Kaisa Ukkonen

IMPROVEMENT OF RECOMBINANT PROTEIN PRODUCTION IN SHAKEN CULTURES

FOCUS ON AERATION AND ENZYME-CONTROLLED GLUCOSE FEEDING
KAISA UKKONEN

IMPROVEMENT OF RECOMBINANT PROTEIN PRODUCTION IN SHAKEN CULTURES
Focus on aeration and enzyme-controlled glucose feeding

Academic dissertation to be presented with the assent of the Doctoral Training Committee of Technology and Natural Sciences of the University of Oulu for public defence in Auditorium IT116, Linnanmaa, on 14 February 2014, at 12 noon

UNIVERSITY OF OULU, OULU 2014
Ukkonen, Kaisa, Improvement of recombinant protein production in shaken cultures. Focus on aeration and enzyme-controlled glucose feeding
University of Oulu Graduate School; University of Oulu, Faculty of Technology, Department of Process and Environmental Engineering

Abstract
Efficient production of biologically functional recombinant proteins is a cornerstone of modern biotechnological research. Laboratory-scale protein production is most commonly accomplished in simple shaking bioreactors such as shake flasks. However, productivity of these cultures is often severely limited by low biomass yield and non-optimal growth conditions regarding medium composition, pH and oxygen supply. In many cases, poor culture performance can constitute a major research bottleneck. This study aims to improve recombinant protein production in shaking *Escherichia coli* cultures by use of enzyme-controlled, fed-batch-like glucose feeding in a rich medium, and by investigating the effects of culture aeration on different aspects of protein production.

The results show that the enzymatic fed-batch medium can provide higher cell densities, volumetric protein yields and, in some cases, improved product solubility or activity compared to traditionally used media. While these improvements could be obtained in ordinary shaking vessels, the results also demonstrate that cultivation in shake flasks with elevated aeration capacity can further improve cell density and volumetric productivity in the fed-batch medium. However, enhanced aeration may also have an adverse effect on the expression of certain proteins such as Fab antibody fragments. Maximum volumetric Fab yield was achieved under reduced aeration rates, and lower oxygen availability also contributed to substantially increased accumulation of periplasmically produced Fab fragments into extracellular medium. Hence modification of aeration conditions and medium composition can be used to control periplasmic/extracellular product localization as outlined in this study. Moreover, high aeration was detrimental to expression in a glycerol-based lactose autoinduction medium, but this strict dependency on aeration level could be mitigated and robustness of expression improved by an autoinduction medium based on the enzymatic glucose feeding as the supporting carbon source instead of glycerol.

The results of this study can be utilized to improve volumetric productivity, protein solubility and control of product localization in small-scale protein production, as well as to facilitate robust and efficient high-throughput protein expression for such applications as structural and functional characterization.

Keywords: aeration, autoinduction, *Escherichia coli*, fed-batch medium, high cell density, oxygen, recombinant protein, shake flask, small-scale culture
Ukkonen, Kaisa, Vierasproteiinituotannon parantaminen ravisteluviljelmissä. Tarkastelussa ilmastus ja entsymaattisesti kontrolloitu glukoossyöttö

Oulun yliopiston tutkijakoulu; Oulun yliopisto, Teknillinen tiedekunta, Prosessi- ja ympäristötieteen osasto
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

**Tiivistelmä**


Tutkimuksen tuloksia hyödyntäen voidaan parantaa vierasproteiinien saantoa, liukoisuutta ja periplasmisen/solunulkoisen kerääntymisen säätelyä, sekä mahdollistaa luotettava ja tehokas proteiinituotto viljelmien suurta lukumäärää vaativiin sovelluksiin, kuten proteiinien rakenteen ja toiminnan tutkimukseen.

**Asiasanat:** autoinduktiio, *Escherichia coli*, happipitoisuus, ilmastus, korkea solutihyys, panossyöttökasvualusta, pienien mittakaavan viljelmät, ravistelupullo, vierasproteiini
Acknowledgements

This study was conducted at Bioprocess Engineering Laboratory, University of Oulu and at BioSilta Oy. I thank BioSilta for the financial and material support of this study. Funding from Finnish Foundation for Technology Promotion is also gratefully acknowledged. I would like to express my sincere gratitude to my supervisors Professor Heikki Ojamo, Professor Peter Neubauer and Professor Juha Tanskanen. I especially thank Heikki for support in the practical matters throughout the whole process of this study; Peter for scientific guidance during my entire research career this far; and Juha for supporting me in the final stages of the thesis process as a Degree Supervisor.

I am grateful to Professor Ursula Rinas and Professor Alexander Frey for careful pre-examination of my thesis and for their insightful comments. I also thank the members of my doctoral follow-up group, Dr. Veli-Pekka Jaakola, Dr. Sanna Taskila and Dr. Vesa Ruotsalainen, for their encouragement as well as for reviewing my thesis. I especially want to acknowledge Sanna for the guidance in practical matters during the final phase of completing this thesis. Elina Ukkonen is acknowledged for the language review.

I warmly thank my BioSilta co-workers Dr. Antti Vasala, Dr. Antje Neubauer, Dr. Craig Fuller, Maria Ruottinen, Tuula Karppinen, Mirja Krause, Eeva-Maija Kolehmainen and Nanna Timonen for their great support. The encouragement from my co-workers was absolutely crucial for completion of this thesis. I also thank Antti, Antje and Mirja for their important scientific contribution. I also want to express my sincere gratitude to BioSilta CEOs Russell Golson and Bob Penney for their support that made my study possible.

I want to thank Docent Tuomo Glumoff, Dr. Tatu Haataja, Dr. Johanna Veijola and Sonja Mayer for their scientific contribution as co-authors in my articles. I also gratefully acknowledge Johanna Panula-Perälä, Ville Sotaniemi, Petri Tervasmäki and Lilja Tuohimaa for their wonderful company at BPEL; thank you for making my summer of thesis writing so nice.

I warmly thank my family – the most important people in my life. I am grateful to my mother Mirja and father Markku for encouraging me to study and all their help and support; and to Elina for being such a great sister. Finally, I want to thank Aleksei for standing by my side during my studies.

Oulu, 11 December 2013

Kaisa Ukkonen
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>24dwp</td>
<td>24 deep well plate</td>
</tr>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BiP</td>
<td>binding immunoglobulin protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CDW</td>
<td>cell dry weight (g l⁻¹)</td>
</tr>
<tr>
<td>CN</td>
<td>complex nutrients</td>
</tr>
<tr>
<td>CRP</td>
<td>cAMP receptor protein</td>
</tr>
<tr>
<td>DOT</td>
<td>dissolved oxygen tension</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>Erl</td>
<td>Erlenmeyer (flask)</td>
</tr>
<tr>
<td>ERp27</td>
<td>27 kDa endoplasmic reticulum protein</td>
</tr>
<tr>
<td>ERp60</td>
<td>60 kDa endoplasmic reticulum protein</td>
</tr>
<tr>
<td>Fab</td>
<td>antigen-binding fragment of immunoglobulin</td>
</tr>
<tr>
<td>FHL</td>
<td>formate hydrogen lyase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>k(g)a</td>
<td>volumetric gas phase mass transfer coefficient (h⁻¹)</td>
</tr>
<tr>
<td>k(l)a</td>
<td>volumetric gas-liquid mass transfer coefficient (h⁻¹)</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>LDS</td>
<td>lithium dodecyl sulfate</td>
</tr>
<tr>
<td>MFE</td>
<td>multifunctional enzyme type 2</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>MSM</td>
<td>mineral salt medium</td>
</tr>
<tr>
<td>NAD⁺/NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP⁺/NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NTproBNP</td>
<td>N-terminal prohormone of brain natriuretic peptide</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OD(_{600})</td>
<td>optical density at 600 nm</td>
</tr>
<tr>
<td>OTR</td>
<td>oxygen transfer rate (mmol l⁻¹ h⁻¹)</td>
</tr>
<tr>
<td>OTR(_{\text{max}})</td>
<td>maximum oxygen transfer rate (oxygen transfer capacity; mmol l⁻¹ h⁻¹)</td>
</tr>
<tr>
<td>OUR</td>
<td>oxygen uptake rate</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PDI</td>
<td>a-domain of protein disulphide isomerase</td>
</tr>
<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>SB</td>
<td>Super Broth</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TB</td>
<td>Terrific Broth</td>
</tr>
<tr>
<td>U</td>
<td>enzyme activity unit</td>
</tr>
<tr>
<td>UY</td>
<td>Ultra Yield (flask)</td>
</tr>
<tr>
<td>ZYM-5052</td>
<td>Studier’s autoinduction medium</td>
</tr>
</tbody>
</table>
List of original articles

This dissertation is based on the following original articles which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.


*Equal contribution.

Article I has previously been included in the following dissertation:
Haataja T (2011) Peroxisomal multifunctional enzyme type 2 (MFE-2). The catalytic domains work as independent units.

The author’s contribution
I K. Ukkonen was responsible for the experimental work, except for the larger-scale MFE2 expression and purification, in cooperation with M. Krause. She wrote the article together with the other authors.

II–IV K. Ukkonen wrote the article, is the corresponding author and interpreted the results. She was responsible for all experimental work.
# Contents

Abstract  
Tiivistelmä  
Acknowledgements  7  
Abbreviations  9  
List of original articles  11  
Contents  13  

## 1 Introduction  15  
1.1 Background ................................................................. 15  
1.2 Objectives and scope of the research ................................. 17  

## 2 Literature review  19  
2.1 Aeration in shaking bioreactors .............................................. 19  
2.1.1 Oxygen transfer in shake flasks ..................................... 20  
2.1.2 Oxygen transfer in multiwell culture plates ..................... 23  
2.2 Determination of OTR and $k_La$ in shaking bioreactors .......... 26  
2.2.1 Sulfite oxidation method ............................................. 26  
2.2.2 Dynamic methods ..................................................... 27  
2.2.3 RAMOS ..................................................................... 28  
2.2.4 Enzyme-based methods ............................................. 28  
2.3 Oxygen in *E. coli* cultivations ............................................. 29  
2.3.1 Metabolic consequences of anoxic conditions ................. 29  
2.3.2 Oxygen availability and recombinant protein expression .... 31  
2.4 Culture media for protein expression .................................... 31  
2.4.1 Complex media ......................................................... 31  
2.4.2 Defined and minimal media ........................................... 33  
2.4.3 Autoinduction media .................................................. 34  
2.5 Fed-batch cultivation ........................................................ 36  
2.5.1 The fed-batch principle ................................................ 36  
2.5.2 Fed-batch in shaken cultures ....................................... 36  

## 3 Materials and methods  39  
3.1 Host strains and expression vectors .................................... 39  
3.2 Media ........................................................................... 39  
3.2.1 Enzymatic fed-batch .................................................. 39  
3.2.2 Reference media ...................................................... 40  
3.3 Cultivation conditions ...................................................... 40  
3.4 Cell density measurements ............................................... 40
4 Results

4.1 Influence of aeration conditions on protein yield in enzymatic fed-batch medium

4.1.1 DOT and protein production under moderate aeration

4.1.2 Enhanced aeration and the effects on protein yield

4.2 Influence of aeration and medium composition on extracellular accumulation of periplasmic Fab fragments

4.3 Robustness of lactose autoinduction media at different aeration conditions

4.4 Recombinant protein yield in enzymatic fed-batch media compared to other expression media

4.4.1 IPTG induction

4.4.2 Lactose autoinduction

5 Discussion

5.1 Effects of aeration on recombinant protein production

5.2 Improvement of protein yield by enzymatic fed-batch medium

6 Conclusions

References

Original articles
1 Introduction

1.1 Background

Simple shaking bioreactors, such as shake flasks, multiwell culture plates and shaking tubes, are the most commonly applied vessels for growing microorganisms in submerged cultures. It has been estimated that more than 90% of all biotechnological cultivation experiments worldwide are accomplished in shaken vessels, amounting to several millions of such cultures performed annually ( Büchs 2001). Cultivation in shaking bioreactors is also the most common approach for production of recombinant proteins for structural characterization (Fox et al. 2008, Gräslund et al. 2008), functional genomics and proteomics research (Larsson et al. 2000, Lesley 2001), use as antigens for antibody generation (Tegel et al. 2009), screening of enzyme libraries (Seelig & Szostak 2007) and other applications that require high-throughput cultivation platforms.

Protein expression cultivations in shaking bioreactors are often hampered by two main problems: a low volumetric yield necessitates the use of high culture volumes inexpedient for high-throughput (Lesley 2001), or the target protein is not obtained in a soluble and biologically functional form at all. Common approaches to solve these issues and improve the product yield employ modification of the expression construct at the genetic level ( Sørensen & Mortensen 2005). While the genetic strategies are often effective in improving the specific yield and/or correct folding of the protein, they do not address the problems of low cell density attainable in conventional culture media (Sezonov et al. 2007) and physiologically unfavorable oxygen and pH conditions that often prevail in shaken cultivations (Losen et al. 2004). Maximization of cell density while maintaining high productivity per biomass inevitably requires optimization of the cultivation conditions (Peti & Page 2007).

Previously introduced enhanced protein expression media enable moderately high Escherichia coli cell densities in the range of OD_{600} ~15–30 (cell dry weight ~4.5–9 g l^{-1}) and corresponding increases in the volumetric protein yield compared to conventional low cell density cultures (Losen et al. 2004, Peti & Page 2007, Sivashanmugam et al. 2009, Studier 2005). However, this is still far below the cell densities that can be obtained in stirred tank bioreactors by the glucose-limited fed-batch strategy, which controls by-product formation and metabolic activity of the culture (Lee 1996, Shiloach & Fass 2005). The EnBase
cultivation principle (enzymatic fed-batch) introduced by Panula-Perälä et al. (2008), which is based on slow glucose release by biocatalytic degradation of a polysaccharide, enables simple fed-batch-mimicking cultivation in practically any scale and vessel and is therefore a promising new approach for recombinant protein expression in shaking bioreactors. In the present study, the enzymatic fed-batch was complemented with more nutrients to overcome nitrogen limitation and provide metabolic control of pH, thus providing a novel medium to support high cell density growth and high specific productivity of recombinant proteins in shaken cultures.

Besides medium composition, oxygen availability is a key environmental factor contributing to the success of recombinant protein expression. It is widely acknowledged that the oxygen transfer capacity of shaking bioreactors is limited and often insufficient to maintain aerobic cultivation conditions (Büchs 2001, Vasala et al. 2006), and that severe oxygen limitation may emerge especially in rich media designed for reaching higher cell densities (Losen et al. 2004). Control of the oxygen demand by fed-batch-type cultivation might also attenuate these problems. On the other hand, while it is clear that maximization of cell density requires non-limiting oxygen supply and that oxygen limitation can have detrimental metabolic consequences, it is possible that the influence of oxygen supply on recombinant protein production is more complex than that. Shake flasks and shaking multiwell plates have been well characterized regarding the factors affecting oxygen transfer capacity (Duetz & Witholt 2004, Gupta & Rao 2003, Hermann et al. 2003, Maier & Büchs 2001, Maier et al. 2004) and mathematical models have been presented to predict OTR$_{max}$ from the vessel geometry and shaking parameters (Doig et al. 2005, Maier & Büchs 2001, Schiefelbein et al. 2013), but information on the actual influence of aeration conditions on recombinant productivity in shaken E. coli cultures is much more scarce. Some earlier reports (Qoronfleh 1999, Tomazetto et al. 2007) suggest that cultivation of E. coli under reduced aeration and DOT levels may be beneficial for the expression of certain proteins, but disadvantageous for others. Especially in autoinducing media, high oxygen availability can contribute to a remarkably impaired expression level (Blommel et al. 2007). Oxygen availability is also known to influence the composition of outer membrane proteins (Rolfe et al. 2011) and lipids (Arneborg et al. 1993, Knivett & Cullen 1965), which might have interesting implications on the extracellular leakage of recombinant proteins directed to E. coli periplasmic space. On this basis, investigation of the aeration effects was a central objective of this study.
1.2 Objectives and scope of the research

This study investigates the influence of aeration conditions and culture media with enzyme-controlled glucose feeding on the production of recombinant proteins in shaken *E. coli* cultures. The aim is to present new methods and information to improve small-scale recombinant protein expression by modification of the cultivation conditions. The focus of improvement is on the aspects of volumetric yield of soluble/active protein, final localization of periplasmically secreted target protein, and robust autoinduced expression.

The enzymatic fed-batch medium was used for IPTG-induced and lactose-autoinduced expression of prokaryotic and eukaryotic model proteins in industrially relevant *E. coli* host strains, and compared to commonly used batch media. Enhancement of aeration was achieved by use of specially designed cultivation flasks, which were characterized regarding the oxygen transfer capacity. Additionally, aeration conditions were modified by varying shaking frequency and culture volume.

The research objective is divided into four research questions (RQ):

**RQ1:** Is the aeration in ordinary shake flasks sufficient for good growth and protein expression in the enzymatic fed-batch medium, and how is volumetric productivity influenced by the improved aeration obtained in Ultra Yield shake flasks?

**RQ2:** What is the influence of aeration and medium composition on leakage of periplasmic Fab fragments?

**RQ3:** How does aeration influence protein expression in lactose autoinduction media with enzymatic fed-batch or glycerol as the supporting carbon source?

**RQ4:** Can cultivation in the enzymatic fed-batch medium improve recombinant product yields compared to different batch media?

These research questions are addressed by four original articles and supporting unpublished results as indicated in Table 1.
Table 1. Contribution of the articles to answering the research questions.

<table>
<thead>
<tr>
<th>Research question</th>
<th>Article I</th>
<th>Article II</th>
<th>Article III</th>
<th>Article IV</th>
<th>Unpublished data</th>
</tr>
</thead>
<tbody>
<tr>
<td>RQ1</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>RQ2</td>
<td></td>
<td></td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RQ3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>RQ4</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

18
2 Literature review

2.1 Aeration in shaking bioreactors

Culture aeration in shaking bioreactors relies on the dissolution of oxygen from headspace air into the liquid culture medium, enhanced by constant agitation of the liquid by shaking on a rotationally moving platform. Mass transfer at the gas-liquid interface is described by the equation:

\[ \text{OTR} = \text{kLa} \cdot (c_{\text{O}_2}^* - c_{\text{O}_2}) \]  

(1)

where OTR (mmol l⁻¹ h⁻¹) is the oxygen transfer rate, kLa (h⁻¹) is the volumetric mass transfer coefficient (product of the mass transfer coefficient kL and the specific gas exchange area a), and \( c_{\text{O}_2}^* - c_{\text{O}_2} \) is the driving force (\( c_{\text{O}_2}^* \) is the maximum oxygen concentration at the gas-liquid interface, and \( c_{\text{O}_2} \) is the dissolved oxygen concentration in the bulk liquid) (Enfors & Häggström 2000). Maximum oxygen transfer rate is obtained when dissolved oxygen in the bulk liquid is zero:

\[ \text{OTR}_{\text{max}} = \text{kLa} \cdot c_{\text{O}_2}^* \]  

(2)

Maximum oxygen transfer capacity (OTR<sub>max</sub>) is thus dependent on maximum oxygen solubility, which is influenced by temperature and electrolyte concentration, and on the physico-chemical properties of the liquid, properties of the culture vessel and the shaking parameters through their influence on the volumetric mass transfer coefficient (Maier & Büchs 2001). The influence of temperature is negligible in the range of normal growth temperatures, as the reduction in oxygen solubility with increasing temperature is counteracted by simultaneous increase in the diffusivity of oxygen (Enfors & Häggström 2000). It should also be noted that \( c_{\text{O}_2}^* \) is dependent on the partial pressure of oxygen in the flask headspace, which may be below air saturation if the rate of gas transfer through the sterile barrier used to seal the vessel is not sufficient to match the rate of gas-liquid mass transfer (Nikakhtari & Hill 2006, Schultz 1964).

Oxygen transfer rates and coefficients have been experimentally determined in both shake flasks (Glazyrina et al. 2011, Gupta & Rao 2003, Maier & Büchs 2001) and multiwell plates (Duetz & Witholt 2001, Duetz & Witholt 2004, Hermann et al. 2003, Hermann et al. 2001, Kensy et al. 2005). Also various model correlations have been described in the literature for prediction of kLa.

2.1.1 Oxygen transfer in shake flasks

OTR\textsubscript{max} obtained in a shake flask culture is typically in the range of 10–100 mmol l\textsuperscript{-1} h\textsuperscript{-1} (Enfors & Häggström 2000). In non-baffled Erlenmeyer flasks OTR\textsubscript{max} settles into the lower half of this range (Maier & Büchs 2001). Composition of the liquid medium naturally influences OTR\textsubscript{max} through the electrolyte concentration and viscosity, but as the medium composition is dictated by the nutritional requirements of the organism it is usually not amenable to significant modifications. The \(k_{l,a}\) and OTR\textsubscript{max} can however be readily influenced by a number of other factors discussed below.

Shaking conditions and flask geometry

In a non-baffled Erlenmeyer flask the orbital movement of the shaker platform and the resulting centrifugal force distribute the liquid medium into a sickle shape between the flask wall and a rotational paraboloid formed by the liquid surface (Maier & Büchs 2001). This distribution can be quantitatively described and the mass transfer area calculated by a mathematical model (Büchs et al. 2007). Besides higher shaking frequency, larger diameter (offset, amplitude) of the movement provides higher centrifugal force and higher OTR\textsubscript{max} (Maier & Büchs 2001). Strangely enough, the standard diameter found in most incubator shakers is 25 mm (or one inch), while much higher oxygen transfer capacity would be easily provided by shaking diameter of 50 mm or more.

Presence of baffles in the flask changes the liquid flow into more turbulent and chaotic, and thus in principle increases the gas-liquid mass transfer and promotes higher cell density (Brodsky & Cronin 2006, Gupta & Rao 2003). However, the opinion on the use of baffled flasks is divided. While cultivation in baffled flasks has been recommended as the method of choice for example in Structural Genomics Consortium consensus protocol for recombinant protein expression (Gräslund et al. 2008), others have advised against baffled flasks (Büchs 2001). The drawbacks of the baffles include possibly impaired reproducibility of experiments due to small variations in the size and position of baffles introduced manually as indentations into the glassware (McDaniel et al. 2008).
1965), difficulty of modeling the chaotic flow patterns as opposed to the simplicity of modeling non-baffled Erlenmeyers (Büchs 2001), excessive foaming, and the wetting of flask closure by liquid spilling and consequent blockage of airflow (Büchs 2001, McDaniel & Bailey 1969). In addition, baffled flasks may constitute a risk of operating cultures in the so-called out-of-phase conditions, whereby majority of the liquid does not follow the rotating movement of the shaker and the mixing efficiency and gas-liquid mass transfer are greatly reduced (Büchs et al. 2001, Peter et al. 2004). The risk of running into out-of-phase situation increases with the number and size of the baffles as well as viscosity of the medium. No information seems to exist on whether the out-of-phase risk is affected by the placement of baffles at the flask bottom (horizontal indentations) as opposed to the traditional set-up with baffles on the flask walls (vertical indentations). All publications to date characterizing the out-of-phase phenomenon in baffled flasks appear to deal with vertical baffles (Büchs et al. 2001, Peter et al. 2006).

As the gas-liquid mass transfer increases with increasing liquid surface to volume ratio, the flask fill volume in relation to the flask nominal volume is obviously an important parameter regarding culture oxygen supply (Büchs et al. 2007, Dahlgren et al. 1993). Curiously though, the emergence of out-of-phase conditions may in baffled flasks result in a counterintuitive situation where mixing efficiency is actually reduced at lower culture volumes (Büchs et al. 2001). The possible occurrence of this phenomenon is more likely at lower shaking diameters.

\[ \text{OTR}_{\text{max}} \text{ decreases with increasing flask size when the geometry and relative fill volume are kept constant (Maier et al. 2004). Large flasks with nominal volume } \geq 2 \text{l may also be susceptible to running into out-of-phase situation even without baffles and at the low viscosities attained in non-filamentous cultures (Büchs et al. 2001). This is mainly a problem at low shaking diameters such as the standard 25 mm, and at low fill volumes.} \]

\textit{Liquid film formation and surface properties}

The mass transfer area in a shake flask is constituted by the liquid surface and a thin liquid film that forms on the flask walls as the bulk of liquid circulates in the flask (Gaden 1962, Maier & Büchs 2001, Maier et al. 2004). The liquid film accounts for a considerable fraction of the total mass transfer area, and thus the material properties of the inner flask wall affect the oxygen transfer capacity.
Hydrophilic flask materials such as glass contribute to higher OTR\text{max} than hydrophobic plastics with reduced film formation (Maier & Büchs 2001). Interestingly, due to the pivotal contribution of the liquid film to the mass transfer area, higher viscosity of the cultivation broth may actually promote higher OTR\text{max}. At low viscosities, the liquid film is thin and has oxygen concentration larger than 0 mmol \text{l}^{-1} even if oxygen concentration in the bulk liquid is zero, while higher viscosity contributes to increased liquid film thickness on the flask wall and consequently larger driving concentration gradient at the gas-liquid interface of the film (Giese et al. 2014). This counterintuitive increase in OTR\text{max} with increasing viscosity in shake flasks was reported by Giese et al. for moderate viscosities up to 10 mPa·s, after which the maximum driving gradient is reached and further increase in viscosity beyond 20 mPa·s will contribute to gradually decreasing OTR\text{max} due to decreasing oxygen solubility and diffusivity. These recent findings have interesting implications for cultivation of non-filamentous organisms such as \textit{E. coli}, which do not reach viscosities above 10 mPa·s until very high cell densities (dry cell weight 60 g \text{l}^{-1} or more) (Ma et al. 2010).

\( k_{La} \) is affected by the liquid surface tension, which in turn is reduced by biological surfactants present in complex culture media and produced by the cultured organism at undetermined concentrations (Seletzky et al. 2007). Decrease in surface tension results in reduced diffusivity at the gas-liquid interface and consequently reduced value of \( k_{L} \) (Jamnongwong et al. 2010). On the other hand, reduced surface tension contributes to increased mass transfer area in bubble-aerated systems as a result of reduced bubble size and increased gas hold-up (Toye et al. 2009), which may be relevant in baffled flasks provided that the shaking frequency is sufficient to introduce air bubbles into the liquid. While this phenomenon has no relevance in non-baffled flasks where no bubbles are formed and oxygen transfer takes place via surface aeration only, lower surface tension might influence the mass transfer area via effects on the liquid film formation on the flask wall (Doig et al. 2005).

\textit{Flask closure}

Besides mass transfer resistance at the gas-liquid interface, oxygen transfer capacity of a shake flask can be influenced by the mass transfer resistance of the closure used for keeping the flask sterile (Gupta & Rao 2003, Mrotzek et al. 2001). Under typical cultivation conditions the resistance of the closure (1/k\text{c,a}) is small compared to the resistance of the gas-liquid interface (1/k\text{L,a}) (Mrotzek et al. 2001).
2001), but under conditions providing high $k_{L}a$ (e.g. baffled flasks) the closure resistance can become a limiting factor in the oxygen transfer capacity of the flask (Mrotzek et al. 2001, Nikakhtari & Hill 2006). Naturally, also in case of completely non-permeable closure materials such as aluminium foil the closure accounts for the majority of oxygen transfer resistance, and thus the common practice of aluminium foil sealing is not viable regarding oxygen transfer and culture performance (Orozco-Sanchez et al. 2011). Cotton plugs are also widely used as the sterile barrier even if it has been long known that such plugs can limit the overall mass transfer significantly, leading to reduced partial pressure of oxygen in the flask headspace as well as accumulation of carbon dioxide (Schultz 1964). Moreover, the resistance of the plug varies depending on how densely it is packed, and thus hand-made cotton plugs can impair the reproducibility of oxygen transfer conditions. Use of other types of closures can improve the overall oxygen transfer capacity, especially in baffled flasks (Gupta & Rao 2003). However, the geometry of the flask neck (narrow/wide) may have greater impact on the oxygen transfer into the flask headspace than closure material and density (Mrotzek et al. 2001). In principle, gas transfer efficiency at the neck increases with increasing turbulence in the flask. Turbulence increases with decreasing fill volume and increasing shaking speed, but since $k_{L}a$ in a flask with sterile closure reaches a maximum at relatively low turbulence the influence of fill volume and shaking speed on gas transfer is practically negligible under normal cultivation conditions (Nikakhtari & Hill 2006).

### 2.1.2 Oxygen transfer in multiwell culture plates

Similar to shake flask cultures, cultures grown in multiwell plates are typically agitated by orbital shaking. Agitation with a small diameter (1–6 mm) is often used. The reported OTR$_{\text{max}}$ values for microwell and deep well plates under optimal shaking conditions are generally in the same range with Erlenmeyer flasks, approximately 20–50 mmol l$^{-1}$ h$^{-1}$ (Duetz et al. 2000, Duetz & Witholt 2001, Duetz & Witholt 2004, Hermann et al. 2003), and the upper limit is set by liquid spilling from the wells at too high shaking rates. However, very high OTR$_{\text{max}}$ (280 mmol l$^{-1}$ h$^{-1}$) has been reported for a 48 microwell plate at high shaking frequency and 3 mm diameter (Kensy et al. 2005).
Special features of microwell plates

Due to the small liquid volume, capillary and adhesive forces in microwell plates are relatively high in comparison to the centrifugal force provided by the shaking. The smaller the well the higher the relative impact of surface tension (Doig et al. 2005), and consequently OTR\textsubscript{max} in smaller wells are lower compared to larger wells with the same filling height even if the theoretical surface to volume ratio increases with the decreasing volume (Duetz & Witholt 2004). Another characteristic specific to small microwell plates is the biphasic dependency of OTR\textsubscript{max} on shaking speed (Hermann et al. 2003, Kensy et al. 2005). This is accounted to the interfacial surface tension, which prevents liquid movement and any increase in mass transfer at shaking frequencies below a critical value where centrifugal force provided by shaking exceeds the surface tension. Thus OTR\textsubscript{max} remains at a level similar to non-agitated conditions until the critical shaking frequency is achieved, beyond which OTR\textsubscript{max} increases exponentially with shaking frequency. 96 round-well microwell plates are particularly affected by this phenomenon (Hermann et al. 2003), but similar resistance to liquid movement at low shaking frequencies can also be observed in square wells (Duetz & Witholt 2004, Hermann et al. 2003).

Shaking conditions and well geometry

Similar to shake flasks, shaking diameter is a key parameter regarding oxygen transfer in round- and square-well plates. Duetz & Witholt (2001) reported 3-fold higher OTR\textsubscript{max} with 50 mm diameter in comparison to 25 mm. They also observed that the liquid flow in a 2 ml square well at 25 mm was characterized by out-of-phase behavior and poor vertical mixing even at shaking frequency of 300 rpm. By contrast, 50 mm diameter provided turbulent in-phase flow, larger surface area and efficient vertical mixing. Higher shaking diameter also lowers the critical shaking frequency where OTR\textsubscript{max} increases above the basal non-agitated level; for example, approximate critical speeds for a round-well 96 well plate at 50, 25 and 3 mm are 200, 250 and 750 rpm, respectively (Hermann et al. 2003). At lowest shaking diameters (1 mm) it may not be possible to achieve in-phase conditions and OTR\textsubscript{max} different from non-agitated conditions at any feasible shaking frequency (Kensy et al. 2005). However, when out-of-phase conditions can be avoided, small shaking diameter can in some cases provide higher OTR\textsubscript{max}. This was demonstrated by Kensy et al. (2005), who applied
maximum shaking speeds without spill-out at 3 mm and 25 mm diameters. While
the liquid heights were identical, the centrifugal force at 25 mm drove the liquid
to one side of the wells leaving the other half empty. At 3 mm, by contrast, the
liquid formed a circle and covered the walls of the round well completely. Thus
the liquid layer was thinner and mass transfer area larger at 3 mm, resulting in 3-
fold higher OTR$_{\text{max}}$ compared to 25 mm.

The corners in square wells act as baffles to increase turbulence (Duetz et al.
2000), and OTR$_{\text{max}}$ in a square well is approximately two-fold compared to a
corresponding round well under similar shaking conditions (Duetz & Witholt
2004, Hermann et al. 2003). Besides the standard round- and square-shaped
wells, also various other well geometries have been studied (Funke et al. 2009).
Cross-sectional shapes different from round circle introduce varying degrees of
baffling that changes the flow of the rotating liquid in the well. Funke et al.
(2009) reported OTR$_{\text{max}}$ in the range of 100 mmol l$^{-1}$ h$^{-1}$ for baffled wells at 3 mm
shaking diameter and 1000 rpm frequency, which was approximately double as
high as OTR$_{\text{max}}$ in round wells. It was also observed that due to emergence of out-
of-phase conditions, most pronounced baffles lost their benefit at high shaking
speeds and provided lower OTR$_{\text{max}}$ than more moderate baffling.

Other factors

Surface properties of both the well material and the liquid medium also affect the
hydrodynamic flow in multiwell plates. While the standard plate material is
hydrophobic polystyrene, hydrophilic wall surface provides significantly higher
OTR$_{\text{max}}$ at the same shaking conditions by increasing the wetting of the walls
(Hermann et al. 2003). However, in practice the maximum achievable OTR$_{\text{max}}$
cannot be notably increased by use of hydrophilic materials, since the maximum
shaking frequency that can be used without spilling of the liquid from the well is
lower in a hydrophilic plate. As noted by Hermann et al., the main benefit of
using hydrophilic wells is the lowering of critical shaking frequency required to
exceed the surface tension. Also the gas-liquid surface tension influences the wall
wetting, and thus the mass transfer area at given conditions is larger for liquids
with lower surface tension, such as surfactant-containing fermentation broths
(Doig et al. 2005). The wall wetting in a system of certain liquid medium and
well material can be quantitatively described in terms of the parameter wetting
tension introduced by Doig et al. (2005).
Evaporation and volume loss during cultivation is a significant issue with the small culture volumes employed in multiwell plates, and thus the sealing of the plate should not only provide high oxygen transfer but limit the loss of water. According to Zimmermann et al. (2003), commercial adhesive plate seals made of rayon fibers or different plastics allow for OTR\textsubscript{max} comparable to unsealed plates, but as a drawback the water retention is low. As an alternative seal the authors studied polyurethane wound dressings, which typically provided high water retention but considerably reduced oxygen transport, and thus no optimal solution for 96 microwell plate sealing was found in the study. A sandwich silicone spongy cover described by Duetz & Witholt (2001) was reported to fulfill the criteria for both high water retention and sufficient oxygen transfer to the well headspace, though the oxygen permeability of this cover may become a limiting factor at oxygen uptake rates exceeding 20 mmol l\textsuperscript{-1} h\textsuperscript{-1}.

Besides modification of the parameters discussed above, suggested approaches to improve oxygen transfer in multiwell plates include the use of liquid perfluorochemical (PFC) as an auxiliary oxygen carrier (Pilarek et al. 2011). The liquid PFC saturated with oxygen-enriched air settles as a separate phase below the aqueous phase constituted by the culture medium, and effectively releases oxygen into the culture from below. By use of oxygen-enriched PFC in 24 deep well plates, 40% increase in cell density was obtained in recombinant \textit{E. coli} cultures (Pilarek et al. 2011).

### 2.2 Determination of OTR and $k_a$ in shaking bioreactors

Various methods have been described in the literature for determination of OTR and $k_a$ in shaking bioreactors (Suresh et al. 2009). These include the conventional sulfite oxidation and dynamic methods, intermittent online oxygen and carbon dioxide measurement method (RAMOS) and enzymatic methods.

#### 2.2.1 Sulfite oxidation method

The sulfite oxidation method is based on the use of sodium sulfite as a chemical oxygen consumer in the liquid phase. The oxidation of sulfite to sulfate is catalyzed by cobalt or copper salt and follows the stoichiometric equation:

$$SO_3^{2-} + 0.5 O_2 \xrightarrow{catalyst} SO_4^{2-}$$  \hspace{1cm} (3)
If oxygen concentration in the bulk liquid can be considered negligible (i.e. the reaction rate is significantly higher than mass transfer rate), calculation of OTR\textsubscript{max} from the measured reaction time required for complete oxidation of a known sulfite concentration is simple (Hermann et al. 2003, Hermann et al. 2001). However, the reaction parameters, namely catalyst concentration, pH and temperature, have to be carefully adjusted to keep the reaction within non-accelerated reaction regime (Suresh et al. 2009). Traditionally OTR was determined by iodometric titration of the sulfite concentration at multiple time points (Corman et al. 1957), but with modern equipment less laborious methods are available. The end of oxidation can be detected either as a sharp increase of DOT, or a sharp decrease of pH. The pH drop can be visualized by bromothymol blue as a color indicator and recorded by a CCD camera, provided that the vessel is transparent (Hermann et al. 2001, Kensy et al. 2005). An alternative two-compartment method introduced by Glazyrina et al. (2011) enables non-invasive measurement of OTR\textsubscript{max} in any type of shake flask without the need for any instrumentation other than a simple electrochemical oxygen electrode.

2.2.2 Dynamic methods

In the dynamic gassing-out method, DOT in the liquid medium is first reduced to zero by bubbling with nitrogen or addition of an oxygen-consuming chemical, and \( k_{La} \) is determined by direct measurement of DOT increase after aeration is started (Van Suijdam et al. 1978). Alternatively \( k_{La} \) can be determined by a dynamic method during cultivation, whereby \( k_{La} \) is calculated from the increase of DOT following a brief interruption of aeration (Bandyopadhyay et al. 1967).

Application of dynamic methods necessitates the use of online DOT probes, which introduce a possible source of considerable error if the probe response time is not adequately accounted for (Tribe et al. 1995). Standard Clark electrodes can be used for DOT monitoring in shake flasks, especially with wireless data recording (Vasala et al. 2006), but since the electrodes act as auxiliary baffles they do not accurately reflect the OTR\textsubscript{max} under normal operating conditions (Hansen et al. 2011, Tunac 1989). Better suited for the purpose are non-invasive, thin optical sensor patches containing oxygen-sensitive luminescent dyes or fluorophores, which are applicable to dynamic \( k_{La} \) measurement in both shake flasks (Gupta & Rao 2003, Schiefelbein et al. 2013, Tolosa et al. 2002, Wittmann et al. 2003) and microwell plates (John et al. 2003). However, these sensor spots may have the drawback of not indicating low DOT levels correctly, since the
sensor is not in constant contact with the rotating bulk liquid unless the fill volume is considerably high (Hansen et al. 2011).

### 2.2.3 RAMOS

Oxygen transfer rates in shake flasks can be measured online during cultivation by an automated system first introduced by Anderlei & Büchs (2001). This system consists of parallel measuring flasks, in which a cycle of rinsing phase and measuring phase is continuously repeated. In the rinsing phase, a gas flow of known composition is supplied into the flasks. This is followed by the measuring phase, whereby gas inlet and outlet are closed and the OTR determined from the decrease of oxygen partial pressure in the flask headspace. This enables the measurement of $OTR_{\text{max}}$ under different operating conditions, as well as monitoring of the oxygen consumption dynamics during cultivation and thus provides information on the physiological state of the culture. The OTR measurement was later complemented with determination of CO$_2$ transfer rate and respiratory quotient to enable more detailed analysis of the culture metabolism (Anderlei et al. 2004) This measuring system was termed RAMOS (respiratory activity analysis system).

### 2.2.4 Enzyme-based methods

Oxygen transfer rates can be also be determined from the rate of rapid enzyme-catalyzed reactions that consume oxygen. These methods are especially suitable to small-scale vessels. In the catechol oxidation method (Ortiz-Ochoa et al. 2005), the enzyme catechol-2,3-dioxygenase catalyses the oxidation of catechol into a bright yellow end product whose formation rate can be quantified by measurement at 425 nm. The rapid consumption of oxygen by the reaction keeps DOT at zero during the measurement period, and thus the linear rate of product formation is proportional to $OTR_{\text{max}}$. Formation of a colored end product as a measure of OTR is also utilized in the glucose oxidase/horseradish peroxidase method applied by Duetz & Witholt (2004) for characterization of oxygen transfer in multiwell plates.
2.3 Oxygen in *E. coli* cultivations

*E. coli* is a facultative anaerobic bacterium, and thus oxygen availability is a key parameter affecting its metabolism and physiology (Smith & Neihardt 1983). Oxygen availability alters the expression of hundreds of genes connected to metabolic, biosynthetic and regulatory functions, transport and cell structure (Salmon *et al.* 2003). Consequently, the productivity of target recombinant proteins is also influenced by oxygen availability, and variations in the time profile of dissolved oxygen are typically the reason behind altered process performance upon scale-up (Konz *et al.* 1998). Moreover, the different metabolic and physiological modes of the cell are not simply restricted to full aerobiosis and full anaerobiosis, but the gene expression profile varies gradually under different levels of intermediate dissolved oxygen concentrations (Rolfe *et al.* 2011).

2.3.1 Metabolic consequences of anoxic conditions

When neither oxygen nor alternative terminal electron acceptors are available, *E. coli* switches from respiration to fermentative metabolism (mixed-acid fermentation) to maintain the regeneration of NAD$^+$ and synthesis of ATP via substrate-level phosphorylation (reviewed by Böck & Sawers (1996)). Acetate, lactate, ethanol, succinate, formate and H$_2$ are secreted as by-products of the mixed-acid fermentation. In order to generate ATP, pyruvate is first cleaved to acetyl-CoA and formate in a reaction catalyzed by the anaerobic enzyme pyruvate formate-lyase (PFL), followed by conversion of acetyl-CoA to acetate and concomitant ATP formation. NAD$^+$ is regenerated by reduction of pyruvate to lactate, acetyl-CoA to ethanol and phosphoenolpyruvate to succinate. By modification of the relative amounts of the fermentation products *E. coli* can adjust its metabolism to fermentative growth on various different substrates.

As the ATP yield of anaerobic metabolism is low compared to aerobic respiration and carbon is diverted to the fermentative byproducts, culture growth rate is significantly reduced under oxygen-limited conditions (Smith & Neihardt 1983). Moreover, in the absence of external pH control the accumulation of acetate, lactate, formate and succinate leads to culture acidification and consequent growth inhibition, and the fermentation products also have toxic and growth-inhibitory effects independent of pH (Luli & Strohl 1990, Warnecke & Gill 2005). Acetate and lactate produced during an anaerobic period can be assimilated and used as energy substrates upon recurrence of aerobic conditions.
(Xu et al. 1999), but such oscillations between aerobic and anaerobic metabolism also contribute to reduced biomass and recombinant product yields (Lara et al. 2006, Sandoval-Basurto et al. 2005).

The primary carbon source applied in E. coli cultivations is usually glucose or glycerol. The traditional conception is that unlike glucose and other carbohydrates glycerol is not a fermentable substrate in E. coli. However, in the recent years it has been suggested that E. coli is, in fact, capable of anaerobic glycerol fermentation, and that this fermentation is linked to the availability of CO₂ produced in the oxidation of formate by formate hydrogen lyase complex (FHL) (Dharmadi et al. 2006). Since the functional FHL complex requires the trace elements nickel, molybdenum and selenium as co-factors (Sawers 1994), supplementation with trace element solutions containing these minerals might possibly influence anaerobic metabolism in glycerol-containing media. It is noteworthy that the commonly used protein expression host strain E. coli BL21(DE3) appears to be deficient of FHL activity (Pinske et al. 2011), and should therefore not be able to ferment glycerol. In case of E. coli K-12, supplementation of glucose-based media with nickel, molybdenum and selenium has been demonstrated to prevent formate accumulation under oxygen limitation (Soini et al. 2008). However, the potential benefit of trace metal supplementation and reduced formate accumulation may be attenuated under conditions of permanent oxygen limitation due to concomitant increase of other fermentation metabolites and loss of carbon as CO₂ via FHL activity (Soini et al. 2008).

Under anaerobic conditions, the activity of TCA cycle is switched off and the pathway shifts from cyclical to branched as a result of the activation of ArcA and FNR transcriptional regulator proteins (reviewed by Konz et al. (1998)). Thus the formation of the TCA-derived amino acid precursors 2-oxoglutarate and oxaloacetate decreases, and the biosynthetic capacity of the cell is reduced. Together with the elevated need of amino acids for recombinant protein synthesis, often in vastly different proportions of the individual amino acids compared to native E. coli proteins, this can lead to depletion of aminoacylated tRNA supply in minimal media where preformed amino acids are not available. The lack of charged tRNA induces the stringent response and concomitant synthesis of proteases (Rozkov & Enfors 2004). As a result, recombinant protein yield under anaerobic conditions can be reduced due to not only reduced synthesis but also increased proteolytic degradation (Konz et al. 1998).
2.3.2 Oxygen availability and recombinant protein expression

As discussed above, anaerobic conditions can adversely influence recombinant protein expression through redirection of carbon fluxes to byproduct formation, growth-inhibitory effects of fermentative metabolites, depletion of amino acid precursors and increased product degradation. However, it has been suggested that also high oxygen levels could have a negative impact on certain recombinant proteins and their expression as a result of the oxidative damage caused by reactive oxygen species generated in the cell (Konz et al. 1998). High oxygen levels can lead to unwanted \textit{in vivo} oxidation of susceptible recombinant proteins, such as conversion of the thioether of exposed methionines to methionine sulfoxide. An example of this phenomenon was reported by Berti et al. (1997), who observed excessive oxidation of active site methionine in recombinant cystatin C when expressed in either \textit{E. coli} cytoplasm or periplasm in an aerobic culture. The methionine oxidation was effectively eliminated by expression under anaerobic conditions. In general it appears that oxygen limitation is beneficial for expression of some proteins but detrimental to others (Qoronfleh 1999), though the mechanisms of these effects often remain elusive. In some cases high shear rate may be the actual reason for reduced expression at high aeration rates (O'Sullivan et al. 2001).

Oxygen availability can also influence the stability of the recombinant plasmid, though the literature on this effect seems to be scarce and inconclusive. Hopkins et al. (1987) reported rapid plasmid loss upon exposure of \textit{E. coli} to a drop in DOT down to 5%. By contrast, Tomazetto et al. (2007) reported increased plasmid stability at reduced aeration rates. Also in the study of Li et al. (1992) anaerobic conditions contributed to approximately 3–12 times higher plasmid content compared to aerobic cultivation. Despite this, highest volumetric activities of recombinant protein were obtained under aerobic conditions due to increased cell mass concentration.

2.4 Culture media for protein expression

2.4.1 Complex media

The most common culture media used for recombinant protein expression in small-scale \textit{E. coli} cultures are complex media based on yeast extract and tryptic casein digest (tryptone) or other protein digests. These nutrient sources provide a
complex mixture of amino acids, peptides, carbohydrates, vitamins, trace minerals and nucleotides, which is in principle sufficient to support *E. coli* growth without further supplementation. Commercial yeast extract powders commonly contain traces of lactose at levels sufficient to induce substantial expression of recombinant genes controlled by lac-derived promoters (Nair *et al.* 2009). Thus it may be advisable to include small amount of glucose in the media to repress premature induction.

Perhaps the most widely used medium for small-scale protein expression to date is the simple Lysogeny Broth (LB; also called Luria-Bertani broth) introduced over 60 years ago in the lysogeny study of G. Bertani (1951). While the original recipe included 0.1% glucose, this is typically excluded from the medium. Thus the primary carbon source in LB medium is constituted by catabolizable amino acids, while the concentration of fermentable sugar equivalents provided by 0.5% yeast extract is estimated to be less than 100 µM (Sezonov *et al.* 2007). As a result, growth in LB medium stops around OD$_{600}$ of 7 when the catabolizable amino acids have been exhausted (Sezonov *et al.* 2007), and the amino acid catabolism leads to alkalinization of the medium up to pH 9 due to excretion of excess ammonium (Losen *et al.* 2004, Sezonov *et al.* 2007). On the other hand, attempts to increase cell density by supplementation with glucose can lead to overflow metabolism and growth arrest at even lower cell densities due to acetate accumulation and excessive pH decrease, whereas glycerol supplementation may cause severe oxygen limitation and concomitant acidification (Losen *et al.* 2004). While the low final cell density and physiologically unfavorable pH development are apparent hindrances for recombinant protein production, LB medium still remains the standard expression medium in many laboratories.

Enhancements of the LB formulation have led to various complex media with higher tryptone and yeast extract content, buffering and supplemental carbon sources. A commonly used highly rich medium is Terrific Broth (Tartoff & Hobbs 1987), containing 1.2% tryptone, 2.4% yeast extract, phosphate buffering and glycerol as carbon source. The higher content of utilizable carbon sources can support growth to biomass yields up to five times higher than LB, but also cause more severe oxygen limitation as the oxygen demand is increased to levels that cannot be easily satisfied in shake flasks (Losen *et al.* 2004). Regarding volumetric recombinant protein yields, Terrific Broth is often superior to LB as a result of the higher cell density (Losen *et al.* 2004) and/or increased specific productivity per biomass (Manderson *et al.* 2013). The phosphate buffering
attenuates pH drifts during cultivation (Manderson et al. 2013), though curiously it appears that high phosphate concentration promotes kanamycin resistance in rich media (Studier 2005). This is likely due to the increased osmolarity of the medium, which is known to reduce the susceptibility of gram-negative bacteria to aminoglycosides (Rodriguez et al. 1990). Proper maintenance of selective pressure for kanamycin thus requires elevated concentrations of the antibiotic in phosphate-buffered complex media. Moreover, the buffering capacity is limited and pH in Terrific Broth often increases above 8 when the culture is grown to high density (Studier 2005).

### 2.4.2 Defined and minimal media

As opposed to complex media, the composition of defined media is fully specified regarding the concentrations of each individual substance. In minimal media nitrogen is provided in inorganic form, typically ammonium salts, and amino acids must therefore be synthesized by the cells. In other cases individual amino acids may be included in predetermined concentrations. Glucose or glycerol is added as the carbon source, and additionally the media contain mineral salts and trace elements to provide the necessary nutrients for *E. coli* growth. Compared to complex formulations, growth in minimal media is 2–3 times slower (Studier 2005), and the metabolism is fundamentally different due to the increased need for de novo synthesis of building blocks such as amino acids, nucleotides and vitamins (Tao et al. 1999). Genes associated with the translation process are expressed at lower levels (Tao et al. 1999), and thus the protein synthesis rate is also reduced. Due to the slow growth and protein synthesis, defined/minimal media are usually not the first choice for routine shake flask scale expression of unlabeled proteins. However, these media are widely used in shaken cultures for production of proteins labeled with $^{15}$N and $^{13}$C for nuclear magnetic resonance studies (Jansson et al. 1996, Li et al. 2011b, Zhao et al. 2004), or with selenomethionine for X-ray crystallography (Li et al. 2011b, Stols et al. 2004).

As standard shake flask cultures are operated without pH control, a major drawback of defined media with glucose as a sole carbon source is the production of overflow acetate (Wolfe 2005) and concomitant culture acidification. The extent of acetate accumulation can be reduced by using glycerol as the carbon source (Andersen & von Meyenburg 1980, Hansen & Eriksen 2007). Compared to glucose, glycerol as a carbon source in defined medium has also been reported
to provide improved recombinant product activity in pH-controlled batch cultures (Hansen & Eriksen 2007).

Sivashanmugam et al. (2009) described an enhanced shake flask cultivation method for isotope labeling, which combines initial pre-induction growth in complex medium to OD$_{600}$ of 3–7 and subsequent switch to a minimal medium where protein expression is induced after a short adaptation period. By this protocol, the authors reported final cell densities in the range of OD$_{600}$ 10–20, and 9 to 85-fold higher volumetric yields of unlabeled and triple-labeled recombinant proteins compared to the conventional expression protocol in minimal medium. The minimal medium used in this improved protocol employs glucose as the carbon source, and peculiarly initial pH is adjusted to very high level (8.0–8.2) to account for the pH decrease during cultivation.

2.4.3 Autoinduction media

The principle of autoinducing culture media is based on the metabolic regulation of lac operon (reviewed by Görke & Stülke (2008)) and the resulting diauxic growth of E. coli in a mixture of glucose and lactose. Transcription of the lac operon, encoding the three essential genes lacZ, lacY and lacA for lactose uptake and metabolism, is subject to negative regulation by binding of the repressor protein LacI to lacO operator sites. The operon is induced when allolactose, derived from lactose by the activity of intracellular β-galactosidase (product of lacZ), binds and inactivates the LacI repressor. Thus lac operon is induced as a result of lactose uptake into the cells. In the presence of glucose, induction of lac operon by allolactose is inhibited by the mechanism of inducer exclusion. Uptake of glucose into the cell results in dephosphorylation of the glucose-specific EIIAGlc protein of the PEP-dependent phosphotransferase system, and consequently the function of lactose permease (lacY) in lactose uptake is prevented by binding of dephosphorylated EIIAGlc to the lactose permease. The complex of cyclic AMP and its receptor protein (cAMP-CRP), the global mediator of catabolite repression, is also needed for activation of lac operon, but in light of the most recent findings the cAMP-CRP binding to lac promoter is not as significant factor in catabolite repression of the lac operon as previously thought (Görke & Stülke 2008).

In autoinducing media, glucose is initially utilized as the primary carbon source, which inhibits the expression of recombinant target protein from lac-
controlled promoter systems as described above. When glucose is exhausted from the medium, inducer exclusion is relieved and lac operon derepressed resulting in expression of the target protein. A medium formulation based on this principle was first introduced by Studier (2005). Cell and protein yields from autoinduction are improved by inclusion of a supporting carbon source that is co-utilized with lactose during protein expression (Kunze et al. 2012, Studier 2005). The original Studier formulation included glycerol in this role, and since then glycerol has been the standard supporting carbon source in virtually all published autoinduction media for lactose-inducible promoters. Autoinducing media of both rich (Kunze et al. 2012, Studier 2005) and minimal/defined (Li et al. 2011a, Sreenath et al. 2005, Studier 2005, Tyler et al. 2005) composition have been designed and successfully used for protein production. The minimal autoinduction formulations are applicable to selenomethionine (Sreenath et al. 2005, Studier 2005) and isotope labeling (Sivashanmugam et al. 2009, Studier 2005, Tyler et al. 2005). Corresponding autoinduction media can also be used for promoter systems inducible by other sugars, such as arabinose (Studier 2005), whose utilization is subject to catabolite repression by glucose.

An apparent advantage of the autoinduction principle is the elimination of the manual induction step and need to monitor the culture for an appropriate moment of induction. An additional benefit for high-throughput applications is the automatic induction of all cultures at a similar phase of growth despite variation in growth characteristics between different clones. Lactose autoinduction media have also been reported to provide higher cell densities and target protein yields compared to manual IPTG induction in commonly used media (Gordon et al. 2008, Li et al. 2011a, Studier 2005). On the other hand, the applicability of autoinduction is more limited, as it cannot be used for host strains with deletions in the lac operon genes. Another drawback may be the sensitivity of expression to culture aeration level. High aeration rates have been reported to contribute to reduced expression yields as a result of shift in carbon source preference from lactose to glycerol, and the phenomenon appears to be exacerbated in clones with higher LacI repressor dosage (Blommel et al. 2007).
2.5 Fed-batch cultivation

2.5.1 The fed-batch principle

While typical small-scale protein production in a shaking bioreactor is operated as a batch culture, the usual mode of operation in medium- to large-scale stirred bioreactors is the fed-batch process. The most commonly applied principle is glucose-limited fed-batch, in which feeding of a highly concentrated glucose solution is started after an initial batch phase. The feed is supplied at a rate that limits the growth and oxygen consumption rate of the culture. Control of the growth rate by an appropriate feeding strategy enables minimization of acetate formation via overflow metabolism (Lee 1996, Riesenberg et al. 1991, Shiloach & Fass 2005), and in well-aerated bioreactors very high cell densities (up to more than 100 g l⁻¹ dry weight for *E. coli*) can be attained by the fed-batch technique (Korz et al. 1995). These features make fed-batch the preferred process mode for industrial-scale production of recombinant proteins, whereby maximization of volumetric productivity is a key objective.

2.5.2 Fed-batch in shaken cultures

Efficient bioprocess development and scale-up to the production scale would require fed-batch cultivation mode to be applied already in the very first steps of development accomplished in shaken cultures (Neubauer et al. 2013, Šiurkus et al. 2010). Cultivation in fed-batch mode could also enhance the performance of the small-scale cultures themselves and thus reduce the culture volume needed to produce sufficient amounts of protein for e.g. biophysical characterization. The first report on a fed-batch cultivation in shake flasks was issued by Weuster-Botz et al. (2001), and described a system of 16 parallel flasks fed intermittently by a syringe pump and valve construction. The intermittent substrate feeding and base addition for pH maintenance were controlled by a process computer. In the recent years, a few different approaches have been described that enable fed-batch cultivation in shaken cultures with continuous rather than intermittent feeding, and do not require special instrumentation or computer control. The first such method published was based on diffusion-controlled release of glucose from silicone elastomer discs containing glucose crystals (Jeude et al. 2006). By application of this technique, large increases were achieved in both biomass and
green fluorescent protein yields in *Hansenula polymorpha* cultures. Another diffusion-based fed-batch system is constituted by a feed reservoir system containing concentrated feed solution, and a diffusion tip through which the feed is slowly released into the shake flask culture (Bähr *et al.* 2012). The feeding rate can in this case be adjusted by modifying the concentration of feed solution. Panula-Perälä *et al.* (2008) introduced the first biocatalyst-controlled fed-batch technique ("EnBase") comprising a solid starch gel attached to the bottom of a cultivation vessel, diffusive release of soluble starch into the liquid culture medium, and consequent degradation of the soluble starch into glucose monomers by a glucoamylase biocatalyst in the liquid medium. The glucose feed rate can thereby be controlled by adjustment of the biocatalyst concentration added into the medium. Also in this case, increase in biomass and recombinant protein yield was obtained in comparison to a complex batch medium. The enzyme-based method with starch gel was also successfully applied for microscale process optimization and consequent scale-up to stirred fed-batch bioreactor (Šiurkus *et al.* 2010).
3 Materials and methods

A summary of the materials and methods used in this study is presented below. Detailed descriptions are given in the original articles.

3.1 Host strains and expression vectors

The host strains used in this study include *E. coli* BL21(DE3) (Studier & Moffatt 1986), *E. coli* BL21(DE3)pLysS (Studier 1991), *E. coli* K-12 RB791 and *E. coli* K-12 RV308. Expression vectors pQE30 and pKK233 were used in the strains RB791 and RV308, respectively, while pPAL7 and pKK233 were used in BL21(DE3) and pET23b(+) and pET101/D-TOPO in BL21(DE3)plysS. Selection pressure for all vectors was provided by 100 µg ml\(^{-1}\) ampicillin.

3.2 Media

3.2.1 Enzymatic fed-batch

The enzymatic fed-batch media used in this study comprise three different commercial media (BioSilta Oy, Finland) based on the EnBase substrate release technology (Panula-Perälä et al. 2008): EnBase Flo (Article I), EnPresso (Articles II and III) and EnPresso B (Article IV and online DOT measurements in Article III). The composition of all three media is based on mineral salts, thiamine and trace elements as specified in Article I. Additionally, the media contained 3.6 g l\(^{-1}\) of yeast extract and peptone mixture, and a soluble polysaccharide as the glucose reservoir. The medium Booster (supplying 18–25 g l\(^{-1}\) yeast extract and peptone mixture) was added after overnight cultivation at the time of induction. Glucose was released by the specific biocatalyst, which was added in the beginning of cultivation and as an additional dose at the time of induction to provide increased rate of glucose feed at the higher biomass concentrations. Each of the three fed-batch media contained a different type of soluble polysaccharide, and thus there were also some differences in the applicable biocatalyst concentrations. 

For autoinduction, the basic fed-batch medium was supplemented with glucose and lactose as described in Article IV. For comparison, some cultivations in Articles III and IV were performed without the Booster.
3.2.2 Reference media

Lysogeny Broth (LB; without glucose) was prepared from LB Broth mixture (Sigma-Aldrich), composition of which follows the original recipe (Bertani 1951). Terrific Broth (TB; including 4 ml l⁻¹ glycerol) was prepared according to the Tartoff & Hobbs (1987) recipe. Composition of Super Broth medium with MOPS buffering is described in Article III. ZYM-5052 autoinduction medium was prepared according to Studier (2005) using tryptone instead of N-Z-amine. Trace elements solution used in ZYM-5052 was the same as in the fed-batch media. Minimal medium (Article I) contained the same mineral salt base, trace elements and thiamine as the fed-batch media, and was additionally supplemented with 10 g l⁻¹ glucose.

3.3 Cultivation conditions

Cultivations in 24 deep well plates (24dwp) were performed in square-shaped plastic wells with V-shaped (Article I) or U-shaped (Articles III and IV) bottom. Plates were covered with adhesive air-permeable plate seals (Thomson Instrument) and filled with 3 ml of medium unless otherwise indicated. Shake flask cultivations were performed in 500 ml and 1000 ml Erlenmeyer flasks with 10% fill volume, and in 250 ml and 500 ml Ultra Yield (UY) flasks (Thomson Instrument) with 20% fill volume. All shake flasks were closed with adhesive air-permeable seals (AirOtop; Thomson Instrument).

All cultivations in this study were performed at 30°C in orbital shakers with either 50 mm or 25 mm shaking diameter. Shaking frequencies were mostly 200 rpm with 50 mm diameter and 250 rpm with 25 mm diameter. In Article III comparison was made between 150 and 250 rpm (25 mm) in UY flasks.

3.4 Cell density measurements

The primary method to determine cell density was optical density measurement at 600 nm in cuvettes, or at 490 nm or 590 nm in microwell plates with appropriate dilution in 0.9% NaCl. Correlations were determined to convert OD₆₀₀ and OD₅₉₀ measured with different plate readers to a corresponding OD₆₀₀ in cuvette. All OD₆₀₀ data are presented as the cuvette values. Correlations were also determined to relate OD₆₀₀ to cell dry weight (CDW) of E. coli RB791. For the spectrophotometer used in Article I, CDW (g l⁻¹) = 0.30 · OD₆₀₀, and for the
spectrophotometer used in Articles II–IV, CDW (g l\(^{-1}\)) = 0.27 \cdot OD_{600}. These correlations are most accurate at cell densities OD_{600} \geq 15.

3.5 Cell lysis

Prior to lysis, cell pellets were stored at -20°C. Lysis was accomplished either by 4 \times 30 \text{s} sonication of suspended cells on ice (Article I), or by suspension of the pellet into BugBuster buffer (Novagen) and addition of Lysonase Bioprocessing Reagent (Novagen) containing recombinant lysozyme and nuclease (Articles II–IV).

3.6 SDS-PAGE analysis of expressed protein

Soluble protein fraction for SDS-PAGE was obtained by removal of insoluble material from the cell lysate by centrifugation. The lysate samples were mixed with SDS or LDS sample buffer and β-mercaptoethanol, denatured by heating at 95°C for 5 min and loaded on reducing SDS-PAGE gels. Self-made 12% polycaryaamide gels were used in Articles I and II, while commercial precast 12% gels (Thermo Fisher Scientific) were used in Article IV. Gels were stained with Coomassie Brilliant Blue G-250, and destained in a solution with 40% ethanol and 10% glacial acetic acid. Protein bands were quantified by scanning the gels as grayscale images and applying ImageJ densitometry software (US National Institutes of Health, Bethesda, Maryland, USA) for determination of the band intensities. Application of the ImageJ software for quantification of SDS-PAGE protein bands has been previously described by Damasceno et al. (2004).

3.7 ADH activity assay

Activity of alcohol dehydrogenase (ADH) in cell lysates was determined by a colorimetric assay based on reduction of ethyl-4-chloroacetoacetate by ADH, concomitant oxidation of NADPH to NADP\(^+\), and kinetic measurement of NADPH concentration decrease at 340 nm (Viitanen et al. 2003). ADH activity was calculated from the kinetic data as presented in Article II.
3.8 Fab quantitation by ELISA

Concentration of functional Fab fragments was measured from broth supernatants and cell lysates by an ELISA assay similar to the assay described by Trentmann et al. (2004). Thioredoxin-NTproBNP fusion was used as the coating antigen, and goat anti-mouse Fab-specific immunoglobulin labeled with alkaline phosphatase (Sigma Aldrich) was applied as the detection antibody after Fab binding to the antigen. Alkaline phosphatase activity was detected by addition of p-nitrophenyl phosphate and reading of the signal at 405 nm. Fab concentration was calculated from the absorbance standard curve generated with purified Fab standards of known concentration.

3.9 $\text{OTR}_{\text{max}}$ and $k_l\text{a}$ determination

$\text{OTR}_{\text{max}}$ in 500 ml Ultra Yield and 1000 ml Erlenmeyer flasks were measured by the two-compartment sodium sulfite method introduced by Glazyrina et al. (2011) as outlined in Article II. $k_l\text{a}$ was calculated from the average $\text{OTR}_{\text{max}}$ of triplicate measurements.

3.10 Online DOT measurement

24 round-well plate with integrated optical oxygen sensor spots (OxoDish®, PreSens GmbH, Regensburg, Germany) (Naciri et al. 2008) was placed onto SDR SensorDish® Reader (Presens GmbH), and the plate and reader were fixed to an orbital shaker with 50 mm shaking diameter. Cultivations were performed with 1.1 ml culture volume at 200 rpm with online recording of DOT in 5 minute intervals. The plate was covered with a sandwich silicone cover described by Duetz & Witholt (2001).

500 ml baffled plastic shake flasks with integrated optical oxygen sensor spots (PreSens GmbH) (Schneider et al. 2010) were placed onto SFR Reader (PreSens GmbH) and fixed to an orbital shaker with 25 mm shaking diameter. Cultivations were performed with 100 ml culture volume at 250 rpm with online recording of DOT in 5 minute intervals.
4 Results

4.1 Influence of aeration conditions on protein yield in enzymatic fed-batch medium

4.1.1 DOT and protein production under moderate aeration

In an enzymatic fed-batch medium of minimal composition, oxygen consumption rate of the culture can be strictly controlled by the concentration of glucose-releasing biocatalyst (Panula-Perälä et al. 2008). However, higher cell densities can be obtained by complementation of the medium with peptone and yeast extract, hereafter referred to collectively as complex nutrients (Fig. 1). Provision of complex nutrients (CN) reduces the need for cellular synthesis of amino acids and nucleotides, and therefore enables more carbon and energy to be directed to growth and protein synthesis. The resulting higher growth rate increases the rate of oxygen consumption. In addition, some of the control over oxygen consumption is lost due to availability of supplementary carbon sources, namely catabolizable amino acids and carbohydrates from yeast extract. These effects are demonstrated in Fig. 1, showing representative cases of *E. coli* BL21 and K-12 cultures under moderately high aeration conditions (k_L a 150–200 h⁻¹). In a minimal fed-batch medium (Fig. 1A and 1C), substrate-limited growth is achieved after 11–12 h, as indicated by sharp increase of DOT. Thereafter the culture remains at DOT ≥75%, although addition of more glucose-releasing biocatalyst at induction slightly increases the oxygen consumption. In a medium initially supplemented with a low concentration (3.6 g l⁻¹) of CN (Fig. 1B and 1D), substrate-limited conditions are attained after 8 h and high oxygen saturation (≥80%) is thereafter maintained until induction. Addition of 18 g l⁻¹ CN, along with more biocatalyst, at the time of induction results in increased respiratory activity and reduced DOT level. DOT is lowest during the first three hours of induction, during which the cells may be temporarily exposed to anaerobic conditions. However, cell densities in the range of OD₆₀₀ 30–60 and high yield of soluble/active target protein are obtained despite the low dissolved oxygen concentration (Fig. 1). As shown in Article I, cultivation in the fed-batch medium with CN supplementation under moderately aerated conditions (3 ml culture in 24dwp or Erlenmeyer flask with 10% fill) provides cell densities and volumetric soluble protein yields superior to reference media. Thus, it is concluded that while
cultures with CN supplementation may be exposed to some oxygen limitation after induction, aeration capacity of ordinary shake flasks and culture plates is sufficient to support moderately high cell densities and high volumetric protein yields in the enzymatic fed-batch medium.
Fig. 1. Effect of fed-batch medium composition on dissolved oxygen tension (DOT, % air saturation), cell density (OD$_{600}$) and volumetric recombinant protein yield (unpublished data). A and B: expression of MFE2 in *E. coli* BL21(DE3), C and D: expression of ADH in *E. coli* K-12 RB791. A and C: minimal medium with initially 1.5 U l$^{-1}$ biocatalyst, induction and addition of 5 U l$^{-1}$ biocatalyst at 16.5 h. B and D: complex medium with initially 3.6 g l$^{-1}$ complex nutrients and 1.5 U l$^{-1}$ biocatalyst, induction and addition of 18 g l$^{-1}$ complex nutrients and 1.5 U l$^{-1}$ biocatalyst at 17 h. 100 ml cultivations were performed in mildly baffled 500 ml PET flasks at 30°C and 250 rpm/25 mm shaking. In figure D, DOT recording was not functioning between 8 and 14 h and the dotted part of the graph has been reconstructed on the basis of DOT measurements with corresponding strains.
4.1.2 Enhanced aeration and the effects on protein yield

While recombinant protein could be well expressed in the CN-supplemented fed-batch medium in Erlenmeyer flasks, increase of oxygen supply and consequently relieved post-induction oxygen limitation was expected to support higher cell densities and consequently higher yields of target protein. Very high aeration efficiency was obtained in shake flask scale by using the Ultra Yield (UY) shake flasks. OTR-enhancing characteristics of the UY flask include six horizontal baffles as indentations at the flask bottom and a wide and short neck. On the other hand, the plastic flask material is likely to be non-optimal regarding mass transfer area due to hydrophobicity effects. \( k_{\text{La}} \) for a 500 ml UY flask with 20% fill at 250 rpm/25 mm was determined to be 474.1 ± 29.2 h\(^{-1}\), while \( k_{\text{La}} \) for a 1000 ml Erlenmeyer flask with 10% fill at the same shaking conditions was 122.4 ± 18.1 h\(^{-1}\) (Article II). In *E. coli* K-12 RB791 cultivation with recombinant ADH expression, the higher aeration in UY flask allowed for use of higher biocatalyst concentration without medium acidification and doubled the final cell density compared to cultivation in Erlenmeyer flask, yielding more than 20 g l\(^{-1}\) CDW (Article II). There was no major difference in specific ADH yield (activity per cell dry weight, U mg\(^{-1}\)) between the flasks after 6 hours of induction, but while the same level of specific productivity was maintained in Erlenmeyer flask during overnight expression there was a decrease in UY flask (Fig. 2A). This attenuation of specific productivity slightly reduced the benefit of higher cell density provided by higher aeration, but nevertheless the volumetric yield (U ml\(^{-1}\)) of active ADH was improved by 60% in the Ultra Yield flask (2B). Neither cell density nor volumetric ADH activity were influenced by the flask type in Terrific Broth or ZYM-5052 autoinduction medium. This suggests that final cell density in these media was limited by factors other than oxygen supply, most likely carbon source depletion and the excessive pH increase up to 8.5 in Terrific Broth.
Fig. 2. Yield of recombinant alcohol dehydrogenase (ADH) in Erlenmeyer (Erl) and Ultra Yied (UY) shake flasks. A: Specific ADH activity per mg of cell dry weight. B: Volumetric ADH activity per ml of culture. EB – EnBase fed-batch medium; TB – Terrific Broth; ZYM – ZYM-5052 autoinduction medium. Samples were taken at 6 h and 24 h from induction in EB medium, 5 h and 21 h after induction in TB medium, and 8 h and 24 h after cultivation start in ZYM medium. (Modified from Article II).
Cultivation in the UY flask also improved the yield of recombinant α-domain of protein disulphide isomerase (PDI) in *E. coli* BL21(DE3) compared to Erlenmeyer flask (Fig. 3A). However, in this case increased aeration efficiency had only minor influence on final cell density (Fig. 3B), while the volumetric yield of soluble protein increased by 3-fold (estimate based on densitometric analysis of SDS-PAGE bands). Thus, increase in the volumetric yield was mainly due to improved specific expression of soluble PDI rather than higher cell density.

**Fig. 3.** Expression of recombinant α-domain of protein disulphide isomerase (PDI, 15 kDa) in *E. coli* BL21(DE3)pLysS at different aeration conditions (unpublished data). 50 ml cultures were grown in the fed-bacth medium in 500 ml Erlenmeyer (Erl) and 250 ml Ultra Yield (UY) flasks at 250 rpm/25 mm. Glucose-releasing biocatalyst was used at concentration 0.6 U l\(^{-1}\) at inoculation, and further 1.5 U l\(^{-1}\) was added at induction (17 h) together with CN supplement. A: Soluble protein on SDS-PAGE gel, volumetric yield. B: Cell density (OD\(_{600}\)) and pH.

While increased aeration efficiency had a positive effect on volumetric yields of ADH and PDI, similar benefit may not be obtained in case of all recombinant proteins. This was observed in the study on periplasmic Fab fragment expression in *E. coli* K-12 RV308 and BL21. For three out of four anti-NTproBNP Fab fragments, volumetric yield (mg l\(^{-1}\)) was reduced in both host strains when the shaking speed in UY flask cultivations was increased from 150 rpm (\(k_{l,a} \sim 200 \text{ h}^{-1}\)) to 250 rpm (\(k_{l,a} \sim 500 \text{ h}^{-1}\)) (Article III). However, the data suggest (Article III) that high oxygen availability is less harmful to Fab expression when CN
supplementation is not used and post-induction growth rate is lower, alluding that the influence of aeration might be dependent on Fab translation rate and/or some metabolic or physiological changes in the host cells. Alternatively, it is possible that the yield of functional Fab was adversely influenced simply by the higher growth rate supported by increased aeration in the CN-supplemented medium.

The overall conclusion from these case studies with a few different recombinant proteins is that depending on the target protein, increase of aeration efficiency from moderate (k_La ~100–200 h⁻¹) to high level (k_La ~500 h⁻¹) can result in three distinct outcomes in the CN-supplemented fed-batch medium:

1. Increased volumetric yield due to higher cell density, but no improvement of specific protein yield.
2. Improved specific expression, and consequently higher volumetric yield (whether or not cell density increases significantly).
3. Reduction of specific expression that is large enough to result in lower volumetric yield despite some compensation by higher cell density.

4.2 Influence of aeration and medium composition on extracellular accumulation of periplasmic Fab fragments

Apart from the effect on total yield, as discussed in Chapter 4.1.2, aeration also influenced the localization of recombinant Fab fragments (Article III). In case of Fab expression in *E. coli* K12 RV308 in the enzymatic fed-batch medium, lower aeration efficiency was associated with higher percentage of the Fab product in the extracellular medium. For three out of the four studied Fabs, cultivation in UY flask at 250 rpm/25 mm shaking resulted in retention of the product in periplasm, while at 150 rpm/25 mm majority of Fab activity was detected in the extracellular medium (Fig. 4). As discussed in Article III, this was partly due to increased cell lysis at the lower shaking speed, but the results suggest that also lysis-independent leakage from intact cells increased significantly in response to lower oxygen supply. One replicate cultivation at the lower shaking speed resulted in distinctly lower extracellular yield than the other two replicates (Fig. 4). This may be due to experiment-to-experiment variation in culture oxygen uptake rate (OUR) and consequently changes in DOT during Fab expression.
Fig. 4. Shaking speed and Fab localization. Yields in mg per liter of culture for Fab fragments F1 (a), F16 (b) and F32 (c) expressed in *E. coli* RV308 in the CN-supplemented fed-batch medium in UY shake flasks at two different shaking speeds (150 rpm and 250 rpm after induction; all cultures were incubated at 250 rpm until induction), as measured by antigen-binding ELISA from cell lysate (periplasmic fraction; in black) and broth supernatant (medium fraction; in grey). Samples were harvested 24 after induction. A–C on the horizontal axis refer to independent replicate experiments. (Article III, reprinted with permission of BioMed Central).
The effect of aeration was also studied with one of the Fab fragments in 24dwp, where different aeration rates were obtained by modification of the culture volume. Culture volumes of 1 to 3 ml resulted in retention of Fab product effectively in the periplasm, while with 4 to 5 ml culture volume most of the Fab activity was found in the medium (Fig. 5). This demonstrates that a notable change in Fab localization can be brought about by a relatively small change in aeration efficiency, such as by increasing culture volume by one third. On this ground it is not surprising if operation of the cultures near the critical aeration threshold results in some experiment-to-experiment variation in product localization, as was the case in shake flask cultures at 150 rpm shaking.

![Fig. 5. Culture volume and Fab localization. Yields of Fab fragment F1 in mg per liter of culture in E. coli RV308 cultivations in the fed-batch medium with varying broth volumes (1–5 ml) in 24 deep well plate. Fab quantities were measured by antigen-binding ELISA from cell lysate (periplasmic fraction; in black) and broth supernatant (medium fraction; in grey). Samples were harvested 24 h after induction. The mean periplasmic and extracellular concentrations of two replicate experiments are shown; standard deviations were typically 10–20% of the mean. (Article III, reprinted with permission of BioMed Central).](image)

Composition of the enzymatic fed-batch medium also had a significant influence on the product localization, either due to the change in oxygen saturation of the culture as a result of modified nutrient availability, or changes in growth rate. Addition of complex nutrients and more glucose-releasing biocatalyst at the time of induction resulted in increased respiratory activity, reduced oxygen saturation and high percentage of extracellular Fab (Fig. 6). Without these additional nutrients Fab expression took place under fully aerobic conditions, and the
product was retained in periplasm. Despite high oxygen availability, the total Fab yield did not decrease in comparison to the cultivation in CN-supplemented medium. Therefore, cultivation in the enzymatic fed-batch medium without the additional CN supplementation at induction appears to be the best strategy to obtain maximum Fab yield in the periplasm.

Fig. 6. Influence of fed-batch medium composition on oxygen saturation and Fab production. Dissolved oxygen tension (DOT, % of saturation) was measured online during 24 round-well plate expression of Fab fragment F1 in *E. coli* RV308 in the fed-batch medium. Fab yields in mg per liter of culture were measured by antigen-binding ELISA from cell lysate (periplasmic fraction; in black) and broth supernatant (medium fraction; in grey) at 41 h. a: Cultivation without addition of nutrients at induction. b: Cultivation with addition of complex nutrients and 3 U l\(^{-1}\) biocatalyst at induction (18 h, indicated by arrows). In each case, Fab yields are shown for cultures with initial biocatalyst concentrations of 0.3, 0.6 and 1.5 U l\(^{-1}\). Representative DOT graphs are shown from the cultures with initially 0.6 U l\(^{-1}\) biocatalyst. DOT profiles with initial biocatalyst concentrations of 0.3 and 1.5 U l\(^{-1}\) were essentially similar to the graphs shown. (Article III, reprinted with permission of BioMed Central).

In both *E. coli* RV308 and *E. coli* BL21 the extracellular Fab leakage appears to be initiated at the time when cell growth starts to cease (Fig. 7). The DOT profile (Fig. 6b), though not from the same culture, indicates reduction in respiratory
activity around the same time. However, Fab accumulation in the periplasm continues for several hours beyond the start of leakage (Fig. 7), indicating that the cellular protein synthesis is still active despite the cessation of active growth. These data suggest that the trigger for Fab leakage might be the physiological changes associated with the gradual transition from active growth and high respiration into stationary phase. Nevertheless, in RV308 this growth phase-associated leakage seems to be influenced by the medium composition and aeration level, as the degree of leakage was very low in cultures without CN-supplementation (Fig. 6a) and in cultures under high aeration (Fig. 4 and 5) even if the cultivation time after induction (23–24 h) was sufficient to allow transition into stationary phase.
Fig. 7. Dynamics of Fab accumulation into periplasm and extracellular medium. Fab fragment F1 was expressed in *E. coli* RV308 and BL21(DE3) in the fed-batch medium and Ultra Yield shake flasks at 150 rpm shaking. Fab quantities in cell lysate (periplasmic fraction; solid line with black circle) and broth supernatant (medium fraction; dashed line with triangle) were measured by antigen-binding ELISA, and are shown at different time points as percentage of the final Fab yield (left vertical axis). Cell density was recorded by OD$_{600}$ readings (dotted line with square), which are shown on the right vertical axis. (Article III, reprinted with permission of BioMed Central).
Fab fragment expression in *E. coli* BL21 in the ZYM-5052 autoinduction medium provided generally lower percentage of extracellular Fab than expression in the same strain in the enzymatic fed-batch medium (Article III). At least part of the reason for this is the difference in the degree of lysis. However, also in the autoinduction medium lower aeration contributed to increased extracellular Fab fraction (Article III). This may be partly due to the difference in total Fab yield, as higher Fab transport to the periplasm may interfere with transport of outer membrane structural elements (Shokri *et al.* 2002) and consequently result in a more permeable membrane composition. On the other hand, the influence of aeration on leakage was very clearly seen also for fragment 1B10 that was produced in equal levels at both aeration conditions (Article III).

### 4.3 Robustness of lactose autoinduction media at different aeration conditions

The effect of aeration on protein expression in lactose autoinduction cultures was studied in Studier’s ZYM-5052 medium using glycerol as the supporting carbon source, and in the enzymatic fed-batch medium using slowly released glucose as the supporting carbon source. In line with earlier studies in fed-batch bioreactors (Glascock & Weickert 1998, Hoffman *et al.* 1995, Neubauer & Hofmann 1994), induction by lactose was not inhibited by glucose feeding at a limiting rate, and the enzymatic glucose fed-batch proved to be a feasible carbon source to support high level of target protein expression with 0.5 to 2 g l⁻¹ of supplemented lactose (Article IV).

Initial expression trials in the autoinducing media were performed in 24dwp, after which the cultures were scaled up to 50 ml volume cultivated in both 500 ml Erlenmeyer and 250 ml Ultra Yield shake flasks. Due to the small liquid volume, OTR max for the 24dwp cultures could not be determined by the method applied for shake flasks, but is estimated to be approximately 25 mmol l⁻¹ h⁻¹ for the 3 ml culture at 200 rpm/50 mm shaking by extrapolation from the data of Duetz and Witholt (2004). Therefore, the aeration efficiency in 24dwp is estimated to be slightly lower than in the Erlenmeyer flask (OTR max 30 mmol l⁻¹ h⁻¹ in a 1000 ml flask with 10% fill, Article II).

In the glycerol-based ZYM-5052 medium, recombinant protein expression was highly dependent on aeration conditions. Specific productivity of MFE2 expressed from *lacI*-supplying pPAL7 vector was 75% lower in the UY flask compared to Erlenmeyer. Even though higher cell density provided some
compensation, volumetric yield was reduced by 60% at the higher aeration level (Fig. 8). By contrast, fed-batch-based autoinduction media provided high expression of MFE2 also in the UY flask, and the specific yields were very similar under the different aeration conditions (Fig. 8). It should also be noted that enzymatic fed-batch media with different concentrations of complex nutrients provided similar specific yields. This further highlights the robustness of expression, since OUR is likely to be higher and DOT lower in the high CN medium. The earlier DOT measurements in baffled flasks (see Fig. 1) suggest that at least in the UY flask most of the expression period in low CN medium takes place under fully aerobic conditions. Hence it can be concluded that high level of MFE2 expression is maintained in the enzymatic fed-batch autoinduction medium under highly aerated conditions up to full oxygen saturation, and the adverse influence of high aeration seen in the glycerol-based medium is effectively avoided.
Fig. 8. Shake flask expression of MFE2 from pPAL7 vector in E. coli BL21(DE3) under different aeration rates. Expression was compared in a moderately aerated flask (Erlenmeyer; solid line with open circles) and a highly aerated baffled flask (Ultra Yield; dashed line with black triangles) in the fed-batch medium with high complex nutrient content (left column), fed-batch medium with low complex nutrient content (middle column), and glycerol-based ZYM-5052 medium (right column). Specific protein yield (top row) was determined by running cell lysates (standardized to OD$_{600}$ = 7.0) on 12% electrophoresis gels and quantifying the bands by a densitometry software. Protein yield per ml of culture (middle row) was determined by multiplying specific yield by the dilution factor. Cell density was determined by optical density measurements (OD$_{600}$, bottom row). (Modified from Article IV).

A yield reduction in response to increased aeration in glycerol-based autoinduction medium was also observed in E. coli K-12 RB791, which carries the chromosomal lac$^F$ mutation and thus produces higher levels of the repressor. In this strain, expression of recombinant alcohol dehydrogenase in ZYM-5052 in
24dwp scale provided relatively high yield of active product, while in the shake flask scale the active yield was reduced by 90% (Fig. 9). Also in the fed-batch autoinduction medium active ADH yield decreased when scaling up from the 24dwp to shake flasks, but not nearly as dramatically as in the glycerol-based medium (Fig. 9). Interestingly, the effect of aeration level was not linear and the specific and volumetric yields in Erlenmeyer flask were lower than in either 24dwp or UY flask. The ADH accumulation profiles suggests that especially in Erlenmeyer flask there may be overgrowth of uninduced cells after lactose depletion, and consequently the calculated yield per cell becomes “diluted” (Article IV). In 24dwp this dilution effect may be reduced due to the lower oxygen supply that limits the culture growth to lower final density, and the data from UY flask suggest that the higher aeration level might slow down the utilization of lactose so that depletion is avoided. These phenomena however do not completely explain the differences in volumetric yield. Nevertheless, the scale-related yield reduction in the enzymatic fed-batch media was much smaller than in the glycerol-based medium, and thus it is concluded that also in case of a lacIq mutant K-12 strain the robustness of lactose autoinduced expression can be improved by the use of slow glucose feed as the supporting carbon source.
Fig. 9. Robustness of recombinant alcohol dehydrogenase expression in autoinduction media at different scales. A: Specific ADH activity. B: Volumetric ADH activity. C: Cell density. Samples were taken after 26 h cultivation in 24dwp or 27 h cultivation in shake flask. High CN – enzymatic fed-batch medium with 21.6 g l\(^{-1}\) complex nutrients, Low CN – enzymatic fed-batch medium with 3.6 g l\(^{-1}\) complex nutrients, ZYM – ZYM-5052 medium. (Modified from Article IV).

4.4 Recombinant protein yield in enzymatic fed-batch media compared to other expression media

Though the optimal medium type for protein production may vary depending on the target protein and host strain, the protein expression case studies included in this work enable some general evaluation of the potential of the enzymatic fed-batch media against most commonly used cultivation media. These case examples comprise expression of recombinant proteins from widely used lactose-inducible promoter systems (T7, T7lac, T5lac, tac) in BL21 and K-12 derived host strains.

4.4.1 IPTG induction

The case examples on IPTG-induced expression include 10 different recombinant proteins, four of them different anti-NTproBNP Fab fragments. Table 2 summarizes these case studies and their outcome as quantitative increase in soluble or biologically functional target protein per culture volume by expression in the enzymatic fed-batch medium compared to the reference medium. All comparisons were performed under conditions of moderate aeration efficiency (\(k_{L\alpha}\) 100–200 h\(^{-1}\)).
In comparison to the other IPTG-induced media (LB, TB, SB-MOPS, minimal medium with glucose), the enzymatic fed-batch medium improved volumetric protein yield in all but one case. The exception was ERp27, a 27 kDa eukaryotic protein that could not be expressed in detectable amounts of soluble product in either the fed-batch or LB medium. Magnitude of the yield improvement was very dependent on the target protein and in some cases also on the reference medium (for example, functional avidin was well expressed in LB medium but hardly any biotin-binding product could be obtained in TB). A modest, 1.8 to 4-fold increase in volumetric yield was obtained for PDI (compared to TB), some of the Fab fragments, avidin (compared to LB) as well as BiP, and this magnitude of increase can be mostly attributed to the higher cell density obtained in the fed-batch medium. The other extremity is represented by ADH expression in E. coli RB791, in which case final cell density was increased only by one third in comparison to TB, but drastically improved specific expression of the target protein in active as opposed to insoluble form (Article II) contributed to over 100-fold increase in volumetric product activity. In several cases the volumetric yield improvement, often approximately 10 to 15-fold, was the combined result of significantly higher final cell density and simultaneously improved yield of soluble/active product per unit of cell density.

In a limited number of cases IPTG induction in the enzymatic fed-batch medium was compared with lactose autoinduction in ZYM-5052 medium. According to Studier (2005), autoinduction media can often provide higher product yields than IPTG induction in the conventional expression media, and in line with this it appears that the volumetric yield improvement provided by the enzymatic fed-batch medium in comparison to ZYM-5052 is more modest. At first, almost 80-fold difference in active ADH yield was observed in favor of the fed-batch medium in Erlenmeyer shake flasks (Article II), but it later turned out that ADH expression in ZYM-5052 could be greatly improved when cultivation was performed under lower aeration in 24dw (Fig. 9 and Article IV). Hence the difference in ADH yield is approximately 3.5-fold in favor of the fed-batch medium when expression in ZYM-5052 is performed under optimal aeration conditions (Table 2). Total yields of Fab fragments in E. coli BL21 were, depending on the fragment, similar in the fed-batch and autoinduction media or up to two times higher in the fed-batch medium.

An apparent benefit of the enzymatic fed-batch medium is the generally much higher cell density compared to the conventional IPTG-induced media (Articles I–III). Also, the effective protein expression period is probably longer than in rich
media like LB, TB and SB-MOPS, in which pH increases strongly (Articles I–III) and likely limits the time window for target protein production. It should be noted that the lactose autoinduction medium appears to provide similar benefits regarding relatively high cell density and maintenance of favorable pH. However, the limited data suggests that the fed-batch medium can still provide up to 2–3 times higher volumetric yield of target protein.

Table 2. Relative soluble/active volumetric yields of different recombinant proteins by IPTG induction in the enzymatic fed-batch medium compared to reference media. Expression of each protein in the fed-batch and reference media was performed at the same temperature and shaking conditions, and the same IPTG concentration was used for induction (except for Fab fragment expression in E. coli RV308, in which case lower IPTG concentration was used in SB-MOPS than in fed-batch medium). MFE2 – multifunctional enzyme type 2; PDI – a-domain of protein disulphide isomerase; ADH – alcohol dehydrogenase; ERp27 – 27 kDa endoplasmic reticulum protein; BiP – binding immunoglobulin protein (heat shock 70 kDa protein 5); LB – lysogeny broth; TB – Terrific Broth; SB-MOPS – Super Broth, MOPS-buffered; ZYM-5052 – Studier’s autoinduction medium.

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Host strain</th>
<th>Reference medium</th>
<th>Increase in volumetric protein yield</th>
<th>Quantification method</th>
<th>Data source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFE2</td>
<td>BL21(DE3)</td>
<td>LB</td>
<td>15-fold</td>
<td>SDS-PAGE and densitometry</td>
<td>Article I</td>
</tr>
<tr>
<td>MFE2</td>
<td>BL21(DE3)</td>
<td>TB</td>
<td>14-fold</td>
<td>SDS-PAGE and densitometry</td>
<td>Article I</td>
</tr>
<tr>
<td>PDI</td>
<td>BL21(DE3)pLysS</td>
<td>LB</td>
<td>17-fold</td>
<td>SDS-PAGE and densitometry</td>
<td>Article I</td>
</tr>
<tr>
<td>PDI</td>
<td>BL21(DE3)pLysS</td>
<td>TB</td>
<td>3.5-fold</td>
<td>SDS-PAGE and densitometry</td>
<td>Article I</td>
</tr>
<tr>
<td>PDI</td>
<td>BL21(DE3)pLysS</td>
<td>Minimal medium</td>
<td>13-fold</td>
<td>SDS-PAGE and densitometry</td>
<td>Article I</td>
</tr>
<tr>
<td>ADH</td>
<td>RB791</td>
<td>TB</td>
<td>113-fold</td>
<td>Kinetic activity assay</td>
<td>Article II</td>
</tr>
<tr>
<td>ADH</td>
<td>RB791</td>
<td>ZYM-5052 (autoind.)</td>
<td>3.5-fold</td>
<td>Kinetic activity assay</td>
<td>Unpublished data</td>
</tr>
<tr>
<td>Fab fragments</td>
<td>RV308</td>
<td>SB-MOPS</td>
<td>4 to 16-fold</td>
<td>ELISA</td>
<td>Article III</td>
</tr>
<tr>
<td>Fab fragments</td>
<td>BL21(DE3)</td>
<td>SB-MOPS</td>
<td>4 to 10-fold</td>
<td>ELISA</td>
<td>Article III</td>
</tr>
<tr>
<td>Fab fragments</td>
<td>BL21(DE3)</td>
<td>ZYM-5052 (autoind.)</td>
<td>none or 1.6 to 2-fold</td>
<td>ELISA</td>
<td>Article III</td>
</tr>
</tbody>
</table>
### Table 3: Summary of protein expression in different media

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Host strain</th>
<th>Reference medium</th>
<th>Increase in volumetric protein yield</th>
<th>Quantification method</th>
<th>Data source</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERp27</td>
<td>BL21(DE3)pLysS</td>
<td>LB</td>
<td>none&lt;sup&gt;1&lt;/sup&gt;</td>
<td>SDS-PAGE (visual estimation)</td>
<td>Unpublished data</td>
</tr>
<tr>
<td>Avidin</td>
<td>BL21(DE3)pLysS</td>
<td>LB</td>
<td>1.8-fold&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Avidin-biotin binding assay</td>
<td>Unpublished data</td>
</tr>
<tr>
<td>Avidin</td>
<td>BL21(DE3)pLysS</td>
<td>TB</td>
<td>60-fold&lt;sup&gt;2&lt;/sup&gt;</td>
<td>Avidin-biotin binding assay</td>
<td>Unpublished data</td>
</tr>
<tr>
<td>Avidin</td>
<td>BL21(DE3)pLysS</td>
<td>Minimal medium</td>
<td>13-fold</td>
<td>Avidin-biotin binding assay</td>
<td>Unpublished data</td>
</tr>
<tr>
<td>BiP</td>
<td>BL21(DE3)pLysS</td>
<td>LB</td>
<td>4-fold</td>
<td>SDS-PAGE and densitometry</td>
<td>Unpublished data</td>
</tr>
</tbody>
</table>

<sup>1</sup>No soluble expression in either medium, <sup>2</sup>Fed-batch medium with minimal composition

### 4.4.2 Lactose autoinduction

Comparison of the fed-batch autoinduction medium (lactose 0.5–2.0 g l<sup>−1</sup>) with glycerol-based ZYM-5052 regarding the expression of three different target proteins (Table 3) resulted in three distinct outcomes. In case of ERp60 expression from pET23 vector in *E. coli* BL21(DE3), there was no notable difference in the cell density or specific productivity between the two media (unpublished data), and hence also the volumetric yields of soluble ERp60 were essentially the same. In case of MFE2 expression there was no difference in the specific protein yield, but 70% higher cell density in the fed-batch medium contributed to corresponding increase in volumetric yield (Table 3; see also Fig. 8). In contrast to this, the specific productivity of active ADH per cell density was almost two-fold higher in the fed-batch autoinduction medium, which together with a small increase in cell density contributed to 2.4-fold higher volumetric yield compared to ZYM-5052 (Table 3; see also Fig. 9). Thus it is concluded that the fed-batch autoinduction medium can provide cell and recombinant protein yields similar to or higher than the Studier medium, and that largest yield improvement is obtained when the glucose fed-batch contributes to increased specific productivity of soluble/active target protein.
Table 3. Relative volumetric yields of different recombinant proteins in the enzymatic fed-batch autoinduction medium compared to Studier’s autoinduction medium (ZYM-5052). MFE2 – multifunctional enzyme type 2; ADH – alcohol dehydrogenase; ERp60 – 60 kDa endoplasmic reticulum protein.

<table>
<thead>
<tr>
<th>Target protein</th>
<th>Host strain</th>
<th>Reference medium</th>
<th>Increase in volumetric protein yield</th>
<th>Quantification method</th>
<th>Data source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MFE2</td>
<td>BL21(DE3)</td>
<td>ZYM-5052</td>
<td>1.7-fold</td>
<td>SDS-PAGE and densitometry</td>
<td>Article IV</td>
</tr>
<tr>
<td>ADH</td>
<td>RB791</td>
<td>ZYM-5052</td>
<td>2.4-fold</td>
<td>Kinetic activity assay</td>
<td>Article IV</td>
</tr>
<tr>
<td>ERp60</td>
<td>BL21(DE3)pLysS</td>
<td>ZYM-5052</td>
<td>none</td>
<td>SDS-PAGE and densitometry</td>
<td>Unpublished data</td>
</tr>
</tbody>
</table>
5 Discussion

5.1 Effects of aeration on recombinant protein production

Oxygen limitation is commonly acknowledged as a major obstacle in shaking bioreactor cultivations (Büchs 2001, Zhang et al. 2005). As noted by previous authors, non-limiting oxygen availability during screening is required for feasible bioprocess development (Maier & Büchs 2001, Zimmermann et al. 2006), and $OTR_{\text{max}}$ and $k_{L}a$ are crucial parameters in scaling up and scaling down the process conditions between shaking vessels and laboratory-scale mechanically stirred bioreactors (Freyer et al. 2004, Islam et al. 2008, Micheletti et al. 2006, Seletzky et al. 2007). This study highlights the significance of optimal aeration also in maximizing the small-scale yield of recombinant protein in shaken E. coli cultures. Curiously though, the results suggest that while in some cases enhanced aeration capacity of the shake flask is advantageous regarding volumetric productivity, in other cases it may actually be beneficial to apply lower aeration rates in order to maximize yield or to optimize the final product localization.

Aeration and protein yield

Oxygen transfer capacity of ordinary Erlenmayer flasks and square-well plates at reasonable fill volumes and shaking conditions is in the range of 20–50 mmol l$^{-1}$ h$^{-1}$ (Duetz & Witholt 2004, Freyer et al. 2004, Gupta & Rao 2003, Maier & Büchs 2001). This level of aeration in the enzymatic fed-batch cultures was demonstrated to be sufficient for moderately high cell density growth (final OD$_{600}$ 20–50 depending on the clone) and high expression of soluble/active target proteins. Thus the application of the fed-batch media for recombinant protein expression does not necessarily require any non-standard devices such as the Ultra Yield flask. Without CN supplementation the culture remains fully aerobic during the entire expression period, demonstrating the utility of enzyme-controlled glucose supply in regulating OUR while the culture is still actively growing and respiring. Maximum cell densities and volumetric productivities are obtained with the CN supplementation, which however also increases the OUR right after induction and thus the oxygen transfer capacity of ordinary shake flasks becomes a growth-limiting factor in the early expression phase. Culture vessels with enhanced oxygen transfer capacity, such as the Ultra Yield flask, are
therefore needed to achieve the maximum biomass yield in the CN-supplemented fed-batch medium.

In the best case, cultivation in the enzymatic fed-batch medium in the Ultra Yield flask can provide very high cell densities exceeding CDW 20 g l⁻¹ (OD₆₀₀ >75) during expression of a non-toxic target protein. However, while the high aeration was found to improve specific expression of PDI, reduced protein per biomass was observed in case of Fab fragments and also ADH during prolonged expression period. Thus the benefit of increased cell density may not always translate to a corresponding increase in volumetric protein yield. Some previous reports have described similar adverse effect of high aeration on protein expression in stirred bioreactors (Li et al. 1992, Qoronfleh 1999, Tomazetto et al. 2007), and noted that the influence of oxygen availability on expression level is apparently very protein-specific (Qoronfleh 1999). Further studies would be needed to elucidate whether the reduction in specific ADH and Fab fragment yields at high aeration was possibly due to plasmid loss (Tomazetto et al. 2007), oxidative damage to the target protein (Konz et al. 1998) or adverse influence of increased growth rate. Chemostat studies would be helpful in differentiating between the effects of DOT and growth rate.

Aeration and Fab fragment localization

Modification of aeration conditions was observed to have substantial influence on the extracellular accumulation of recombinant Fab fragments secreted to E. coli periplasm. Extracellular localization of the product is often preferable as it enables simple purification procedures where the steps of protein extraction from cell pellet are bypassed (O’Brien et al. 2002). Cultivation in the CN-supplemented fed-batch medium under moderate oxygen limitation was found to be a straightforward approach for maximizing the extracellular product accumulation in E. coli RV308, though further optimization of the harvest time would be needed to minimize the release of cytoplasmic proteins by lysis. The simplicity of aeration adjustment is an apparent advantage over methods that apply modifications at the genetic level, such as fusion to carrier proteins (Fernández et al. 2000) or co-expression of assisting genes (Wan & Baneyx 1998), to increase extracellular protein accumulation. Moreover, the use of auxiliary chemicals such as Triton X-100 to increase membrane permeability can be avoided. On the other hand, this study also demonstrates simple means for minimizing Fab leakage and maximizing Fab yield in the periplasm for applications where this localization
may be preferred. Control of the final product localization by adjustment of culture aeration rate and/or medium composition with CN supplementation is a feature of high practical utility, and results with E. coli BL21(DE3) in the autoinduction medium suggest that the tendency for increased leakage at lower aeration rates is not limited to one specific strain and medium.

Elucidation of the mechanism by which aeration level affects the leakage would require further studies to differentiate between the effects of DOT, growth rate and medium composition. While E. coli lacks the machinery for active secretion of protein products, passive leakage of certain periplasmic proteins is a well-known phenomenon (Plückthun 1991, Rinas & Hoffmann 2004, Shokri et al. 2003). Aeration is known to influence the lipid and protein composition of the outer cell membrane (Arneborg et al. 1993, Knivett & Cullen 1965, Rolfe et al. 2011), and while growth rate-dependent changes in outer membrane composition have been shown to affect the leakage of periplasmic proteins (Bäcklund et al. 2008, Shokri et al. 2002), the possible association of aeration and periplasmic protein leakage has not been previously studied. It is possible that the effect of aeration observed in the present study is mediated indirectly via the influence of oxygen availability on growth rate, though the extracellular accumulation appears to begin in the phase of stagnating growth rather than during the period of oxygen-limited growth. It has also been suggested that leakage of periplasmic proteins is promoted by stressful conditions such as temperature upshift (Rinas & Hoffmann 2004), and thus it might be that the combined stresses of recombinant product formation (Gill et al. 2000, Hoffmann & Rinas 2004) and oxygen limitation are the cause of increased leakage. Further studies are also implicated regarding whether the same aeration effect applies to a wider range of different Fab fragments and other types of recombinant proteins transported to the E. coli periplasm.

**Aeration and autoinduction media**

Earlier studies have reported high aeration rates to have adverse effect on recombinant protein expression in autoinducing media (Blommel et al. 2007, Studier 2005), though contradictory findings have also been presented (Li et al. 2011a). All these previous reports have dealt with autoinducing media using glycerol as the supporting carbon source, and according to Blommel et al. (2007) oxygen availability might modify the preferred order of lactose and glycerol utilization, resulting in delayed onset of lactose-induced recombinant protein
expression under high oxygen availability. The results of this study are in line with the findings of Studier (2005) and Blommel et al. (2007) regarding the reduced expression level in glycerol-based autoinduction media under high aeration rates. However, while Studier stated that lactose concentration of 2 g l\(^{-1}\) should be sufficient to attenuate this aeration-dependency, this was not the case in this study. Expression of MFE2, ADH and three out of four Fab fragments was drastically reduced at high aeration when cultivation was performed in Studier’s ZYM-5052 medium with 2 g l\(^{-1}\) lactose. Moreover, the findings do not support delayed onset of induction as the cause of reduced expression. Further studies would be needed to uncover the actual cause for the observed aeration-dependency of expression in ZYM-5052 medium.

The improved robustness of expression in the fed-batch autoinduction medium compared to ZYM-5052 is a highly advantageous feature especially for high-throughput expression screening. As the OUR profile can vary considerably from clone to clone (Kunze et al. 2012), different clones cultivated under the same shaking conditions can be exposed to different DOT levels. The higher the robustness of the system regarding oxygen availability, the less the variation in OUR and DOT will confound the screening results. Moreover, as the case example of ADH autoinduction demonstrates, the expression level in a glycerol-based medium may drop very dramatically over a relatively minor change in oxygen transfer capacity. Thus the improved robustness achieved by the fed-batch autoinduction medium could also reduce trial and error attempts when protein production is transferred from one type and/or scale of vessel to another.

5.2 Improvement of protein yield by enzymatic fed-batch medium

The results of this study demonstrate that compared to commonly used expression media such as LB and TB the enzymatic glucose fed-batch medium can provide considerable increase in volumetric yield of soluble/active recombinant protein. Also compared to Studier’s autoinduction medium volumetric yield increases can be achieved by application of either IPTG induction or autoinduction in the fed-batch medium. Previously, Panula-Perälä et al. (2008) have shown the enzymatic fed-batch based on a starch gel and minimal medium composition to yield higher volumetric yield of recombinant protein in comparison to complex reference medium, although in that case specific yield was lower and final cell density limited by nitrogen in the fed-batch medium. In the present study the enzymatic fed-batch was applied primarily in CN-supplemented medium to maximize cell
density and avoid nitrogen limitation. After publication of Article I introducing the CN-supplemented fed-batch medium based on fully soluble polysaccharide, the enzymatic fed-batch with CN supplementation has been applied to recombinant protein production by several others, who have mostly reported results showing increases in E. coli cell density and volumetric protein yield (Ehrmann et al. 2010, Imaizumi et al. 2013, Mahboudi et al. 2013, Nguyen et al. 2011, Peck et al. 2014). Thus the findings of the present study are supported by the work of other groups, though naturally it should be noted that results showing no benefit over standard expression procedures are likely to get reported less frequently than yield improvements. To date, one published study has reported lower cell density and volumetric protein productivity in the fed-batch medium compared to a glucose-based complex batch medium (Hortsch & Weuster-Botz 2011), though this result was obtained in millilitre-scale stirred tank reactors with DO-control rather than simple, uncontrolled shaking vessels.

The results of this study also suggest that the enzymatic fed-batch may improve the expression of certain proteins in soluble and/or functional form. Similar benefit was observed by Panula-Perälä et al. (2008). Hence the fed-batch medium could be advantageous for expression of some target proteins that are difficult to obtain as soluble or functional protein in ordinary media. The likely reason for improved solubility/activity is the relatively low post-induction growth rate and consequently reduced protein synthesis rate in the fed-batch medium. Though growth rate in the fed-batch medium is elevated by the CN supplementation at induction, the resulting post-induction growth rate is still considerably lower than in cultures induced at the exponential growth phase. Reduced synthesis rate is generally considered beneficial for protein solubility and correct folding, as the lower intracellular concentration of folding intermediates reduces the probability of aggregate formation (Georgiou & Valax 1996).

The improved volumetric productivity enables protein production in smaller culture volumes, and is therefore particularly beneficial regarding high-throughput protein expression. Use of smaller flasks enables parallel operation of a higher number of cultures, and robot-operated high-throughput cultivation is facilitated when the required biomass and protein yield can be obtained in micro- or deep well plates instead of shake flasks. The utility of the fed-batch medium in high-throughput applications has already been demonstrated by Tegel et al. (2011), in whose report the enzymatic fed-batch medium enabled microscale high-throughput expression screening by providing sufficient amount of
recombinant protein for small-scale purification and subsequent detection by mass spectrometry in only 150 µl culture volume. Considering the importance of efficient high-throughput recombinant protein production in fields such as structural biology (Goulding & Perry 2003) and functional genomics (Larsson et al. 2000), the enzymatic fed-batch medium could potentially have significant contribution to the time- and cost-efficiency of biotechnological research projects.
6 Conclusions

RQ1: Is the aeration in ordinary shake flasks sufficient for good growth and protein expression in the enzymatic fed-batch medium, and how is volumetric productivity influenced by the improved aeration obtained in Ultra Yield shake flasks?

- Supplementation of the enzymatic fed-batch medium with complex nutrients at induction allows for higher cell densities compared to a non-supplemented medium, but at the same time the increased respiratory activity may lead to some oxygen limitation during the first hours following the induction. Despite the temporary oxygen limitation, ordinary Erlenmeyer shake flasks providing OTR\textsubscript{max} of approximately 30 mmol l\textsuperscript{-1} h\textsuperscript{-1}, as well as deepwell plates with similar magnitude of OTR\textsubscript{max}, can support final cell densities of OD\textsubscript{600} 25–50 in the enzymatic fed-batch medium. Relative to reference media, high specific productivity of soluble target proteins is also obtained, and it is concluded that protein expression in the enzymatic fed-batch medium can be feasibly accomplished in standard shaking bioreactors.

- Depending on the host strain and target protein, the UY flask (OTR\textsubscript{max} 118 mmol l\textsuperscript{-1} h\textsuperscript{-1}) can provide cell densities of up to more than 20 g l\textsuperscript{-1} CDW (OD\textsubscript{600} ≥75) in the CN-supplemented fed-batch medium. Provided that specific productivity is sufficiently maintained, this contributes to increased volumetric yield of the recombinant protein. In some cases, the enhanced aeration may even improve specific productivity in comparison to an Erlenmeyer flask. However, the high aeration can also have adverse effects on the expression of certain proteins, resulting in reduced volumetric productivity despite increased cell density. Further studies are implicated for elucidation of the underlying causes of reduced specific expression at high aeration rates.

RQ2: What is the influence of aeration and medium composition on leakage of periplasmic Fab fragments?

- Reduced aeration was observed to promote higher extracellular accumulation of recombinant Fab fragments secreted to E. coli periplasm. Cultivation of E. coli RV308 in the enzymatic fed-batch medium under high aeration rates contributed to efficient periplasmic retention of Fab fragments, while at lower aeration rates the release of Fab fragments to the extracellular medium by
both lysis and lysis-independent leakage increased significantly. Similar tendency was observed with *E. coli* BL21(DE3) in ZYM-5052 autoinduction medium. Moreover, reduced Fab leakage was achieved in *E. coli* RV308 by omitting medium supplementation with complex nutrients. While these findings have considerable practical utility for controlling the product localization, further studies are implicated regarding the mechanisms of the observed effects on Fab leakage.

**RQ3:** How does aeration influence protein expression in lactose autoinduction media with enzymatic fed-batch or glycerol as the supporting carbon source?

- As reported previously in literature and observed in this study, high aeration rates can have a strong adverse effect on protein expression in a glycerol-based autoinduction medium. The robustness of expression under different aeration conditions is substantially improved by an autoinduction medium based on the enzyme-controlled glucose feed as the supporting carbon source instead of glycerol. Further investigation would be needed to explain the observed aeration-dependency of glycerol-based autoinduction, and to confirm whether the robustness of the enzymatic fed-batch medium is due to the limited availability of the supporting carbon source.

**RQ4:** Can cultivation in the enzymatic fed-batch medium improve recombinant product yields compared to different batch media?

- Compared to conventional expression media (LB, TB) used in shaking cultures, the enzymatic fed-batch medium can provide considerably higher volumetric protein yields. The higher cell density typically results in a 2 to 5-fold increase in volumetric productivity, and in several cases also the specific productivity of protein in soluble/active form is improved, which can lead to a volumetric yield improvement by factor of 10–20 or even more. Compared to ZYM-5052 autoinduction medium, advantages of the fed-batch medium include not only reduced dependency of successful expression on the aeration level but also improvements in the volumetric protein yield.
References


Original articles


Reprinted with permission of BioMed Central (I–III) and Elsevier (IV).

Original publications are not included in the electronic version of the dissertation.
464. Tammela, Simo (2013) Enhancing migration and reproduction of salmonid fishes: method development and research using physical and numerical modelling

465. Yadav, Animesh (2013) Space-time constellation and precoder design under channel estimation errors


468. Remes, Jukka (2013) Method evaluations in spatial exploratory analyses of resting-state functional magnetic resonance imaging data

469. Oravijärvi, Kati (2013) Industry and traffic related particles and their role in human health

470. Czajkowski, Jakub (2013) Optical coherence tomography as a characterization method in printed electronics

471. Hasapainen, Mikko (2013) Dielectrophoretic mobility of a spherical particle in 2D hyperbolic quadrupole electrode geometry


476. Juuso, Esko (2013) Integration of intelligent systems in development of smart adaptive systems: linguistic equation approach

477. Lu, Xiaoja (2013) Resource allocation in uplink coordinated multicell MIMO-OFDM systems with 3D channel models


Kaisa Ukkonen

IMPROVEMENT OF RECOMBINANT PROTEIN PRODUCTION IN SHAKEN CULTURES

FOCUS ON AERATION AND ENZYME-CONTROLLED GLUCOSE FEEDING