexible automated MBR platforms with advanced sensing technology. To meet this demand, a novel system consisting of a BioLector and a liquid-handling robot (termed Robo-Lector) was successfully built and tested (Chapter 5). Three unique exemplary methods were programmed on the Robo-Lector platform that effectively allowed to study in detail high-throughput cultivation processes and the host-vector system *E. coli* BL21(DE3) pRhotHi-2-EcFbFP, expressing the fluorescence protein EcFbFP. With the method 'induction profiling' it was possible to conduct 96 different induction experiments (varying inducer concentrations from 0 to 1.5 mM IPTG at 8 different induction times) simultaneously in an automated way, generating more than 20000 data points. The method 'biomass-specific induction' allowed to automatically induce cultures with different growth kinetics (initial OD 0.03 to 0.16) in a MTP at the same biomass concentration, which resulted in a relative standard deviation of the EcFbFP production of only $\pm 7\%$. In comparison to the product formation (with a relative standard deviation of $\pm 24\%$) of simultaneously induced cultures with similar initial biomass concentrations (OD 0.01 to 0.2; Chapter 3.3), the method 'biomass-specific induction' yielded a clear improvement. The third method 'biomass-specific replication' enabled to generate equal initial biomass concentrations in main cultures from precultures with different growth kinetics. These methods could help to circumvent the problems associated with different growth kinetics encountered in Chapter 3. The interaction between the BioLector and the robot combines high-content data generation (especially for key parameters such as growth and product formation) with systematic high-throughput experimentation in an automated fashion, offering new possibilities to study biological production systems. As the Robo-Lector has a simple but efficient design (MTP format; use of standard liquid-handling robot and hence widespread automation possibilities), it can be easily combined with established techniques for small scale downstream processing and analytical assays. Furthermore, the liquid handling capabilities can be used to extend the applications of the Robo-Lector, e.g. to generate media compositions with subsequent parallel cultivations to optimize cultivation media automatically or to add liquids periodically to MTPs for fed-batch cultivations. In the end, this novel versatile platform can accelerate and intensify research and development in the field of bioprocess optimization, systems biology as well as modelling.

In Chapter 6, high-throughput experimentation tools were applied to study and optimize the T7 expression system *E. coli* BL21(DE3) pRhotHi-2-EcFbFP cultivated under phosphate limitation. With the help of the Robo-Lector platform and the online-monitoring devices BioLector and RAMOS, the effects of a phosphate limitation were

efficiently investigated. Hence, an increase in specific productivity of the target protein EcFbFP from 21 % to 31 % of the total protein content of the cells could be achieved. To our knowledge, such an effect of a phosphate limitation was shown for a T7 expression system for the first time. A combination of the presented high-throughput experimentation tools with 'omics'-techniques would be promising to further investigate the described effects of a phosphate limitation on a molecular basis. This could help to find new targets to further enhance the production capacity of recombinant *E. coli* strains.

In summary, the results of this thesis demonstrated that extensive monitoring of key parameters (i.e. biomass and product formation) in small scale cultivations, in particular MTPs, enables a profound understanding and hence optimization of recombinant expression systems and high-throughput cultivation processes. Here, the BioLector proved to be a valuable tool and offers new applications within the design of the RoboLector platform. Ultimately, this platform can contribute to the envisioned paradigm shift in bioprocess development. This entails switching from rather empirical process development (low-throughput experimentation with no or unsophisticated monitoring) to high-throughput experimentation with very sophisticated yet simple monitoring to gain deeper knowledge of biological production systems.

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Appendix

Appendix A - Overview of typical high-throughput cultivation processes for recombinant protein production in *E. coli*

A review on ten different publications using type 1 clone libraries (Table 1-1, Chapter 1.1) is conducted to evaluate the characteristics of typical high-throughput cultivation processes. The focus of this review is on process-related parameters of the starting material, preculture, main culture and protein expression. Hence, details about cultivation conditions, such as temperature, medium and shaking frequency are omitted here.

parameter	reference →	(Braud et al. 2005)	(Brodsky and Cronin 2006)	(Chambers et al. 2004)	(Knaust and Nordlund 2001)	(Nguyen et al. 2004)	(Page et al. 2004)	(Peti et al. 2005)	(Kohl et al. 2008)	(Chen et al. 2007)	(Berrow et al. 2006)
clone library	host	various, e.g. <i>E. coli</i> /BL21 (DE3)	<i>E. coli</i> /BL21 (DE3)	<i>E. coli</i> /BL21 (DE3) pLysS	<i>E. coli</i> XL-1 Blue	<i>E. coli</i> BL21(DE3) or B834(DE3)	various <i>E. coli</i>	<i>E. coli</i> (DL41)	<i>E. coli</i> /BL21 (DE3)	<i>E. coli</i> /BL21 (DE3)/AI	
	starting material	transformation mix	cryoculture	transformation mix	colonies on agar plates	colonies on agar plates	cryoculture	colonies on agar plates	colonies on agar plates	colonies on agar plates	
preculture	format (also for main culture)	96-well MTP	96-well DWP	24-well DWP	96-well DWP	24-well DWP	96-well DWP	96-well DWP	48-well DWP	96-well DWP	Benchmarking of eight high-throughput processes at different laboratories involved in the Structural Proteomics In Europe-SPINE consortium.
	inoculum	complete transf. mix	NR	complete transf. mix	single colonies	NC	1µL cryoculture	single colonies	single colonies	single colonies	
	time	overnight	overnight	overnight	overnight	NC	overnight	12-15h	16h	overnight	
main culture	inoculum	~ OD 0.05	15µL of preculture	20µL of pelleted and resuspended preculture	complete preculture	single colonies	25µL of pelleted and resuspended preculture	40µL of a 2nd preculture	100µL preculture	1:200 dilution of preculture	
	induction at: OD / time	~OD 0.5 or 1.2 / 3h	~ OD 3,3-4,2 / 4h	~ OD 1 / 3-4h	NC / 1h	NR	~ OD 3 / 1.5h	~ OD 2-3 / ~ 6h	NC / 1.5 or 2.5 or 3.5h	NC / 3-4h	
	induction method	IPTG (1mM) or arab. (0.2%)	IPTG (0.1mM)	IPTG (1 mM)	IPTG (1mM)	autoinduction medium	arabinose (0.2 %)	arabinose (0.2%)	IPTG (1mM)	IPTG (1mM)	
	cultivation time: pre- / post induction (harvest)	3h / 16h	4h / overnight	3-4h / 6-8 h	1h / 4h	24h	1.5h / 4-5h	6h / 16h	1.5 or 2.5 or 3.5h / 12h	3-4h / 3h or 20h	
DWP	deep well plate										
MTP	microtiter plate										
IPTG	isopropyl-β-D-thiogalactopyranoside										
NR	not reported										
NC	not conducted										

Appendix B - Robo-Lector system setup

Figure B-1: Custom-made version of the BioLector. This system consists of two separate units, the incubation unit (left) and the measurement unit (right).

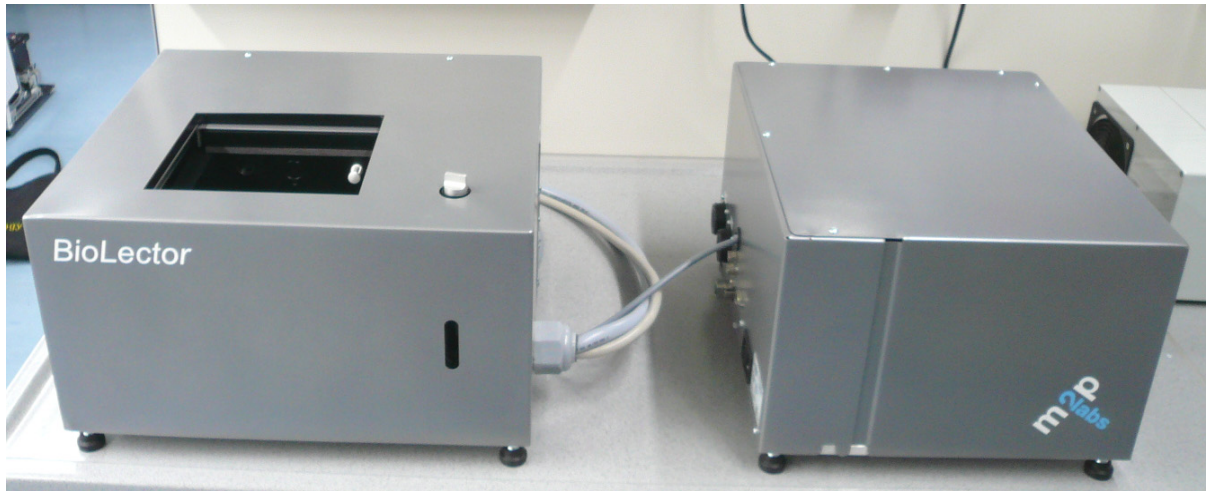


Figure B-2: Robo-Lector system setup. The incubation unit of the BioLector was placed inside and the measurement unit below the pipetting robot. Both units are connected by an optical fiber for signal acquisition. The BioLector is connected to a control computer via a TCP/IP-network; the pipetting robot is linked to the same computer via a USB connection. The Vector software controls the pipetting robot, whereas the BioLector HMI (Human Machine Interface) controls the BioLector. The interaction of both devices is realized via command codes that are sent to the BioLector HMI, which, in turn, sends the status and data of the BioLector to the pipetting robot. Additionally, the BioLector software allows to analyze and visualize data from the MTP-cultivation of the BioLector.

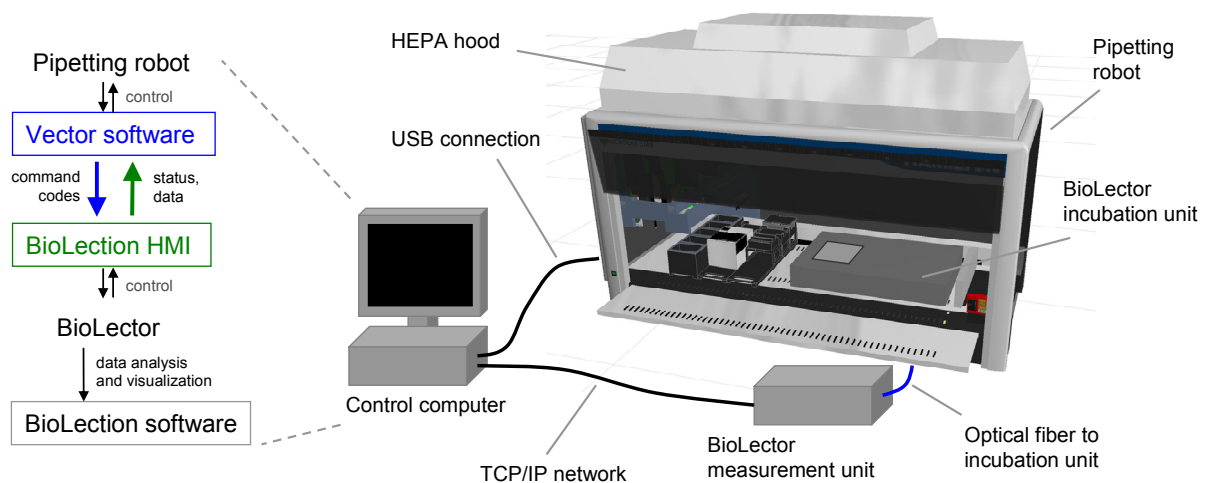


Figure B-3: Exemplary deck layout of the Robo-Lector platform in the Vector software. The tip carrier provides sterile tips of different volume and the gripper enables transport of MTPs on the deck, e.g. to the MTP position of the BioLector. After pipetting, the tips can be removed at the waste bin.

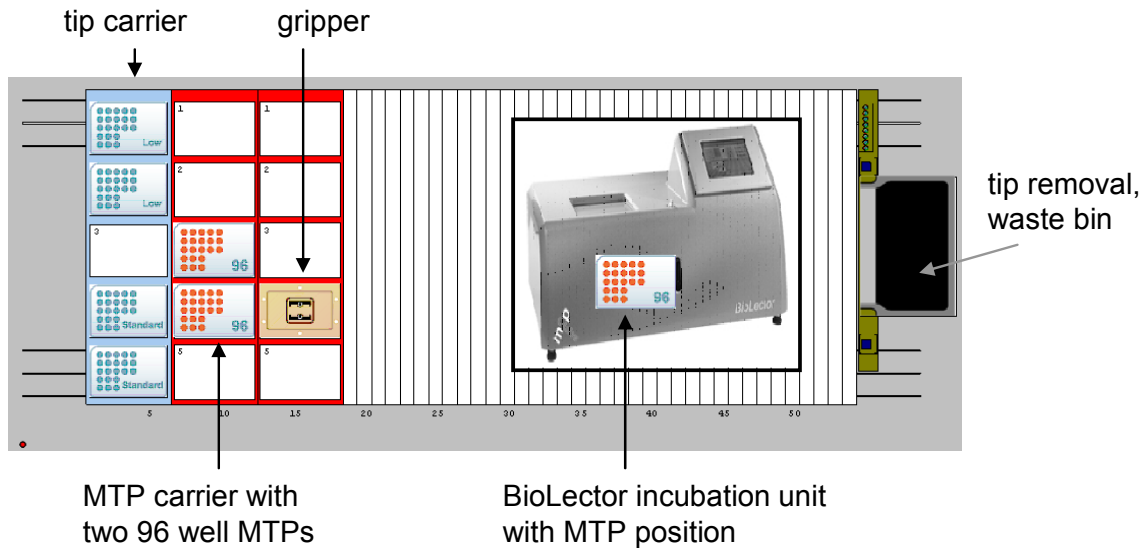


Figure B-4: Exemplary screenshots of the BioLection HMI and the BioLection software. The HMI is used to control the BioLector (left), the BioLection software (right) is used to analyze and visualize cultivation data of the BioLector.

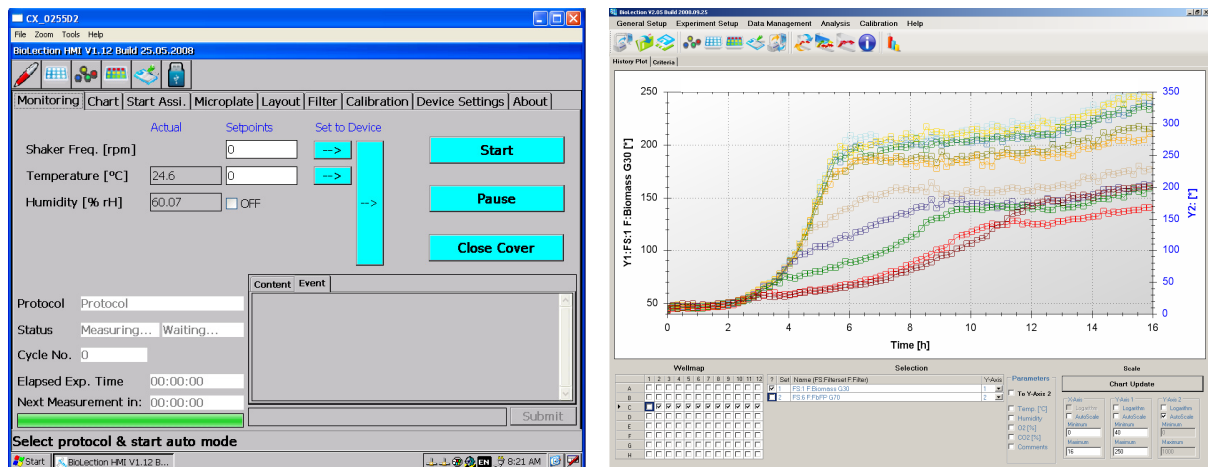
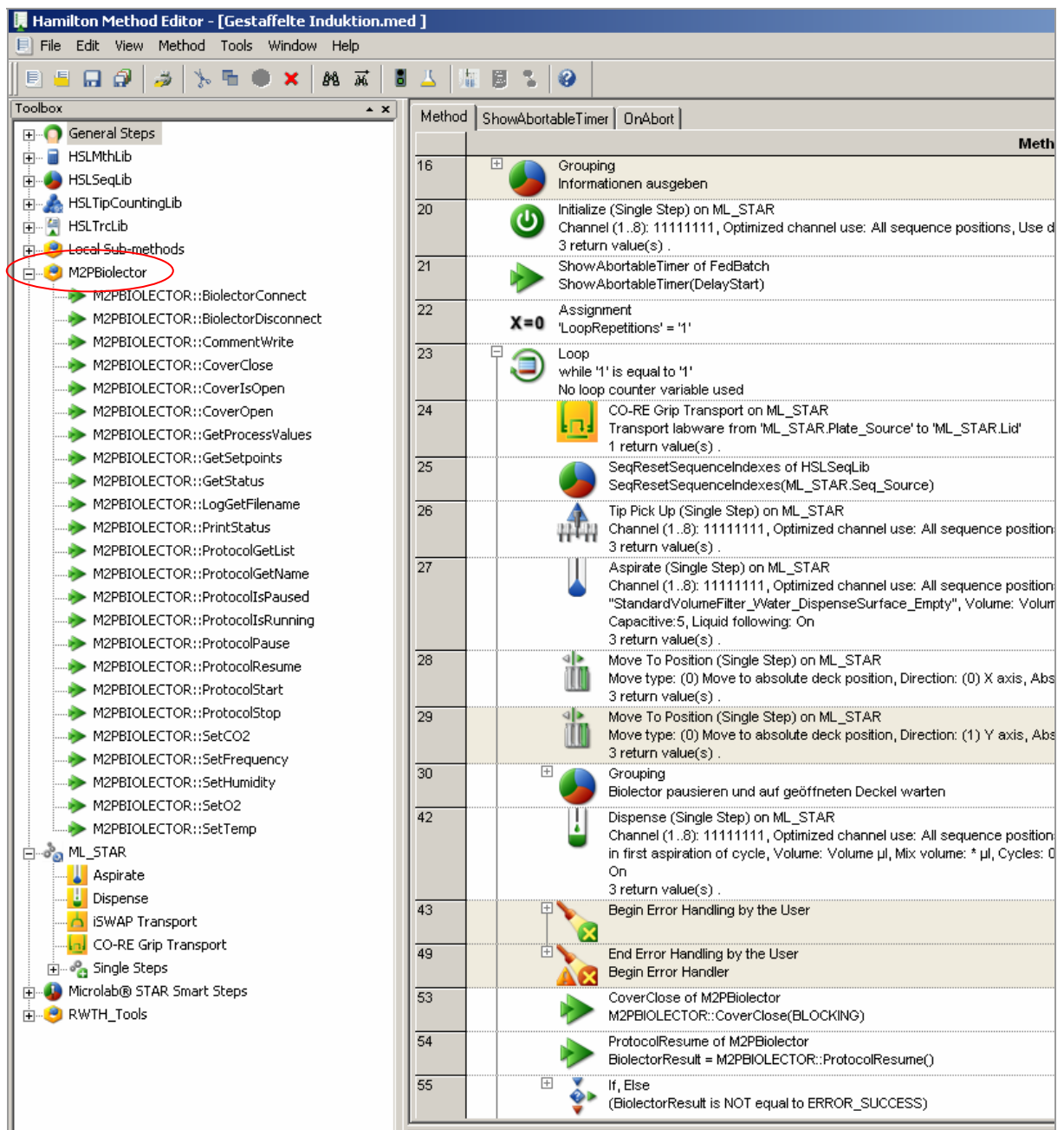


Figure B-5: Exemplary screenshot of the Vector software. Left panel (Toolbox): overview of programmed command codes to control the BioLector (red circle); right panel (Method): command lines for actions of the pipetting robot and the BioLector in the method 'biomass-specific induction'.



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