Impacts of oscillating cultivation conditions on the quality of recombinant inclusion bodies in *Escherichia coli*
Impacts of oscillating cultivation conditions on the quality of recombinant inclusion bodies in *Escherichia coli*

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For Natascha –

my precious wife, my best friend, my true love
Abstract

The controlled use of microorganisms in the pharmaceutical, (bio-)chemical and life science industries to produce recombinant proteins or other organic compounds in large-scale bioreactors is a standard procedure today. However, predicting the process performance after scaling up from the development scale to the final production scale is still a critical problem in modern bioprocess development. With increasing cultivation scale inhomogeneities start to appear. Due to insufficient mixing caused by limited power input and the use of highly concentrated and viscous feeding solutions, substrate excess in a zone near the feeding point can be detected, triggering higher metabolic activity and thus oxygen limitation. The limited mixing also leads to starvation conditions in other compartments of the bioreactor, where almost no substrate can be detected. Close to the aeration inlet, this may lead to cells experiencing starvation combined with oxidative stress.

To study the effects of these oscillating cultivation conditions on a miniproinsulin producing *Escherichia coli* K-12 strain two- and three-compartment scale-down bioreactors were used in this thesis. The second compartment represents a “feeding zone” with high substrate availability and oxygen limitation and the third compartment additionally incorporates oscillating starvation conditions. The third compartment could be aerated to combine substrate starvation either with oxygen limitation or oxidative stress, respectively. The results showed increased production of metabolites from the mixed acid fermentation and overflow metabolism pathways. Furthermore, we detected accumulation of the non-canonical amino acids norvaline, norleucine and β-methyl-norleucine and the misincorporation of these amino acids into the recombinant miniproinsulin under oscillating conditions.

These results implicate that oscillating cultivation conditions should be already applied at the screening stage at the beginning of bioprocess development to identify production clones with highest productivity and robustness, i.e. product quality under process-like conditions. However, as it is not feasible to use a multi-reactor-scale-down setup for screening many candidate strains due to the complexity of setup and experiments, a further scale-down was performed by using cyclic pulsed-feeding and/or repeated short-time shaker stops in shake flask and multi-well plate one-compartment set-ups.
Also, a fluorescence-based assay for at-line characterization and quantification of recombinant miniproinsulin based inclusion bodies on culture- and single-cell-level was developed, which opens at-line monitoring of protein formation as a basis for a novel process analytical tool and process parameter for bioprocess control.

Keywords: scale-down, large-scale, fed-batch cultivation, non-canonical amino acids, norvaline, norleucine, β-methylnorleucine, recombinant proteins, inclusion bodies, misincorporation, insulin, *E. coli*
Zusammenfassung


Diese Beobachtungen legen nahe, dass inhomogene Kultivierungsbedingungen bereits frühzeitig in der Bioprozessentwicklung bedacht und angewendet werden sollten, um bereits beim Screening Klone zu identifizieren, die unter Prozess-simulierenden Bedingungen und

Christian Reitz
Zusammenfassung

Christian Reitz


Schlagworte: Scale-down, Industriemaßstab, nicht-kanonische Aminosäuren, Norvalin, Norleucin, ß-Methyl-Norleucin, rekombinante Proteine, Inclusion bodies, Fehleinbau, Insulin, E. coli
Acknowledgements

Although only one gets the honor of receiving a doctoral degree, this work would not have been possible without all the valuable contributions over the past years. I want to use this opportunity to show my appreciation to these people.

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I also thank Dr. Stefan Junne for his productive feedback and proposals he had during our discussions.

I need to thank my dear colleague Dr. Ping Lu for the excellent teamwork on the bioreactor cultivations. Due to the complexity of scale-down-cultivations as well as cultivations times over 20 h succeeding these cultivations would not have been possible without such a partner as her, supporting and encouraging no matter how early we started or during the races for the last metro in the night. I also appreciate her support in the preparation of the uncountable number of samples for analysis and the endless discussions we had about the scientific data and their interpretation. Also, I want to thank the students who supported me during the cultivations – namely Franziska Vera Ebert, Ongey Elvis Legala, Christoph Klaue, and Qin Fan – and analysis, especially Sergej Trippel and Robert Spann for introducing me into GC-MS analysis.

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Contributions

Besides the author (Christian Reitz), this dissertation thesis would not have been possible without contributions from Ms. Dr. Ping Lu, Ms. Franziska Vera Ebert, Ms. Qin Fan and Ms. Houda Kalot.

Dr. Ping Lu and Qin Fan participated in the Scale-Down cultivations, supporting sampling and GC-MS analysis.

Franziska Vera Ebert performed and analyzed the cultivations in the 2 L scale and supported the multi-well-plate experiments.

Houda Kalot supported the cultivation experiments of the optimization and calibration of the fluorescence assay. In addition, Anika Bockisch and Markus Fiedler performed the flow-cytometry and fluorescence microscopy analyses.
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## Abbreviations

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<th>Meaning</th>
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<tbody>
<tr>
<td>aa-AMP</td>
<td>Aminoacyl-adenosine monophosphate</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>Acetyl Coenzyme A</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CAA</td>
<td>Canonical amino acid</td>
</tr>
<tr>
<td>BCAA</td>
<td>Branched-chain amino acid</td>
</tr>
<tr>
<td>DCW</td>
<td>Dried cell weight</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOT</td>
<td>Dissolved oxygen tension</td>
</tr>
<tr>
<td>FADH₂</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IB</td>
<td>Inclusion bodies</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>Met</td>
<td>Methionine</td>
</tr>
<tr>
<td>β-MetNle</td>
<td>β-Methylnorleucine</td>
</tr>
<tr>
<td>MSM</td>
<td>Mineral salt medium</td>
</tr>
<tr>
<td>MTBSTFA</td>
<td>N-(tert-butyldimethylsilyl)-N-methyl-trifluoro-acetamide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>Nva</td>
<td>Norvaline</td>
</tr>
<tr>
<td>Nle</td>
<td>Norleucine</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical density at the wavelength of 600 nm</td>
</tr>
<tr>
<td>OTR</td>
<td>Oxygen transfer rate</td>
</tr>
<tr>
<td>OUR</td>
<td>Oxygen uptake rate</td>
</tr>
<tr>
<td>OTS</td>
<td>Orthogonal translation system</td>
</tr>
<tr>
<td>o-tRNA</td>
<td>Orthogonal transfer RNA</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PFR</td>
<td>Plug flow reactor</td>
</tr>
<tr>
<td>pO₂</td>
<td>Dissolved oxygen partial pressure</td>
</tr>
<tr>
<td>RID</td>
<td>Refractive index detector</td>
</tr>
<tr>
<td>RQ</td>
<td>Respiration quotient</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel</td>
</tr>
<tr>
<td>STR</td>
<td>Stirred tank reactor</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid cycle</td>
</tr>
<tr>
<td>TCR</td>
<td>Two-compartment reactor</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
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</table>
1. Introduction

Nowadays, the research on and production of recombinant proteins has increased demand for more applications in numerous pharmaceutical and industrial areas developed to economic scale. By early 2009, over 150 recombinant protein-based pharmaceutical compounds have been licensed by the U.S. Food and Drug Administration and European Medicines Agency (Ferrer-Miralles et al. 2009). One of the most important engineered host strain for recombinant production of pharmaceutical proteins at the commercial scale is *Escherichia coli*. It is well-known and established as a robust cell factory. Numerous studies describe its use for heterologous production of many natural products originating from other bacteria, fungi or plants via molecular biology tools (Schmidt 2004; Huang, Lin, and Yang 2012).

The critical aim for bioprocess engineering with a heterologous product in mind is to obtain a high-quality yield of the product in combination with optimum process efficiencies and ultimately lower the production costs of the final product. A highly-optimized process is crucial as bioprocess conditions affect not only the amount of produced recombinant proteins but also their solubility and posttranslational modifications and so the complexity of downstream processing. Also, the most important economic discussion in bioprocess development is the scale-up and transfer of a bioprocess from the laboratory scale to the final industrial production scale (Bylund et al. 1998). However, a finally up-scaled bioprocess is then set and later modifications and optimizations not seen in the lab-scale cannot be applied anymore due to economic reasons and approval restrictions. A full scale-up from lab to production scale is also long-lasting and so connected to a financial risk. In the end, a bioprocess scale-up is limited on focusing on the main bioprocess parameters defined by mechanical restrictions, technical limitations, or simply economic reasons (D. Wang 1979). Several scale-down methodologies have been developed to research and understand critical scale-up parameters and their impacts on the cultivation process (Neubauer and Junne 2016). New insights on physiological responses of cells towards gradients in industrial scale like environments and on optimal growth as well as process conditions under large scale conditions aiming at highest productivity were gained (Lemoine et al. 2015).
Using *E. coli* as engineered production host introduces well-known and characterized advantages into bioprocess development, such as:

1. Simple cultivation conditions and fast growth rates
2. Whole genome sequence and manifold genetic engineering tools available
3. Grows on low-cost substrates
4. Well-characterized metabolic and regulation pathways
5. Easily scalable cultivation techniques
6. Numerous strains engineered for specific expression tasks already available

However, *E. coli*, as well as other prokaryotic organisms as host for the heterologous production of complex metabolites, has also several drawbacks. The most important are codon usage bias between the source strain and the expression host, incorrect folding of the target protein, lack of posttranslational modifications, and inefficient secretion. Furthermore, necessary precursors needed for correct protein expression can be missing in the production host. Consequently, producing recombinant proteins in *E. coli* leads often to protein aggregates instead of correctly folded heterologous proteins, which are harvested as inclusion bodies (Neubauer, Hauke, and Antonio 2006). Also, due to limited mixing capacities, gradients concerning the nutrient and oxygen availability develop when scale and cell density reach a critical level in an industrial scale bioreactor. In *E. coli*, a high substrate concentration in combination with oxygen limitation triggers an increased production of metabolites based on pyruvate due to overflow metabolism and mixed-acid fermentation. Furthermore, a higher flux into the branched-chain amino acid pathway can be seen leading to an increased production of branched-chain amino acids including non-canonical amino acids like norvaline (Soini, Ukkonen, and Neubauer 2011). Non-canonical amino acids can be incorporated into proteins, e.g. norvaline as a substitute for leucine. Also, methionine is known to be exchanged by norleucine (Randhawa et al. 1994). The aim of this thesis is a better understanding of impacts of oscillating cultivation conditions on misincorporations of non-canonical amino acids into a leucine-rich recombinant miniproinsulin expressed as Inclusion bodies. Also, we discuss how to transfer these oscillations into the screening scale.
2. Literature review – Part I: Introduction into E. coli physiology

2.1. Central carbon metabolism in *Escherichia coli*

The term metabolism sums up all biochemical processes which take place in a cell or an organism. Characterizing bacterial metabolism focuses in general on uptake and utilization of organic and inorganic molecules to generate energy or endogenous compounds for cell growth and maintenance catalyzed by enzymatic systems. Heterotrophic metabolism by bacteria describes the breakdown of organic molecules with the aim to conserve energy in adenosine triphosphate (ATP) and to produce other organic metabolites usable as a precursor for further biosynthetic or assimilatory processes inside the cell. In addition to respiration, fermentation is a special metabolic pathway used by several microorganisms under oxygen limitation. Here, not oxygen but organic molecules are the final acceptor for electrons and hydrogen ions leading to not completely oxidized substrates and so a decreased yield of energy from the substrate and decreased growth. In microbial cells growing under fermentative conditions phosphorylation at the substrate-level is the most common reaction for ATP generation via a transfer of phosphate group from a high-energy organic compound to ADP. In Figure 2.1 a detailed picture of the major metabolic pathways (respiratory and fermentative) and the catalyzing enzymes responsible for the distribution of carbon between catabolism, anabolism and energy supply inside *Escherichia coli* cells are illustrated.
2.1.1. Mixed-acid fermentation

The mixed-acid fermentation is an anaerobic metabolic reaction pathway catalyzing the breakdown of a hexose (mostly glucose) into a complex mixture of organic acids. Mixed acid fermentation is common in bacteria and a characteristic feature of the Enterobacteriaceae family, which includes *Escherichia coli* (Madigan et al. 2014). During a limited supply of dissolved oxygen or under anaerobic cultivation conditions, it is the metabolic pathway of choice for ATP generation in *E. coli*. Using glucose as carbon substrate mixed-acid fermentation is a two-stage process: First, glucose is converted to pyruvate via the glycolysis pathway. In addition to pyruvate, four moles of ATP and two moles of NADH are produced per mole glucose. Second, the produced NADH is then reoxidized by reducing pyruvate to one or more products of the mixed-acid fermentation.
Literature review – Part I: Introduction into E. coli physiology

In general, the product pool of mixed-acid fermentation in *E. coli* consists of acetate, ethanol, formic acid, lactate, and succinate. Also, formic acid is further lysed into the gases carbon dioxide and hydrogen via an active formic acid hydrogen lyase enzyme complex (Förster and Gescher 2014). Figure 2.2 illustrates the pathways for anaerobic mixed-acid fermentation and aerobic overflow metabolism (see Chapter 2.1.2) in *E. coli*.

Formic acid production is catalyzed by an anaerobic pyruvate formic acid lyase, encoded by the *pflB* gene, which cleaves non-oxidatively pyruvate into formic acid and Acetyl-CoA (B. Xu et al. 1999). The production and accumulation of formic acid is a critical indicator for oxygen limitation in cultivations (Knappe and Sawers 1990), as the expression of the *pflB* gene and so the presence of pyruvate formic acid lyase is regulated by pyruvate accumulation under oxygen limitation (Sirko et al. 1993). As mentioned before, *E. coli* cells can metabolize formic acid even further to CO₂ and H₂. These reactions are catalyzed by a formic acid hydrogen lyase enzyme complex (FHL) to control the intracellular pH value under anaerobic conditions (Mnatsakanyan, Bagramyan, and Trchounian 2004). This enzyme complex consists of a formic acid dehydrogenase (FDH) and six further enzymes encoded by the *hyc* operon. The FHL complex is depending on a suitable presence of the trace elements molybdenum, nickel, and selenium in the cultivation broth (Biermann et al. 2013).
Like formic acid, lactate is a product of pyruvate conversion. A lactate dehydrogenase (LDH) encoded by *ldhA* catalyzes this reaction. Also, this response reoxidizes one mole NADH back to NAD⁺ per mole lactate produced. Both, lactate dehydrogenase and pyruvate formate lyase, are essential enzymes for mixed-acid fermentation as their reactions establish the entry points for the pathway. Also, their enzyme activity is inhibited by the availability of dissolved oxygen and strongly regulated by enzyme feedback repression (Kessler and Knappe 1996).
Ethanol formation starts from Acetyl-CoA, a by-product from the cleavage of formic acid from pyruvate, catalyzed by an alcohol dehydrogenase complex (ADHE) encoded by \textit{adhE}. The reactions also reconvert two molecules of NADH into NAD\textsuperscript{+} per molecule ethanol produced. Also, Acetyl-CoA in \textit{E. coli} can also be a precursor for acetate production in a two-stage reaction. Chemical conversions via a phosphate acetyltransferase (encoded by \textit{pta}) and an acetate kinase (encoded by \textit{ackA}) generate one mole ATP per mole acetate using substrate-level phosphorylation (Lin and Iuchi 1991).

Synthesis of succinate begins with phosphoenolpyruvate (PEP) as a precursor, an intermediate of glycolysis. The first step is an enzymatic carboxylation of PEP into oxaloacetate by a phosphoenolpyruvate carboxylase encoded by \textit{ppc} (Kai, Matsumura, and Izui 2003). Oxaloacetate is then first converted into malate catalyzed by a malate dehydrogenase (encoded by \textit{mdh}) and further dehydrated into fumarate via a fumarate hydratase (encoded by \textit{fumB}) (Thakker et al. 2012). Finally, \textit{E. coli} reduces fumarate to succinate enzymatically using a fumarate reductase (encoded by \textit{frd}) oxidizing NADH to NAD\textsuperscript{+}. Remarkably, Ingledew and Poole described this reaction as anaerobic respiration. It utilizes electrons linked to a NADH dehydrogenase and the electron transport chain forming an electrochemical gradient in the cells, which can be used to produce ATP by an ATP synthase (Ingledew and Poole 1984).

The mixed acid fermentation and its products are the most significant possibility to maintain a balanced redox state during the metabolization of glucose via glycolysis under anaerobic cultivation conditions. Variable environmental conditions control this complex reaction network (Clark 1989). Produced amounts of each product of the mixed acid fermentation pathway depend on the enzymatic activity of key catalysts and environmental factors such as dissolved oxygen availability, state of substrate oxidation, presence of redox agents, and the change of pH due to accumulation of fermentation products (H. Liu et al. 2011).
2.1.2. Overflow metabolism
Overflow metabolism describes the phenomenon of fermentation of substrate instead of using respiration for energy production and so accepting a loss in yield although sufficient oxygen is present. This metabolic pathway enables the possibility to prevent pyruvate accumulation under conditions with a high carbon flux through glycolysis via by-product formation, if the TCA cycle and aerobic respiration reactions cannot completely oxidize all pyruvate originating from glycolysis (Hollywood and Doelle 1976). In *E. coli* acetate is the main product of glucose triggered overflow metabolism (B. Xu et al. 1999). First, a pyruvate dehydrogenase complex decarboxylates pyruvate to Acetyl-CoA. The produced Acetyl-CoA can then be a precursor for acetate formation instead of entering the TCA cycle. The same phosphotransacetylase and acetate kinase as in the mixed acid fermentation catalyze the conversion of Acetyl-CoA to acetate (Dittrich, Bennett, and San 2005). The expression of the multimeric pyruvate dehydrogenase enzyme complex is down-regulated under oxygen limitation, and its activity is controlled by the pyruvate concentration inside the cell (Quail, Haydon, and Guest 1994) and the NADH/NAD⁺ concentration ratio (De Graef et al. 1999). In general, Acetyl-CoA is further metabolized within the TCA cycle and finally respiration under aerobic conditions, as this yields significantly more ATP and reducing equivalents.

During the last decades, research indicated a link of acetate accumulation due to overflow metabolism and high growth rates of *E. coli* under aerobic cultivation conditions. Starting at an accumulated concentration of 0.5 g L⁻¹ acetate has a significant impact regarding decreased growth rate, reduced biomass yields and so decreased maximum achievable cell numbers, and product yield due to inefficient use of carbon and formation of by-products instead of biomass in high cell density cultivations using *E. coli* (Eiteman and Altman 2006). A set specific growth rate of μ = 0.14 h⁻¹ in an *E. coli* fed-batch process triggered the formation of acetate (Korz et al. 1995). In 2010, Valgepea et al. discovered a novel regulation mechanism for the overflow metabolism pathway in *E. coli* using a systems biology approach including characterization of the transcriptome, proteome, and metabolome. This study indicated a downregulation of Acetyl-CoA synthetase by carbon catabolite repression, and so reduced consumption of acetate produced via the phosphotransacetylase leading to a disturbed acetate recycling over the PTA-ACS node in *E. coli* (Valgepea et al. 2010). Lately, a new study showed that metabolic phenomena like overflow metabolism can be accurately illustrated and quantified via
application of proteome resource allocation (Basan et al. 2015). Overflow metabolism in *E. coli* could be a global metabolic response to balance varying proteomic demands regarding biomass synthesis and energy supply under changing environmental conditions. Interestingly, only *E. coli* K-strains suffer on acetate accumulation due to overflow metabolism. *E. coli* B-strains show in cultivations less acetate accumulation due to higher consumption rates of acetate caused by higher activities of the Acetyl-CoA synthetase and the glyoxylate shunt pathway (Phue et al. 2005).

### 2.1.3. Biosynthesis of amino acids

Proteins are essential building blocks of life carrying out numerous important tasks like structural support, transport carrier, and catalyzing biochemical reactions as enzymes. Their basic building blocks are amino acids, organic acids containing at least one functional carboxylic and amino group. If amino acids are not provided sufficiently by the cultivation medium, *E. coli* is capable of synthesizing them either from precursor compounds in the medium or intermediates of glycolysis and the TCA cycle combined with the assimilation of inorganic nitrogen for efficient cell growth and recombinant protein production (Madigan et al. 2014). Based on the carbon skeleton precursor used for amino acid synthesis all canonical amino acids can be categorized into five families: serine, aromatic, alanine, aspartate, and glutamate based. An overview of these families is summed up in Table 2.1.

**Table 2.1: Amino acid families depending on the carbon skeleton precursor molecules extended to synthesize amino acids**

<table>
<thead>
<tr>
<th>Amino acid family</th>
<th>Carbon skeleton precursor</th>
<th>Amino acids produced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine family</td>
<td>3-phosphoglycerate</td>
<td>Serine, Cysteine, Glycine</td>
</tr>
<tr>
<td>Aromatic family</td>
<td>Phosphoenolpyruvate</td>
<td>Phenylalanine, Tyrosine, Tryptophan</td>
</tr>
<tr>
<td>Alanine family</td>
<td>Pyruvate</td>
<td>Alanine, Valine, Leucine, Isoleucine</td>
</tr>
<tr>
<td>Aspartate family</td>
<td>Oxaloacetate</td>
<td>Aspartate, Asparagine, Homoserine, Methionine, Threonine, Isoleucine, Lysine</td>
</tr>
<tr>
<td>Glutamate family</td>
<td>α-ketoglutarate</td>
<td>Glutamate, Glutamine, Proline, Arginine</td>
</tr>
</tbody>
</table>
Figure 2.3 illustrates the biosynthetic pathways of amino acids related to the glycolysis and the TCA cycle. 3-phosphoglycerate is the precursor for amino acids from the serine family, which is an intermediate of glycolysis. Also, the reactions of this biosynthetic pathway provide a crucial ratio of the carbon flux required for the formation of purines and thymine. Aspartate and glutamate family based amino acids are products from intermediates of the TCA cycle. Glutamate, for example, is directly catalyzed from α-ketoglutarate via amination and so supporting a balanced TCA cycle. Also, transamination reactions with glutamate and glutamine as a source for amino groups realize the introduction of nitrogen into metabolic pathways, so the biosynthesis of glutamate and glutamine is essential for the assimilation of inorganic nitrogen in a defined cultivation medium. Glutamate provides almost every amino group for freshly synthesized amino acids. Furthermore, the role of glutamate as nitrogen provider is so fundamental it is one of the highest concentrated compounds solved inside an *E. coli* cell and further serves as an osmotic stabilizer between the cytosol and extracellular medium. The precursor for aspartate and relatives is oxaloacetate, also an intermediate of the TCA cycle. Oxaloacetate conversion into aspartate with an amino group provided by glutamate leads to α-ketoglutarate as a by-product of this reaction. Aspartate itself can act as a precursor for the formation of asparagine, lysine, methionine, and threonine, which is also enzymatically deaminated to provide the basis for modified branched-chain amino acids. Alanine is a product of transamination of pyruvate, which is also one precursor in the biosynthesis of the branched-chain amino acids including isoleucine, leucine, and valine (Umbarger 1996).

### 2.2. Formation of canonical and modified branched-chain amino acids

Of the defined proteinogenic 20 amino acids canon three amino acids can be categorized as branched-chain amino acids (BCAAs): isoleucine, leucine, and valine. The most obvious aspect of BCAAs should be the relaxation and growth stimulating effect in human muscles, if supplemented after training (Shimomura et al. 2004). Similar effects could be seen in rat SK muscles (Balage et al. 2001). Sufficient BCAA concentrations can also increase the glucose uptake in liver and SK muscle tissues as well as increase the glycogen production due to higher activities of the glycogen synthetase (Nishitani et al. 2004). Furthermore, an effect on lipid oxidation during exercise was observed reducing fatigue and so support degradation of body fat (Qin et al. 2011). They also play a major role in the brain and may have a direct or indirect regulatory effect on the biosynthesis and function of brain proteins and neurotransmitters like...
dopamine, norepinephrine, and serotonin (Fernstrom 2005). Consequently, BCAAs are in discussion to be used as a treatment of numerous neurological disorders (Batch, Hyland, and Svetkey 2014). Furthermore, serum concentration levels of BCAAs and related metabolites could provide a novel biomarker for cardiometabolic health issues independent from other standard factors like the body-mass-index (Batch et al. 2013).

2.2.1. Biosynthesis of branched-chain amino acids
Similar biochemical reactions build the base for the biosynthesis pathways for the amino acids isoleucine and valine because the same set of enzymes catalyze the production of both amino acids from different precursors (see Figure 2.3). The synthesis starts with a decarboxylation of pyruvate to an active acetaldehyde bound to the thiamin pyrophosphate prosthetic group of the pyruvate decarboxylase in the pyruvate dehydrogenase enzyme complex. This active acetaldehyde is then bound to another acetaldehyde to form α-acetolactate, the precursor for valine production, or with α-ketobutyrate, derived from a deamination of threonine, to form α-aceto-α-hydroxybutyrate, the precursor for isoleucine production.

These synthesis reactions are catalyzed by an acetohydroxy acid synthase (AHAS). Three different isozymes of this enzyme were found in wild-type strains of Escherichia coli and Salmonella typhimurium encoded by the genes ilvBN (I), ilvGM (II) and ilvIH (III). Each of these isozymes catalyzes above-mentioned thiamin-pyrophosphate-dependent decarboxylation of pyruvate and the transfer of the remaining acetaldehyde to α-ketobutyrate or pyruvate. Also, all three isozymes need Flavin adenine dinucleotide (FAD) as a cofactor and prosthetic group. Interestingly, FAD separates from isozyme I and II and needs to be added as a cofactor in in-vitro assays, but is strongly bound to isozyme III (Sella et al. 1993). Expression levels of AHAS enzymes are regulated via product feedback inhibition by one or more branched-chain amino acids (Umbarger 1996).

Based on the influences to the feedback control AHAS II and III are more likely to catalyze α-aceto-α-hydroxybutyrate resulting in isoleucine formation compared to AHAS I. In addition, the synthesis of isozyme I and III is inhibited by high valine concentrations, whereas isozyme II expression is not influenced by valine concentrations. Unfortunately, AHAS II is inactive in E. coli K-12 strains due to a frame-shift mutation in the ilvG gene encoding the large subunit of this isozyme (Lawther et al. 1981). This loss of AHAS II in E. coli K-12 strains leads to the so-
called “valine toxicity phenomenon”: A high intracellular concentration of valine inhibits the expression and activity of AHAS I and III. As AHAS II cannot be synthesized, this leads to a total breakdown of leucine and isoleucine production (D. Andersen et al. 2001). Therefore, the supplementation of valine in E. coli K-12 cultivations can trigger a stringent response in the cells as the presence of valine leads to leucine and isoleucine starvation (Hecker, Schroeter, and Mach 1983).

The synthesis of α-ketobutyrate is in general enzymatically catalyzed by a transamination of threonine via a biosynthetic threonine deaminase encoded by the ilvA gene in E. coli and S. typhimurium. There is a second catabolic threonine deaminase encoded by tdcB (Umbarger and Brown 1957). Both enzymes are inactivated by high concentrations of serine (“serine toxicity”), but only the activity of the biosynthetic threonine deaminase is feedback regulated by isoleucine. Further, isoleucine formation is controlled by the concentration of isoleucine itself and so prevents accumulation of isoleucine in the cells. A C-terminal regulation domain realizes feedback regulation (Taillon, Little, and Lawther 1988). Though, there is a proposed shortcut in the formation of α-ketobutyrate deriving from pyruvate via a direct carbon chain extension catalyzed by the leuABCD operon enzymes alternating the carbon flux from pyruvate directly into the synthesis pathway of isoleucine (Bogosian et al. 1989). This proposal is based on enzymatic kinetic studies on the α-isopropylmalate synthase from Salmonella typhimurium (Kohlhaw, Leary, and Umbarger 1969) and Serratia marcescens (Kisumi, Sugiura, and Chibata 1976a) and braced by recent studies (Soini et al. 2008).
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Figure 2.3: Schematic overview of the biosynthesis pathway of the branched-chain amino acids valine, leucine and isoleucine starting at pyruvate in connection to glycolysis, tricarboxylic acid cycle and from threonine in E. coli AHAS, acetohydroxy acid synthase including three isozyme forms encoded by ilvBN, ilvGM and ilvIH; DH, dihydroxy acid dehydratase encoded by ilvd; PMS, α-isopropylmalate synthase encoded by leuA; IR, acetohydroxy acid isomeroreductase encoded by ilvC; IPMD, β-isopropylmalate dehydrogenase encoded by leuB; ISOM, α-isopropylmalate isomerase encoded by leuCD; TD, threonine deaminase encoded by ilvA; TrB, transaminase B encoded by ilvE; AK, aspartokinase; ASAD, aspartate β-semialdehyde dehydrogenase; HSAT, homoserine acyltransferase; HSD, homoserine dehydrogenase; HSK, homoserine kinase; TS, threonine synthase; TrC, transaminase C encoded by avtA; tyrB, a gene encoding aromatic transaminase which is tyrosine repressible. Dashed lines represent selected feedback regulation pathways of synthesized branched-chain amino acids. (adapted and modified from Kisumi, Komatsubara, and Chibita 1977; Umbarger 1978; Nelson and Cox 2008).
α-ketoisovalerate, an intermediate of valine formation, is also the precursor for the synthesis of leucine (Umbarger 1996). The production of leucine includes a carbon chain extension of α-ketoisovalerate to α-ketoisocaprate followed by a transamination resulting in leucine (Fig. 2.3). In detail, leucine synthesis starts with the transfer of an acetyl group of Acetyl-CoA to α-ketoisovalerate to form α-isopropylmalate. This reaction is catalyzed by a α-isopropylmalate synthase (PMS) encoded by leuA and feedback inhibited by high concentrations of leucine (Leary and Kohlhaw 1970). Then α-isopropylmalate is isomerized into β-isopropylmalate with dimethyl-citraconate as intermediate via a α-isopropylmalate isomerase (ISOM) encoded by the genes leuC and leuD (Fultz and Kemper 1981). Finally, β-isopropylmalate is converted into α-ketoisocaprate via an oxidative decarboxylation catalyzed by a β-isopropylmalate dehydrogenase (IPMD) encoded by leuB and using NAD+ as a cofactor and hydrogen acceptor (Parsons and Burns 1969). Although the main substrate for PMS is α-ketoisovalerate, the enzyme shows promiscuity towards other α-ketoacids and accepts them as a substrate for carbon chain elongation. These α-ketoacids can include pyruvate, α-ketobutyrate or α-ketovalerate and result in modified non-canonical amino acids derived from the leucine synthesis pathway, like the commonly known modified branched chain amino acids norvaline, norleucine, and β-methyl-norleucine (Apostol et al. 1997; Sycheva et al. 2007; Soini et al. 2008).

The final reaction in the formation of isoleucine and valine is a transamination with glutamate as a donor of the amino group catalyzed by a transaminase B encoded by the ilvE gene. Here, α-keto-β-methylvalerate or α-ketoisovalerate is converted into isoleucine or valine, respectively (Rudman and Meister 1953). This enzyme shows a higher affinity for α-keto-β-methyl-valerate than for α-ketoisovalerate. Interestingly, valine can also be produced via transamination using an amino group of alanine or α-aminobutyrate catalyzed by the transaminase C encoded by avt (Whalen and Berg 1982). Consequently, strains with a mutated ilvE gene lacking transaminase B activity can still produce valine but are auxotroph for isoleucine (Berg et al. 1988).
The last step in the biosynthesis of leucine is a transamination of \( \alpha \)-ketoisocaproat to leucine with glutamine as a donor of the amino group catalyzed by former mentioned transaminase B or an aromatic transaminase encoded by tyreb, which is feedback regulated by tyrosine. ilvE mutants in addition to the earlier mentioned possibility to still produce valine are also able to produce leucine and not dependent on leucine supplementation for sufficient growth (Vartak et al. 1991).

### 2.2.2. Biosynthesis of modified non-canonical branched-chain amino acids

Only 20 amino acids build the base for the known proteinogenic amino acid canon, although over 300 individual amino acids have been identified in nature. As the occurrence of these amino acids is rare and they are no standard building blocks for protein synthesis, they are defined as non-canonical amino acids. Mostly, non-canonical amino acids are used to produce secondary metabolites via non-ribosomal peptide synthesis (Shoji and Sakazaki 1970). Some non-canonical amino acids can be misincorporated into cellular and recombinant proteins in bacterial cells (Bogosian et al. 1989).

The first study mentioning a non-canonical amino acid was published in 1953, revealing norvaline as a compound of an antifungal peptide secreted by Bacillus subtilis (Nandi and Sen 1953). During the last decades, research has shown that the non-canonical amino acids norleucine and \( \beta \)-methyl-norleucine are by-products in deregulated Serratia marcescens mutants overproducing isoleucine. Further, comparable to norvaline synthesis, \( \alpha \)-ketobutyrate is the common precursor (Kisumi, Sugiura, and Chibata 1976a).

The first hypothesized pathway for the synthesis of norvaline was described in 1976 starting from \( \alpha \)-ketobutyrate as a precursor with \( \alpha \)-ketovalerate as intermediate compound catalyzed by the enzymes normally producing leucine encoded by the leuABCD operon (Kisumi, Sugiura, and Chibata 1976a). Later, this suggested “\( \alpha \)-ketoacid-chain-elongation pathway” in E. coli was enhanced with a synthesis route for norleucine (Kisumi, Sugiura, and Chibata 1976a). It is in discussion, whether a broader substrate specificity range of the enzymes encoded by the leuABCD operon causes an alternative activity towards the “\( \alpha \)-ketoacid-chain-elongation pathway”. Conditions demanding an increased leucine synthesis would trigger the production of non-canonical branched chain amino acids (Bogosian et al. 1989). Figure 2.4 sums up the biosynthesis pathways of norvaline, norleucine, and \( \beta \)-methyl-norleucine. These amino acids
are products of enzymatic reactions starting from α-ketobutyrate catalyzed by α-isopropylmalate synthase (PMS) (leuA), α-isopropylmalate isomerase (ISOM) (leuCD), α-isopropylmalate dehydrogenase (IPMD) (leuB) and concluded by a transamination. A detailed description of these catalyzed reactions was given earlier (see chapter 2.2.1.).

As mentioned before and shown in Figure 2.4, PMS condenses α-ketobutyrate, also the precursor for isoleucine synthesis, and Acetyl-CoA to α-ketovalerate, which is the common precursor for norvaline, norleucine, and β-methylnorleucine. The carbon chain of α-ketovalerate elongates in combination with isomerization and reduction reactions resulting in the formation of α-ketocaproate (final intermediate before norleucine formation) or α-keto-β-methyl-caproate (final intermediate before β-methyl-norleucine formation). Norvaline is synthesized via a direct transamination of α-ketovalerate into norvaline (Kisumi, Sugiura, and Chibata 1976a). α-ketobutyrate is the main compound in the synthesis of these non-canonical amino acids. Accumulation of α-ketobutyrate is a prerequisite, since the affinity of 2-IPMS for α-ketobutyrate is an order of magnitude lower compared to its natural substrate α-ketoisovalerate as detected for S. marcescens (see Table 2.1) (Sycheva et al. 2007).

<table>
<thead>
<tr>
<th>α-ketoacid</th>
<th>(K_m) (M)</th>
<th>(v_{max}) (n mole CoA min(^{-1}) mg(^{-1}) protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-ketoisovalerate</td>
<td>7.7 x 10(^{-4})</td>
<td>49</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>3.4 x 10(^{-3})</td>
<td>33</td>
</tr>
<tr>
<td>α-ketobutyrate</td>
<td>7.7 x 10(^{-3})</td>
<td>64</td>
</tr>
<tr>
<td>α-ketovalerate</td>
<td>9.0 x 10(^{-3})</td>
<td>16</td>
</tr>
<tr>
<td>α-keto-β-methyl-valerate</td>
<td>-</td>
<td>1</td>
</tr>
</tbody>
</table>

Due to a frameshift mutation in the \(ilvG\) gene, encoding a subunit of the first enzyme of the Ile pathway, AHAS II, which has the highest affinity for α-ketobutyrate, E. coli K-12 strains could favor the accumulation of α-ketobutyrate in comparison to other strains. The synthesis pathway for α-ketobutyrate is well-known. It derives from the oxaloacetate over aspartate, homoserine, and threonine. Interestingly, the formation of norvaline and norleucine could not be prevented by knocking out the \(ilvA\) gene encoding the threonine aminase converting
threonine into α-ketobutyrate in *E. coli* (Sycheva et al. 2007). Hence, an alternative pathway based on other major carbon metabolites must exist for the formation of α-ketobutyrate. In literature, a “shortcut” reaction pathway from pyruvate towards α-ketobutyrate is discussed and described in detail in chapter 2.2.1. (Sycheva et al. 2007).

![Schematic view of predicted biosynthetic pathway of the modified branched-chain amino acids](image)

**Figure 2.4:** Schematic view of predicted biosynthetic pathway of the modified branched-chain amino acids including norvaline, norleucine and β-methylnorleucine from pyruvate via the so called “ketoacid chain elongation pathway” over α-ketobutyrate and α-ketovalerate to α-ketocaproate facilitated by the promiscuous enzymes of the (iso)-leucine biosynthetic pathway in *E. coli* (based on data from Kohlhaw, Leary, and Umbarger 1969; Masahiko Kisumi, Komatsubara, and Chibita 1977; Bogosian et al. 1989; Muramatsu, Misawa, and Hayashi 2003; Soini et al. 2008).
2.2.3. Misincorporation of non-canonical amino acids into recombinant proteins

Non-canonical amino acids (NCAAs) like norvaline, norleucine and β-methyl-norleucine gained an increased interest because they could be incorporated in minor concentrations into recombinant proteins in *E. coli*, which would alter the quality of the target protein and is such an unwanted crucial factor in the expression of pharmaceutical proteins. Incorporation of NCAAs into recombinant proteins occurred as substitution of proteinogenic amino acids and was proven for different recombinant proteins. Norvaline replaced leucine during the production of recombinant hemoglobin (Apostol et al. 1997). Norleucine was shown to be falsely incorporated instead of methionine into a recombinant produced human brain-derived neurotrophic factor (Sunasara et al. 1999) as well as interleukin 2 (L. Tsai et al. 1988). β-methyl-norleucine was shown to be a substitute for isoleucine during expression of a recombinant hirudin (Muramatsu, Misawa, and Hayashi 2003). Table 2.2 gives a detailed summary.

In general, the misincorporation of non-canonical amino acids can be observed in *E. coli* under cultivation conditions, which derepress the branched-chain amino acid pathway and especially during expression of a leucine-rich recombinant protein (Fenton et al. 1997). The former mentioned recombinant hemoglobin, for example, had a leucine ratio of 13% while Interleukin-2 even has a leucine ratio of 17%.
Table 2.2: Summary of references for the incorporation of modified amino acids derived from the branched chain amino acid pathway into heterologous proteins.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Product</th>
<th>Comment</th>
<th>AA composition</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL</td>
<td>Hirudin in E. coli</td>
<td></td>
<td></td>
<td>(Muramatsu, Miura, and Misawa 2002)</td>
</tr>
<tr>
<td>NL</td>
<td>Bovine somatotropin in E. coli</td>
<td>NL in Met positions</td>
<td>27 leu/191aa</td>
<td>(Bogosian et al. 1989)</td>
</tr>
<tr>
<td>NL</td>
<td>human brain derived neurotrophic factor in E. coli</td>
<td></td>
<td></td>
<td>(Sunasara et al. 1999)</td>
</tr>
<tr>
<td>NL</td>
<td>interleukin-2 in E. coli</td>
<td>NL in Met positions</td>
<td>26 Leu/152 aa</td>
<td>(Lu et al. 1988; L. Tsai et al. 1988; Fenton et al. 1997)</td>
</tr>
<tr>
<td>NL</td>
<td>Met-rich vaccine candidate</td>
<td>NL in Met positions</td>
<td></td>
<td>(Ni et al. 2015)</td>
</tr>
<tr>
<td>ß-MNL</td>
<td>Hirudin in E. coli</td>
<td>In Ile positions</td>
<td></td>
<td>(Muramatsu, Miura, and Misawa 2002; Muramatsu et al. 2002; Muramatsu, Misawa, and Hayashi 2003)</td>
</tr>
<tr>
<td>HIL</td>
<td>Coiled-coil peptide A1 in E. coli</td>
<td>Homoisoleucine in Leu positions</td>
<td></td>
<td>(Van Deventer, Fisk, and Tirrell 2011)</td>
</tr>
</tbody>
</table>


2.2.4. Incorporation mechanism for non-canonical amino acids into heterologous proteins

Non-canonical amino acids are separated into two particular groups. NCAAs which show isostructural characteristics to canonical amino acids (CAA) can be falsely be recognized and utilized by the cell own protein synthesis machinery. NCAAs, which are interesting for protein engineering but show no similarities to CAA, are called orthogonal to the host cell, as the microorganism cannot transfer these amino acids into their translational system. Replacement of a specific CAA against an isostructural NCAA is done via supplementing the
NCAA into cultivations using a host strain auxotrophic against the desired CAA. This forces the host strain to utilize the supplemented NCAA (Link, Mock, and Tirrell 2003). Incorporation of orthogonal NCAAs is harder, as the translational synthesis machinery of the host cell needs reprogramming. This approach includes the transfer of the aminoacyl-tRNA synthetase (aaRS) and tRNA corresponding to the desired NCAA as well as changing a stop or non-occupied codon to an amino acid related codon (L. Wang et al. 2001). These so-called orthogonal translation systems (OTs), consisting of an aminoacyl-tRNA synthetase (o-aaRS) and tRNA (o-tRNA) are usually gained from phylogenetically distant organisms (Y. Xu et al. 2014). Reprogramming of the genetic code has also been shown in nature for the two natural proteinogenic NCAAs, selenocysteine, and pyrrolysine. Stop codons used with a low frequency were reprogrammed by the host to accept these amino acids for synthesis of homologous and recombinant proteins (Hoesl and Budisa 2012).

![Figure 2.5: Leucine, isoleucine and methionine in comparison to their isostructural analogues norvaline, β-methyl-norleucine and norleucine.](image)

The earlier mentioned norvaline and norleucine are known to be produced and accumulated due to the minor substrate specificity of the branched chain amino acid synthesis enzymes catalyzing reactions with different structural related α-ketoacids as substrates. In combination with the also, not absolute substrate specific leucyl-tRNA synthetase mischarged norvalyl-tRNA\textsubscript{Leu} is produced, which is not recognized during translational proofreading and results in norvaline-containing proteins (Apostol et al. 1997). Problematic is that oscillating oxygen
limitation in *E. coli* cultivations comparable to industrial scale conditions increases the intracellular accumulation of pyruvate and triggers the biosynthesis of norvaline (Soini et al. 2008).

**Table 2.3 Alternative substrates for aminoacyl-tRNA synthetases**

<table>
<thead>
<tr>
<th>tRNA synthetase</th>
<th>Other analogues accepted</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MetRS</td>
<td>Norleucine, cis-crotylglycine, 2-aminoheptanoic acid, norvaline, 2-butynylglycine, allylglycine</td>
<td>(Kiick, Weberskirch, and Tirrell 2001)</td>
</tr>
<tr>
<td>LeuRS</td>
<td>Norvaline</td>
<td>(Apostol et al. 1997; Tang and Tirrell 2002)</td>
</tr>
<tr>
<td>IleRS</td>
<td>ßMNL, (Val)</td>
<td>(Umbarger 1996)</td>
</tr>
</tbody>
</table>

A similar mechanism is a base for the exchange of methionine by norleucine. An overview on the substrate promiscuity of aminoacyl-tRNA synthetases can be seen in Table 2.3.

2.2.5. **Toxicity and characteristics of incorporated non-canonical amino acids**

Incorporation of non-canonical amino acids into recombinant proteins could lead to altered protein structures, and so changed chemical properties or even new functions. Already in the 1970s, it was described how the misincorporation of NCAAs could lead to extreme physiological changes inside of *E. coli* cells. Supplementing canavanine, a NCAA which could replace arginine in protein biosynthesis, leads to spontaneous cell lysis due to a metabolic breakdown caused by the accumulation of non-functional enzymes (J. Hewitt and Kogut 1977). In general, most of the NCAA spectrum induces growth inhibition in microorganisms, if the incorporation rate exceeds a certain level. As mentioned before, norleucine is isosteric like methionine, and it was early shown, that norleucine could completely replace methionine in recombinant proteins (Anfinsen and Corley 1969). Further, the presence of norleucine can also have a toxic effect on cell growth. Interestingly, *E. coli* is under certain cultivation conditions able to stable produce and accumulate norleucine to down-regulate the leucine biosynthesis pathway, a behavior not seen under standard lab growth conditions (Bogosian et al. 1989).
These undesired toxic effects often result because of the competition of chemically similar amino acids and NCAAs for transport systems or permeases or accidental conversion into a toxic compound. It is known, that structural properties and specificity of the methionine permease can accept methionine and norleucine as substrate. Furthermore, norleucine accumulation shuts off the synthesis of methionine due to the erroneous feedback regulation of the homoserine succinyltransferase, the entry reaction into the methionine synthesis pathway (Kisumi, Sugiura, and Chibata 1977). It is further under discussion if the misincorporation of NCAAs into recombinant proteins is an influential factor for protein misfolding and so aggregation like it can be seen for many human proteins produced recombinantly in high concentrations in *E. coli* cultivations (Baneyx 1999).

On the other side, NCAAs and their incorporation into proteins open the door for new challenging possibilities to design and produce innovative kinds of enzymes, therapeutics or biopolymers with new activities and characteristics, which are difficult or impossible to synthesize using other chemical or biotechnological approaches. Already known changes via incorporation of NCAAs are immobilized enzymes, protein based polymers, selenoproteins, phosphoproteins, antibody drug complexes or modified therapeutics. These examples show the possibilities behind NCAAs for adapting proteins towards new functions or environments and so they are expanding the chemistry of life.

### 2.2.6. Novel approaches to limit misincorporation of non-canonical amino acids into recombinant proteins

Until today it is not completely clear, which cultivation conditions lead to the incorporation of NCAAs in *E. coli* processes. There are several conventional and straightforward methods and strategies established to prevent norleucine incorporation into recombinant proteins produced in *E. coli* as a host. Known examples are the removal of methionine residues from the protein via changing the DNA sequence, coexpression of norleucine degrading enzymes or the knock-out of genes involved in norleucine synthesis from the host genome (Bogosian et al. 1989). Another simple application to prevent misincorporation is to supplement the cultivation medium with analog isostructural compounds like 2-hydroxy-4-methylthiobutanoic acid or methionine. Continuous feeding of methionine is applied in recombinant *E. coli* cultures, where norleucine incorporation would be critical. Based on this observation it was discussed that an environment with high leucine concentration could
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minimize norvaline incorporation into recombinant proteins (Bogosian et al. 1989), which could be shown around ten years later (Apostol et al. 1997). These studies demonstrate the potential of media supplementation as a powerful tool for ensuring the quality of recombinant proteins. However, especially in the biotechnological industrial scale additional feeding solutions would increase operational complexity and process costs as well as impact process efficiency as additional feeding unlikely dilutes the cultivation medium, and so reduces yields of biomass and recombinant protein.

Strain engineering could be another approach to gain bioprocesses with higher qualities of recombinant protein due to less incorporation of NCAAs. A proof of principle was published, in which an E. coli host was genetically engineered towards a higher activity of the biosynthesis of methionine via several chromosomal mutations. Overproducing methionine via mutations in genes linked to synthesis and regulation of methionine (metA, metK, and metI) prevents norleucine incorporation in this strain without impacting cultivation performance or yield rates negatively (Veeravalli et al. 2015). Another promising approach to prevent norleucine incorporation into recombinant proteins in E. coli is the exchange of the methionyl-tRNA synthetase with a variant from a different organism, which shows no acceptance of norleucine as substrate (Perona and Hadd 2012). Unfortunately, no scientific data identifying a methionyl tRNA synthetase without norleucine activity is known.

The composition of the cultivation medium can also have a strong influence the accumulation of NCAAs. Norvaline and norleucine accumulation is seen in recominant E. coli cultivations under conditions combining glucose excess with oxygen limitation. The addition of the trace elements molybdenum, selenium, and nickel, reduces the accumulation of both amino acids significantly (Biermann et al. 2013). These trace elements are co-factors for the catalyzed reactions of the formic acid-hydrogen lyase metalloprotein complex, which is one of the essential enzymes in the anaerobic mixed-acid fermentation pathway to reduce pyruvate accumulation in E. coli (Yoshida et al. 2007). Also, formic acid accumulation in high cell density cultivations is prevented, as this enzyme converts formic acid into CO₂ and H₂ (Soini, Ukkonen, and Neubauer 2008).
2.3. Inclusion bodies based production of recombinant proteins in *Escherichia coli*

*Escherichia coli* is the most widely used industrial host regarding the expression of recombinant proteins (Baneyx 1999). There are several advantages, which turn *E. coli* into an appreciated host for the commercial production of heterologous proteins. Established and fast cloning techniques are available as well as low-cost cultivation and expression techniques leading to the straightforward and robust production of high concentrations of protein. Nevertheless, *E. coli* is not known for efficient secretion of proteins to the cultivation medium in high concentrations. Furthermore, induced active and vigorous heterologous protein expression in *E. coli* often triggers the aggregation of the target protein into almost pure intracellular inclusion bodies (Fahnert, Lilie, and Neubauer 2004). These inclusion bodies can be formed inside the cytoplasmic as well as the periplasmic compartments of *E. coli* cells. Inclusion bodies of recombinant proteins usually are non-native insoluble aggregates showing no biochemical activity. Consequently, purification of recombinant proteins in inclusion bodies requires not only the separation from cell material but further efficient methods of solubilization of aggregated proteins and refolding them into their native and active form (Vallejo and Rinas 2004). Development and optimization of chemical, enzymatic, mechanical, and physical methodologies for inclusion bodies downstream processing results in yields over 40% of natively folded and active target protein from aggregates (Neubauer, Hauke, and Antonio 2006).

Nonetheless, recombinant protein expression as inclusion bodies in *E. coli* is until today an appreciated production technique and widely applied for heterologous protein production in the commercial scale regardless of the efforts in downstream processing (Walsh 2014). Several advantages of the formation of heterologous inclusion bodies outweigh drawbacks in downstream processing. Using the agglomeration of recombinant proteins as inclusion bodies, expression of high concentrations of the target protein is combined with the easy purification of inclusion bodies from the cultivation broth and cell material. At the same time aggregated proteins are isolated against cellular proteases and are already highly pure (Georgiou and Valax 1999). Further applications of bacterial inclusion bodies and methods to force aggregation of recombinant proteins are summarized and discussed in Rinas et al. (2017).
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Inclusion bodies formation is not only caused by heterologous proteins. Incorrect protein folding during the posttranslational processing is caused by several reasons like stressed cells due to nutrient depletion or a heat shock (Kopito 2000). Protein aggregation triggers stress responses inside the cell and so the increased expression of chaperones, like the hsp70 and hsp100 family, to “rescue” and refold non-native protein structures (Mogk, Kummer, and Bukau 2015). Commercialization of inclusion bodies formation started in the early 1980s, as a recombinant human insulin produced in E. coli was released for the medical treatment of diabetes as the first pharmaceutical compound developed and produced using recombinant DNA (Ladisch and Kohlmann 1992). The precursor for insulin-like the A- and B-chain or a proinsulin are often produced as cytoplasmic inclusion bodies with a final amount of up to 20% of the cellular volume of an E. coli cell during the phase of highest protein expression (Williams et al. 1982).

A major drawback is the loss of productivity in the industrial scale. In a scale-down study using a two-compartment scale-down simulator to research the influences of oscillating oxygen availability on metabolic responses and expression of a recombinant pre-proinsulin in an E. coli host, it was shown that the growth performance of the cells was negatively influenced. The yield of recombinant pre-proinsulin was significantly decreased by oscillating aerobic and anaerobic conditions (Sandoval-Basurto et al. 2005).

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3.1. Microbial bioprocesses

The main purpose of bioreactor cultivations in research and industrial processes is to increase the biomass yields into high cell densities linked to a high yield of desired recombinant protein, as high cell densities are a requirement for maximized volumetric yields of recombinant products in E. coli (Riesenbergs and Guthke 1999). In general, the fed-batch technique is chosen as an approach to gain high cell densities and so high productivities in E. coli cultivation processes. This chapter will give an overview on and discuss important factors, which influence the efficiency of microbial bioprocesses and their control.

3.1.1. Chemically defined cultivation media and the EnBase® technology

The most crucial factor for designing an efficient bioprocess is the choice and composition of the cultivation medium as well as its optimization towards biomass and product yield, as it
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defines the chemical environment during the process and consists of a substrate mixture essential for growth and product formation (Soini, Neubauer, and Ojamo 2012). High cell density cultures have additional nutritional demands. Concentrations of compounds supplying macro elements (mainly C, H, N, O, P) need to be increased while in contrast micro elements like K, S, Mg or trace elements and other growth factors need to be supplied but should not exceed a concentration of 1 %. In general, complex cultivation media are easier to prepare than defined media. An exact composition of these media is usually not known, as they consist of yeast extract, protein hydrolysates or lignocellulose feedstocks, containing all essential nutrients, growth factors and vitamins (Zhang and Greasham 1999). The popular complex three-component Luria-Bertani (LB) broth can supply E. coli cells up to a final dried cell weight (DCW) of 1 g L\(^{-1}\) under temperature, oxygen controlled and pH-regulated conditions. The significant impact of the medium composition on the yield of recombinant proteins in shake flask cultures of E. coli was proven and shown by Ukkonen et al. (2013).

In the early 1990s maximum concentrations of essential medium components for E. coli were established, like 50 g L\(^{-1}\) for glucose, 3 g L\(^{-1}\) for ammonium, 1.15 g L\(^{-1}\) for iron, 8.7 g L\(^{-1}\) for magnesium, 10 g L\(^{-1}\) for phosphorous as well as 0.038 g L\(^{-1}\) for zinc. Higher concentrations would inhibit growth (Riesenberg et al. 1991). A defined medium of exact chemical definition optimized for high cell density growth with the maximum non-inhibiting concentrations of medium components yields to 15 g L\(^{-1}\) DCW of E. coli biomass. In conclusion, an optimized medium composition is necessary for improved process scale-up as well as upstream and downstream processing of cultivations (Lee 1996; Zhang and Greasham 1999). Genetic engineering created E. coli strains able to grow on glucose concentrations up to 100 gL\(^{-1}\) (Lara et al. 2008).

A particular form of the chemically defined cultivation medium is the mineral salt medium consisting only of simple inorganic salts and a defined carbon source (Neidhardt, Bloch, and Smith 1974). A mineral salt medium is usually the medium of choice to produce recombinant proteins in bioreactors in research and industrial processes (B. Xu, Jahic, and Enfors 1999). Though, their application in non-controllable shake flask cultivations is limited as exhausted nutrients cannot be supplemented with a feed. Increasing the concentrations of medium components is not possible as elevated levels of ammonia or magnesium do not only inhibit
growth but can also be toxic to the cells. Also, medium components could precipitate after reaching certain levels. Choosing an alternative carbon source for high cell density cultivations of *E. coli* has shown to reduce or even prevent the accumulation of acetate, which has a beneficial impact on cell growth (Martínez-Gómez et al. 2012).

To overcome the limitations of mineral salt media and application of the fed-batch technique in shake flasks and multi-well plates the enzyme based substrate delivery system EnBase® was developed and is widely used for increased biomass and product yields in shaken cultures. Simulating a fed-batch process in cultivation volumes from the µL to mL and liter scale the EnBase® technology could successfully be applied in high throughput screenings, for recombinant protein expression optimization and simplified plasmid DNA production (Krause, Neubauer, and Neubauer 2016). The fed-batch principle of an EnBase® medium is based on the enzymatically-controlled release of glucose from a complex polysaccharide substrate. The controlled limited availability of free glucose inhibits glucose overflow metabolism in *E. coli*. Thus, the decreased formation of growth inhibiting by-products leads to high cell densities in shaken cultures and increased product protein yields. Also, higher ratios of correctly folded soluble recombinant proteins inside the cells are gained (Krause, Neubauer, and Neubauer 2016).

As mentioned before some medium components are known to possibly precipitate due to the formation of non-soluble complexes of metal-ammonium phosphates, magnesium phosphates or other phosphates depending on the ion concentrations in the cultivation medium (Dean 1990). Precipitation can be prevented by controlling the phosphate concentration in the cultivation medium, e.g. by applying a polyphosphate glass, which allows a slow release of phosphate into the medium via diffusion (Curless, Baclaski, and Sachdev 1996). Precipitation can also be triggered during the cultivation process by the accumulation of organic acids or increased carbon dioxide concentrations.

3.1.2. Cultivation strategies for high cell density bioprocesses

Bioprocesses must fulfill various criteria to become economically feasible, including a high final product yield, a high volumetric productivity, stability, reproducibility and robustness combined with low costs for substrates and operation. In recombinant processes with genetically engineered microorganisms also legal barriers need to be considered.
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Consequently, all industrial bioprocesses derive basically from basically three different operation modes for cultivation based bioprocesses, which are applied to produce recombinant proteins or other compounds in industrial biotechnology, namely the batch, fed-batch and continuous cultivation technique.

A batch culture is per definition a closed system containing all growth-related nutrients at their maximum concentrations initiated by the inoculum and is usually temperature, pressure, pH and aeration controlled. Due to the high nutrient levels in the starting phase of the cultivation, catabolite repression events can be triggered. Also, high growth rates due to highly available substrates may result in overflow metabolism and formation of undesired by-products. Aerobic *E. coli* cultures at maximum growth rates and high concentrations of available carbon lead to the formation of acetate. Accumulating acetate influences cell growth and decreases the yield of biomass and recombinant products through an undesired loss of carbon into acetate (Carneiro, Ferreira, and Rocha 2013). Therefore, batch cultivation approaches cannot be recommended for industrial scale production processes, if these drawbacks impact the process feasibility.

In continuous cultivations, fresh medium is added to the bioreactor over the cultivation time. To keep the reaction volume constant, culture broth is removed from the bioreactor at the same rate as fresh medium is added. These types of bioprocesses can have a reduced operation cost and, as the cultivation can run longer, reactor downtimes for cleaning and sterilization have a decreased impact on process efficiency. However, prolonged cultivation times with access to the bioreactor from the outside increases the risk of contamination or spontaneous mutation of the host lowering or even inhibiting efficient product formation (Kazemi Seresht et al. 2013).

Combining the easiness of the batch with prolonged run times like in continuous cultures is the fed-batch cultivation technique. It is until today the most favored standard process to produce compounds and proteins in bioprocesses. Two of its principal advantages improve the microbial physiological behavior in industrial scale cultivations. The first one is the controlled supply of fresh and usually highly concentrated nutrients. Feeding of substrates opens the possibility to run the process at optimum oxygen transfer conditions. Regulation of microbial growth results in decreased effects of substrate excess on cell physiology, growth,
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and product formation as well as undesired by-product formation (Han 2002). Running a process at the point of maximum oxygen transfer also inhibits the formation of organic acids in *E. coli* under oxygen limitation (Enfors et al. 2001). Furthermore, anaerobic growth under oxygen limitation provides less energy for metabolic reactions in *E. coli*, like the synthesis of proteins. Applying a fed-batch approach for controlled growth under conditions for optimum oxygen supply can surpass several probable process restrictions due to limitations in bioreactor design or power input (Castan and Enfors 2000). The second main benefit is the possibility to design fed-batch processes as high cell density cultivations with maximized space and time yields end increased total volumetric productivity due to extended cultivation periods (Aucoin et al. 2006).

After all, also the fed-batch technique has drawbacks, preventing it from being the perfect process procedure. Feeding to maintain limiting concentrations of the main substrate could trigger cellular starvation responses in high cell densities cultivations, which in extreme cases can lead to loss of performance and product formation due to cell death and lysis (Andersson, Strandberg, and Enfors 1996). Therefore, an optimized composition of the feeding solution, as well as an appropriate feeding strategy, needs to be developed for a feasible bioprocess performance. Several feeding techniques have been established and applied in recombinant *E. coli* fed-batch cultivations including constant feeding, exponential feeding, step-wise increased feeding, short-pulsed feeding and feeding controlled by feedback to maintain constant dissolved oxygen and pH levels (Babaeipour et al. 2008; Tripathi 2009).

Acetate accumulation due to overflow metabolism is triggered in *E. coli* at high growth or glucose uptake rates under aerobic conditions. The major advantage of an exponentially increased feed in a fed-batch cultivation is that the cells can be controlled grown at a desired specific growth rate, which is below the maximum specific growth rate or critical glucose uptake rate to prevent acetate formation in *E. coli* high cell density cultivations (De Mey et al. 2007). Exponential increasing feed is defined as a constant start feeding rate exponentially increasing over the feeding time and can be determined using substrate balance equations (Åkesson et al. 1999).
3.1.3. Process monitoring and control

Nutrient-limited feeding is not the only way to control and maintain a microbial cultivation at the desired growth rate. Feeding strategies can also be based on numerous other physical factors including pH and dissolved oxygen tension (Johnston, Cord-Ruwisch, and Cooney 2002), temperature (Schaepe et al. 2011), or carbon dioxide production (Taherzadeh, Niklasson, and Lidén 2000). In the early 2000s, it was discussed that every growth influencing physical factor or medium compound has the potential to be used as process controlling parameter if there is a way to monitor this parameter with sensitive and reliable sensor technique in the bioreactor (Y.-C. Liu, Wang, and Lee 2001).

The German company PreSens GmbH could apply dissolved oxygen and pH sensitive fluorescent materials into shake flasks. This enhanced labware allows the on-line monitoring of oxygen levels and pH inside the shake flask on a special sensor reading device installed in the incubator without the need for sampling. This system is widely and efficiently applied not only for shake flask experiments but also with the aim of process development (W.-L. Tsai et al. 2012). Furthermore, this sensor technique was additionally transferred into 24-well plates allowing on-line pH and DOT monitoring in a cultivation scale up to 1 mL (Kensy et al. 2005) as well as single-use bioreactors.

Particularly for recombinant bioprocesses, it would be a significant advancement if the product protein synthesis rate could be used for process development, optimization, and control. A drawback here is that the product synthesis rate can usually be calculated after sampling, purification and quantification of the target compound. Approaches have been established with the aim to monitor product formation at-line and to turn the product synthesis rate into a process analytical tool. Proteins, which are usually expressed as soluble recombinant proteins, can be fused to a GFP. The fluorescence of cells is related to the amount of soluble product. The fusion to GFP has no impact on solubility (Waldo et al. 1999). This method is not applicable to recombinant proteins, which do not fold correctly in the host cell and agglomerate into inclusion bodies, as agglomeration would inhibit fluorescence of the GFP. Furthermore, for small recombinant proteins like insulin or Interleukin-2 the size of the fusion partner would surpass the size of the desired product. Interestingly, bacterial inclusion bodies share structural and biological features with amyloid plaques (L. Wang et al. 2008).
could be shown, that an amyloid binding fluorescent dye like Thioflavin-S can penetrate *E. coli* cells and stain bacterial inclusion bodies (Espargaró et al. 2016).

3.2. Scale-up of microbial bioprocesses

In the last decades, the reputation of industrial scale bioprocesses increased all over the world, and biotechnological developments and applications are pushed due to high demand not only of novel biopharmaceuticals but also of innovative chemical non-pharmaceutical compounds worldwide (Festel 2010; Neubauer 2011). Based on the anticipation, efficiently available capacities of industrial scale bioreactors are an essential key point for optimization. Furthermore, the consistent scale-up of productivity and yield rates from the development to the final production scale is the most crucial task in the elaboration of a bioprocess (Neubauer and Junne 2016).

3.2.1. Consistent bioprocess development

The bioreactor vessel is the central point in the upstream processing of cultivations for industrial bio-production. In general, stainless steel stirred-tank bioreactors with final volumes up to 500 m³ are used in large-scale pharmaceutical production. Consistent bioprocess development strategies are necessary for optimum performance in the final scale (Neubauer et al. 2013). Commonly, such strategies consist of three distinct phases summed up in Figure 3.1.

The first step in the development of a novel microbial bioprocess is focused on screening for and engineering of a suitable production strain and definition and optimization of the cultivation medium. These steps are usually performed in shake flasks or mini-bioreactors without excessive monitoring of environmental parameters. The second phase is the engineering of the cultivation procedure via cultivations in lab-scale bioreactors to detect and optimize environmental cultivation conditions. In contrast to the screening scale, these cultivations allow monitoring and control of oxygen levels, pH, temperature and other detectable process parameters. After definition and validation of an optimized cultivation protocol for the desired host, the last phase of development begins – scale-up to the final scale. A pilot-plant is built with a capacity for 100 – 1000 L cultivations designed to match the optimized cultivation conditions to study cellular responses to the scale-up to verify the
preliminary bioprocess development. If these studies approve the economic feasibility of the desired process, it is transferred to its final production scale.

Figure 3.1: Scale-up bioprocess development for industrial pharmaceutical production from lab scale to industrial large-scale. (1) Screening of the production strain and medium development, (2) Lab scale bioreactors cultivations for optimization of the cultivation conditions, (3) Large-scale production plant level (based on data of D. I. C. Wang and others 1979; Bylund et al. 1998).

3.2.2. Scale-up impacts on microbial bioprocesses
In general, the scale-up of a microbial bioprocess is limited due to complex hydrodynamics inside the bioreactor vessel and their influences on various cell metabolic mechanisms. As these effects can vary with the bioreactor scale, microbial behavior during the scale-up is tough to predict. Thus, the main aim of bioprocess development should be the transfer of cultivation performance without a loss across all scales to gain new efficient industrial scale production processes. Hence, impacts of scale-up are defined as the differences between the “ideal” lab scale environmental conditions compared to the conditions existent in the production scale bioreactor. These scale-up impacts can be categorized into three categories including biological, chemical and physical impacts (Takors 2012).

3.2.2.1. Biological impact factors
Increasing the scale of a microbial cultivation also increases the total number of cell divisions due to extended phases of pre-cultivations and longer process times. Increased run times may
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lead to a loss of the plasmids encoding the desired product as well as an increased probability of mutations in the host strain or contaminations. Also, a bioprocess gets more complicated during scale-up as environmental factors start to get unstable with certain cultivation volumes and could so limit growth. Recombinant production strains need to be screened and designed for robustness against stress conditions caused by the scale-up of the cultivation volume to resist long-lasting regulatory inhibitions (Junker 2004).

3.2.2.2. Chemical impact factors
Scaling up the volume of a microbial cultivation can set special demands on preparation and composition of the cultivation medium. For instance, using salt-free titration agents (ammonia solution) or gasses (ammonia gas) for pH controlling is crucial for effective downstream processing to prevent salt accumulation formed by sodium or potassium hydroxide solutions. Also, an increased cultivation scale leads to the higher solubility of gasses including oxygen and carbon dioxide, which could influence the formation of all carbonic ions and so the buffer capacity. Foaming due to interactions of air bubbles with hydrophobic molecules accumulating in the medium can be boosted in large-scale bioreactors. An effective antifoam agent needs to be chosen during the process development, which is also inexpensive and can be applied in the final production scale without harming the economic viability (Vardar-Sukan 1998; Junker 2004).

3.2.2.3. Physical impact factors
Increasing the bioreactor scale can cause many physical changes to the cultivation environment cells experience. Obviously, a large-scale production process with a reaction volume of over 100 m$^3$ could have a vertical hydrostatic pressure gradient of 1 bar and higher depending on the reactor height. The hydrostatic pressure impacts gas solubilities and transport mechanisms. Also, oxygen gradients caused by high cell metabolic activities and technical limitations in gas transfer are even a graver concern. It was shown, that mixing times in industrial bioreactors in the m$^3$-scale are significantly higher than needed for efficient oxygen supply to prevent oxygen limitation due to microbial metabolism (Junker 2004). Accordingly, cells transported through the bioreactor are continuously exposed to inhomogeneous environmental conditions, especially oscillating distribution of dissolved oxygen, carbon and nitrogen substrate concentrations, pH and temperature (Enfors et al. 2001). Furthermore, limited heat transfer complicates the evacuation of microbially
generated heat from microenvironmental zones with higher metabolic activities with increasing cultivation scale (Junker 2004).

3.2.3. Scale-up parameters for bioprocesses
The difficulty of scaling up the bioreactor volume is that not all process defining parameters can be transferred to the new reactor design at the same time. Also, appearing gradients or other scale-up impacts might influence the process performance during the process transfer from lab scale to production scale (Neubauer and Junne 2016). Therefore, it is necessary to define physical scale-up criteria, which are today the most applied strategy to design a scale transfer based on the principle to keep the most critical factor constant across different scales.

Table 3.1: Frequently physical process parameters applied as scale-up criteria in bioprocesses (from Peter Neubauer and Junne 2016)

<table>
<thead>
<tr>
<th>Scale-up physical criteria</th>
<th>Determination equation</th>
<th>Typical range in a large-scale fermenter of over 20 m³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geometric similarity</td>
<td>$H/D$</td>
<td>Up to 8:1</td>
</tr>
<tr>
<td>Volumetric power input</td>
<td>$\frac{P}{V_1} = \frac{2\pi n M}{V_1}$</td>
<td>1 – 2 kW m⁻³</td>
</tr>
<tr>
<td>Volumetric oxygen mass transfer coefficient</td>
<td>$K_L a = c_0 * \left(\frac{P}{V_1}\right)^{c_1} * w_{gas}^{c_2}$</td>
<td>ca. 400 h⁻¹</td>
</tr>
<tr>
<td>Mixing number</td>
<td>$\Theta_{95} = n \tau$</td>
<td>100</td>
</tr>
<tr>
<td>Impeller tip speed</td>
<td>$u = \frac{2\pi nd}{1}$</td>
<td>Smaller than 7 m s⁻¹</td>
</tr>
<tr>
<td>Volumetric gas flow rate</td>
<td>$\frac{Q}{V_1}$</td>
<td>1</td>
</tr>
<tr>
<td>Ratio of the local to the mean specific energy dissipation rate</td>
<td>$\frac{e_l}{\overline{e_T}} = \left(\frac{P}{V_1}\right)/\left(\frac{\overline{P}/(V_1^2 \rho_1)}{1}\right)$</td>
<td>70</td>
</tr>
</tbody>
</table>

with $c_1$, $c_2$: empirical constants; $c_0$: dissolved oxygen saturation concentration; $D$: bioreactor vessel diameter; $H$: bioreactor vessel height; $P$: power input; $V_1$: local liquid volume inside the bioreactor vessel. $n$: impeller speed; $M$: torsion of liquid; $K_L a$: volumetric oxygen mass transfer coefficient; $w_{gas} O_2$: oxygen velocity; $\Theta_{95}$: mixing number; $\tau$: mixing time; $u$: circumvental velocity; $Q$: gas flow rate; $e_T$: energy dissipation; $p$: density of growth medium; $\rho_1$: local density of liquid.
Several widely applied physical aspects (scale-up factors) suitable to compare and estimate differences in process conditions are summarized in Table 3.1. These factors include geometric similarity, volumetric power input, volumetric oxygen mass transfer coefficient, mixing number or circulation time, tip speeds of the impeller, volumetric gas flow rate, ratio of the local to the mean specific energy dissipation rate and the Reynolds number (Neubauer and Junne 2016)

The Reynolds number is an essential factor in fluid dynamics to describe dynamic similarities between different fluid flow conditions. Also, it can categorize different types of flow regimes within comparable fluids like laminar or turbulent flow. Due to low viscosities of the cultivation medium, the flow in bioreactor vessels is turbulent independent from the bioreactor scale with Reynolds numbers as high as $10^4$ and above. For scale-up design, the influence of the Reynolds number is, therefore, insignificant, but can be useful for applying turbulent flow theories to analyze fluid dynamics across increasing scales (Schmidt 2005).

Optimized oxygen transfer and so oxygen availability defines the release of microbial heat linked to the activity of aerobic metabolism of cells. The temperature of the cultivation broth must be monitored and controlled to prevent cellular responses to heat stress. Produced heat needs to be evacuated by efficient heat transfer at a similar rate via the cooling surface of the bioreactor. At the industrial production scale, efficient heat transfer is problematic due to the limited power input for efficient mixing and insufficient areas of cooling surfaces, as the cultivation volume is increased with the cubed diameter of the bioreactor whereas cooling surfaces only scale with the squared bioreactor diameter during a scale-up in geometrically similar systems. Consequently, more complex cooling systems like cooling coils or cooling baffles inside the bioreactor need to be applied to support heat transfer with increasing cultivation scale (C. Hewitt and Nienow 2007).

In the last decades, numerous studies discussed the link between cellular metabolic behavior and the successful operation of industrial scale bioprocesses. Also, it is also suggested to include microbial physiology parameters into fluid dynamic studies of bioreactors for a better scale-up design and optimization of process parameters (Votruba and Sobotka 1992). Some critical physiological parameters, which need to be considered in microbial cultivations, are summed up in Table 3.2. As limitations in the gas mass transfer, gradients of nutrient supply
or pH or other scale induced phenomena can directly have an impact on cellular metabolism and physiological fitness of microbial cells, comparable cultivation conditions during scale-up should lead to predictable microbial behavior. Hence, understanding the connection between fluid characteristics in bioreactor designs and their influence on microbial physiology opens new possibilities for efficient scale-up of industrial bioprocesses (Y. Wang et al. 2009).

Table 3.2: Microbial physiological system specific parameters (C. Hewitt and Nienow 2007)

<table>
<thead>
<tr>
<th>Parameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth and productivity</td>
</tr>
<tr>
<td>Nutrient and other additive requirements including oxygen</td>
</tr>
<tr>
<td>Carbon dioxide evolution and respiration quotient</td>
</tr>
<tr>
<td>Sensitivity to oxygen and carbon dioxide concentration</td>
</tr>
<tr>
<td>pH range and sensitivity</td>
</tr>
<tr>
<td>Operating temperature range</td>
</tr>
<tr>
<td>Shear sensitivity</td>
</tr>
</tbody>
</table>

It is no surprise then that many transferable analytical tools have been developed to characterize and define physiological parameters of cultivations and the physiological state of single cells. This tool box includes flow cytometry, chromatography, spectroscopy, electric tongues, artificial noses, lab-on-a-chip techniques as well as on-line or in-situ monitoring with chemical or biological sensors (Lemoine et al. 2017). Also, development of advanced computational fluid dynamic simulation tools illustrating and modeling fluid behavior in bioreactors help to detect non-optimum flow areas in bioreactor designs. Their application to support industrial scale-up contributed to new bioreactor designs optimized towards more homogeneous cultivation conditions and could help to gain more detailed information about the influences of flow dynamics on the metabolic and physiological state of microbial cells (Lapin, Schmid, and Reuss 2006).

Another approach to cellular state characterization is the use of fluorescent proteins. Since their discovery the green and other fluorescent proteins developed into a pillar of cellular and molecular biology research. Thousands of published scientific studies describe their use in any imaginable way (Chudakov et al. 2010). The use of fluorescent proteins as reporter or marker
proteins opens new possibilities for characterizing cellular states as they are easily detectable both in bulk samples and on the single cell level using fluorescence microscopy and flow cytometry. More obvious advantages of fluorescent proteins include no need for a prosthetic group for activity, functional and detectable within intact cells, relatively small and producible by a large variety of cells including bacterial and mammalian cell cultures. The application of fluorescent proteins as biosensor for environmental changes offers a potential approach to characterize physiological responses of cells to changing micro-dynamics of flow in bioreactors during scale-up (Vizcaino-Caston, Wyre, and Overton 2012).

3.2.4. Gradient formation in industrial scale bioreactors
The fed-batch cultivation is mostly the preferred operational mode for large-scale industrial production of recombinant pharmaceutical proteins. Additionally, recombinant protein production processes are usually designed as high cell density cultivations for maximized spatial and time-based efficiency and volumetric product yields. The present effect of reduced mixing quality with increasing bioreactor sizes is linked to insufficient power input compared to the liquid volume. Especially in high cell density cultivations this leads to formation of a dynamic environment consisting of characteristic zones in a conservative designed large-scale fed-batch process, where substrate is fed highly concentrated usually from the top and the reactor is aerated from the bottom (Enfors et al. 2001).

Figure 3.2: Gradients of dissolved oxygen, pH, glucose, dissolved carbon dioxide and stress present in an industrial-scale fed-batch high-cell-density cultivation bioprocess fed from the top and aerated from the bottom. In general, substrate availability nearly depletes in areas away from the feeding inlet point. These alternating substrate concentrations cause altered metabolic activities inside cells which lead to an opposite oriented dissolved oxygen concentration gradient. A pH gradient is influenced by high mixing times of its controlling agents. The dissolved carbon dioxide concentration is increased in zones of high metabolic activity and in the bottom part due to hydrostatic pressure.
Due to the insufficient mixing, heterogeneous conditions can be detected near the point of feed addition, at the gas inlet, and close to the addition point of any external added controlling agent like acids or base (Figure 3.2). In general, substrate excess conditions occur around the top feeding point as feeding solutions are added at very high concentrations to minimize dilution effects. At the same time, cells in this area experience oxygen limitation due to increased metabolic activity, while cells in the bulk area or at the bottom of the bioreactor are suffering due to substrate starvation (Larsson et al. 1996). A reverse gradient for the availability of dissolved oxygen is formed in industrial scale bioreactors caused by altering substrate availability and so varying metabolic activity in the different environmental zones. Moreover, in large scale bioreactors gradients regarding pH, dissolved carbon dioxide, and temperature can be detected simply caused by increased mixing times due to insufficient power input (Enfors et al. 2001).

3.3. Scale-down of microbial bioprocesses
A bioprocess needs to be scaled up through several phases, as each volume increase can introduce new changes on process parameters due to physical limitations. A selected recombinant host and the bioprocess procedure developed and validated under laboratory and pilot scale conditions could be neither optimum nor practical at the final production scale. Based on economic reasons, cultivations to proceed the research on bioprocess development and verification cannot be performed in pilot or even production-scale facilities. Consequently, scale-down approaches to simulate environmental perturbations found under production conditions in large-scale bioreactors could be a cost-effective and practical tool to be applied in the laboratory development phase of bioprocesses. Several scale-up approaches mostly focus on classic engineering parameters of bioreactors like constant tip speeds, constant power unit per volume unit, and constant mixing time (Vrábel et al. 2000). However, an efficiently and successfully scaled up bioprocess should be on a level of robustness so that it can be transferred from one production facility to another although its changed engineering properties. The scale-up approach needs to include cellular physiological and metabolic parameters as well as space-time dynamics of environmental parameters, which define specific cellular responses and need to be investigated for each microbial organism. For years now, several scale-down approaches formed on concepts of such whole regime characterizations became powerful tools to receive new insights on scale-up problems, to
investigate future large-scale performance and to identify significant parameters, which influence microbial culture behavior (Neubauer and Junne 2016).

3.3.1. Scale-down approaches for imitating large-scale perturbations (single- and multi-compartment systems)

Many scale-down bioreactor systems have been developed and successfully applied to get a better understanding how cells respond and adapt to the gradient formation in large scale bioreactors (Neubauer and Junne 2010; Neubauer and Junne 2016). In principle, two basic approaches of scale-down bioreactors are used nowadays to simulate industrial scale perturbations: (1) stirred tank reactors with additional external compartments used to decrease mixing efficiency and (2) single compartment reactors with additional installations inside, which disturb the mixing profile and so limit sufficient mixing. Consequently, numerous known scale-down simulators, which mimic industrial scale conditions, based on these two principles gained popularity in research. One-, two-, or more compartment bioreactor cultivation systems consisting of classic stirred tank reactors (STR), modified STRs, STRs connected to plug-flow reactors (PFR), STRs connected to PFRs equipped with axial static mixers or tubular closed-loop airlift reactors are well known. These systems are illustrated in Figure 3.3 a – g.
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Figure 3.3: Schematic overview on different scale-down simulator approaches. (A-C) Scale-down simulators as one-reactor systems. (A): tubular closed-loop air-lift reactor; (B): stirred tank reactor (STR) with internal disks for increased mixing times; (C): STR with oscillatory pulsed feeding profile; (D-F): Scale-down simulators with two-compartment reactor systems. (D): two STRs; (E): STR connected to a simple plug flow reactor (PFR); (F): STR combined with an enhanced PFR that contains static mixer modules; (G): STR connected to two enhanced PFRs (based on Neubauer and Junne 2010; Neubauer and Junne 2016).

Scale-down techniques using a single compartment approach include designs of tubular single-loop closed air-lift reactors (Fig. 3.3a), modified STRs with internal modifications to disturb mixing (Fig. 3.3b), and STRs with oscillatory applied pulsed feeding strategies (Fig. 3.3c). The mentioned tubular air-lift reactor design equipped with stirrers, aeration and feeding points was already used in the 1970s to investigate successfully responses to induced oscillatory intracellular NADH concentrations in the yeast *Trichosporon cutaneum* as well as impacts on the productivity of n-paraffin produced in *Candida tropicalis* under oscillating respiratory activity and intracellular ATP levels (Katinger 1976). Constructing disks inside an
STR to disturb vertical mixing leads to increased mixing times and so gradients like in large-scale bioreactors can be applied to cultivations (Schilling et al. 1999). The easiest scale-down strategy to be implemented within a single STR is the pulsed feeding profile strategy, which opens the possibility to investigate responses to oscillating substrate availability or oxygen supply on biomass and product yields (Chassagnole et al. 2002).

The most obvious designs for more complex scale-down simulators consist of two individual reactor compartments coupled as one system including either two STRs (Fig. 3.3d) or an STR connected to a PFR (Fig. 3.3e, f) and are used to characterize cellular responses to perturbations in industrial scale bioreactors. In the last decade, the STR-STR approach was applied to study impacts caused by oscillating dissolved oxygen concentrations (Sandoval-Basurto et al. 2005) as well as oscillating supply of CO₂/HCO₃⁻ in the cultivation medium (Buchholz et al. 2014). One well-known example of a two-compartment scale-down bioreactor system using the STR-PFR approach was first described by George, Larsson, and Enfors (1993). In this system, a certain part of the cultivation broth is transported from a stirred tank reactor through a plug flow reactor and re-entering the main reactor again. The PFR is acting as a bypass loop. Due to the lack of external aeration and mixing in the PFR module, cells in the PFR module are exposed to oxygen limitation conditions. Therefore, zones with insufficiently mixed substrate conditions could be simulated by adjusting the residence time in the PFR part of the scale-down system. The originally described scale-down simulator using the STR-PFR design consisted of an aerobic held STR and a “simple” STR as illustrated in Fig. 3.3e. Lately, many studies redesigned the PFR module and enhanced it with built-in static mixers to improve horizontal mixing and plug-flow behavior even under aerated conditions. This improved STR-PFR approach was applied to successfully study cellular responses to oscillating gradients and availability of glucose, oxygen and pH for several microorganisms like *Saccharomyces cerevisiae* (George, Larsson, and Enfors 1993), *Escherichia coli* (Soini, Ukkonen, and Neubauer 2011), *Bacillus subtilis* (Junne et al. 2011), or *Corynebacterium glutamicum* (Lemoine et al. 2016). Furthermore, this STR-PFR approach was further enhanced to develop an innovative three-compartment scale-down simulator consisting of a standard STR connected to two PFR modules. In this setup, one PFR module can simulate the substrate excess feeding zone whereas the other PFR module can serve as a simulated starvation zone in large scale bioreactors (Fig. 3.3g) (Lemoine et al. 2015).
3.3.2. Inhomogeneity studies using *Escherichia coli*

Studies on the impact of stressful environmental cultivation conditions on the physiology of *Escherichia coli* have been described for oxygen supply (De León et al. 2003), temperature (Caspeta et al. 2009) as well as starvation due to limitation of carbon (Matin 1991) or other energy sources. Metabolic and physiological stress responses of *E. coli* cells caused by extracellular environmental conditions are triggered by short- or long-term exposition of cells to oscillating glucose availability in large-scale bioreactors. These oscillations lead to a decreased biomass yield due to a reduced cellular stability and accumulation of unwanted by-products (Enfors et al. 2001). It is additionally indicated, that expression of recombinant introduced heterologous genes lead to the destruction of ribosomes, increasing the dynamic cellular behavior and triggered stress responses (H. Schweder, Lin, and Jürgen 2002).

Much research was and is invested in developing new bioreactor designs optimized towards minimized stress responses of *E. coli* cells. Stress responses are influenced by the used strain, the chosen growth rate, and the glucose concentration at the feeding point. To be clearer, the glucose concentration at the feeding point in industrial scale bioreactors defines the residence time of cells in the formed feeding zone (Sunya et al. 2012). Several studies have proven, that the cellular response towards glucose fluctuations is at least biphasic (Lara et al. 2009), in which the first phase linked to glycolysis is shorter than 5 seconds (De Mey et al. 2010). In general, the maximum capacity for metabolizing glucose is dependent on the current specific growth rate but also on the history of the cultivated cells. In simulated feeding zones during scale-down experiments using *E. coli* an increased specific glucose uptake capacity and so a high specific growth rate was observed in the cultivations (Neubauer, Haggstrom, and Enfors 1995).

Usually, a two-compartment approach is used for scale-down studies involving *E. coli* including both the STR-STR (Lara, Leal, et al. 2006) as well as the STR-PFR systems (Neubauer et al. 1995). Here, the main metabolic response of *E. coli* cells to oscillating glucose conditions is the formation and accumulation of overflow by-products from glycolysis. In addition, oxygen limitation results in accumulation of the complete mixed-acid fermentation product profile including acetate, formic acid, lactate, succinate and further carbon dioxide and hydrogen inside the compartment simulating industrial scale perturbations (B. Xu et al. 1999). Though, accumulating by-products are metabolized in other parts of the scale-down system, where the
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Glucose concentration is limiting or completely consumed triggering starvation (B. Xu et al. 1999).

Onyeaka, Nienow, and Hewitt (2003) investigated the cellular responses of *E. coli* in a STR-PFR approach towards heterogeneities of pH, glucose and dissolved oxygen concentrations simultaneously and concluded that this setup reproduces excellent biomass yield and cell viability of a large-scale cultivation (volume level 20 m³), if the residence time in the PFR and so oxygen limitation and glucose excess was set to below 50 seconds. Especially the addition of the pH controlling agent at the PFR, leading to oscillating pH conditions, triggered a reduced viability of the culture and a significant loss of biomass yield (Onyeaka, Nienow, and Hewitt 2003). In addition, a strong connection between the residence time and productivity of recombinant protein expression and probability of plasmid loss is indicated (Ying Lin and Neubauer 2000). Also, also the role of the heating rate for thermo-inducible processes was studied in a scale-down system via a varying temperature increase as industrial scale bioreactors have a limited heat transfer capacity (Caspeta et al. 2009). It was shown that the slowest applied heating rate (0.4 °C min⁻¹), which mimics the heat transfer inside a 200 m³ bioprocess, resulted in the highest productivity of the recombinant product expression indicating a slow increase towards the induction temperature allows the cells a better adaption to the new cultivation conditions. This observation was also proven on metabolic and transcriptomic levels.

In addition to the accumulation of conventional mixed-acid fermentation products, accumulation of pyruvate was detected in *E. coli* cells during cultivations including oscillating concentrations of glucose in combination with oxygen limitation. Pyruvate accumulation can enhance the metabolic carbon flux from pyruvate into the connected biosynthesis pathways of amino acids and lead to an accumulation of alanine, branched-chain amino acids like leucine or valine, and in increased production of non-canonical amino acids like norvaline or norleucine. Norvaline accumulation in *E. coli* is known to be linked to glucose overflow metabolism and pyruvate accumulation (Huang, Lin, and Yang 2012). Furthermore, a down-shift of dissolved oxygen concentration in a two-compartment scale-down bioreactor has a proven as significant impact on triggering the biosynthesis of non-canonical amino acids.
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produced in the branched chain amino acids pathways due to pyruvate accumulation in recombinant *E. coli* cultivations (Soini, Ukkonen, and Neubauer 2011).

3.4. Research motivation and objectives

The microbial strain *Escherichia coli* K12 W3110 is widely used as production host in industrial processes and the base strain for genetically strain improvement approaches. It is known that *E. coli* K12 can synthesize non-canonical amino acids, which accumulate in the cultivation medium under glucose excess and anaerobic conditions. This formation unavoidably leads to misincorporation of non-canonical amino acids into proteins, which is critical for recombinant proteins produced for pharmaceutical use. Furthermore, expressing leucine-rich proteins triggers the upregulation of the branched-chain amino acids biosynthesis. As the synthesis of non-canonical amino acids is tightly connected to the activity of the branched-chain amino acid pathway production of a leucine-rich protein intensifies the problem.

The aim of this doctoral dissertation study is to investigate cellular responses of a recombinant *E. coli* W3110 to oscillations of substrate and oxygen availability in a Two-CR and Three-CR scale-down system. These two factors simulate certain stress conditions in large-scale bioprocesses due to limited mixing capabilities. A particular focus is set on misincorporation of the non-canonical amino acids norvaline, norleucine, and β-methyl-norleucine into a produced recombinant miniproinsulin as heterologous inclusion bodies. Furthermore, the feasibility of transferring these effects into smaller and simplified approaches to introduce oscillating cultivation conditions into the screening scale was studied. Thus, the main aims of this thesis are:

- Deeper insight into the impacts of oscillating cultivation conditions on recombinant processes regarding production and misincorporation of non-canonical amino acids using two- and three-compartment scale-down reactor setups (**Chapter 4.1**)
- Simplification of the multi-compartment setup to a single STR approach and so comparison of segmental oscillations to pulsed feeding of the whole culture (**Chapter 4.2**)
- Applying oxygen oscillations at the multi-well-plate scale (**Chapter 4.3**)
- Monitoring inclusion bodies formation at-line (**Chapter 4.4**)

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4. Results

4.1. Impacts on cell physiology and product quality of recombinant *Escherichia coli* caused by oscillating cultivation conditions in a Two- and Three-Compartment Scale-Down Bioreactor

4.1.1. Abstract

Increased mixing times due to limited achievable power input cause appearing gradients in oxygen and nutrient supply in large-scale bioreactors. To investigate potential influences of these gradients on microorganisms in the lab-scale, scale-down strategies are utilized. In this study nutrient-limited fed-batch cultivations of a recombinant *Escherichia coli* strain overexpressing a leucine-rich miniproinsulin performed in two-compartment and three compartment bioreactor setups are compared. These setups consist of a stirred tank reactor and one or two attachable plug-flow reactor modules. In two-compartment cultivations the PFR compartment either mimics a feeding zone of a top-fed large-scale bioreactor (high nutrient concentration/oxygen limitation), starvation conditions (low nutrient concentration/oxygen limitation) or conditions near the bottom of a large-scale bioreactor (low nutrient concentration / aerobic oxygen levels) in several two-compartment cultivations. Furthermore, we combined the feeding zone and bottom zone setup to a three-compartment reactor.

Our research results show a decreased biomass and increased production of metabolites deriving from pyruvate based on overflow metabolism and mixed-acid fermentation (acetate and lactate) under oscillating conditions when the feeding loop setup is applied. Furthermore, a flux into the branched-chain amino acid pathway can be seen contributing to an increased production of branched-chain amino acids including non-canonical amino acids like norvaline. Non-canonical amino acids can be incorporated into proteins, e.g. norvaline as a substitute for leucine. We see increased incorporation of the non-canonical amino acids norvaline, norleucine, and ß-methyl-norleucine when oscillating cultivation conditions are applied, influencing the quality of the recombinant product and underlining the importance of process optimization.
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4.1.2. Introduction
Until today, the fed-batch is the most applied cultivation technique in industrial scale bioprocesses, and until today the scale-up of a bioprocess from development to final production scale is bound to unsolvable limitations. It was shown already in the 1970s that increasing the cultivation volume scale by a factor of 1000 results in increased mixing times (D. Wang 1979). The fed-batch technique, cultivating microorganisms or cells under limited substrate growth via external feeding of highly concentrated feeding solutions, is applied to control cellular metabolism and circumvent technical limitations or by-product formation (Larsson et al. 1996). Nevertheless, several studies have revealed, that a scale-up of cultivation volume leads to a loss of biomass and product yield in *E. coli* cultivations compared to experiments in lab scale vessels.

Increased mixing times in combination with the use of highly concentrated feeding solutions added at one point, pH controlling agents and single gas inlet – the classic and common fed-batch bioreactor setup – will result in gradient formation regarding substrates, pH and dissolved oxygen and carbon dioxide. Microbial cells experience oscillating cultivation conditions via passing the reactor volume repeatedly over time (Enfors et al. 2001).

Scale-down studies revealed that oscillating excess substrate availability in combination with oxygen depletion triggers several physiological responses in *E. coli* cells. Accumulation of acetate, formic acid, lactate, and succinate – products of the mixed acid fermentation – or formation and metabolization of acetate via overflow metabolism can be seen (B. Xu et al. 1999). Effects of oscillating cultivation conditions on growth and physiology have also been characterized for other industrial relevant microorganisms like *Saccharomyces cerevisiae* (George et al. 1998) as well as *Corynebacterium glutamicum* (Lemoine et al. 2015).

A loss of biomass and product yield due to scale-up can be acceptable from the economic point of views. Critical – especially for pharmaceutical purposes – are influences on the quality of expressed recombinant proteins in *E. coli*. It has been described before that *E. coli* cells can produce non-canonical amino acids in detectable amounts under certain environmental conditions as side products of the branched chain amino acid synthesis pathway in addition to leucine, isoleucine, and valine (Bogosian et al. 1989; Muramatsu, Misawa, and Hayashi 2003). Especially the synthesis of the amino acids norvaline and norleucine has been proven
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to be closely connected to the synthesis pathway of leucine (Kisumi, Sugiura, and Chibata 1976a). It is also known, that the non-canonical amino acids norvaline, norleucine and β-methyl-norleucine can be incorporated into native and recombinant proteins replacing the chemically similar canonical amino acids leucine (Apostol et al. 1997), methionine (Muramatsu et al. 2002) and isoleucine (Muramatsu, Misawa, and Hayashi 2003).

Several factors are known to provoke the formation of these specific amino acids. The upregulation of the branched chain amino acid synthesis pathway due to overexpression has been shown to result also in increased production of these non-canonical amino acids (Bogosian et al. 1989). Another important trigger is the dissolved oxygen availability under glucose excess conditions (Soini et al. 2008). Both can be detected in large-scale fed-batch bioprocesses. Essential – especially for pharmaceutical purposes – is the possible change of structural and functional changes in proteins due to misincorporation of non-canonical amino acids (Gilles et al. 1988). For more efficient bioprocesses in industrial scale bioreactors research on large scale dependent physiological impacts on cells needs to be part of bioprocess development. Numerous scale-down approaches to mimic oscillating cultivation conditions in lab scale vessels have been recently summarized and reviewed (Neubauer and Junne 2016). E. coli has been widely applied in scale-down studies to reveal effects of cultivation heterogeneities on biomass yield, recombinant expression productivity, cellular physiology, transcriptome, respiratory activity and formation of by-products (Neubauer and Junne 2010).

This study is the first focusing on the quality of a recombinant expressed miniproinsulin in E. coli via analyzing the misincorporation of the non-canonical amino acids norvaline, norleucine, and β-methyl-norleucine into the protein in several STR-PFR scale-down approaches. In two-compartment cultivations the PFR compartment either mimics a feeding zone of a top-fed large-scale bioreactor (high nutrient concentration/oxygen limitation), starvation conditions (low nutrient concentration/oxygen limitation) or conditions near the bottom of a large-scale bioreactor (low nutrient concentration/aerobic oxygen levels – additional air supply at the PFR) several two-compartment cultivations. Furthermore, the feeding zone and bottom zone setup were combined to a three-compartment reactor. The residence times in the PFR mimic
a 10 % cultivation volume area like it is described for feeding zones in industrial scale reactor vessels (B. Xu et al. 1999).

The goal of this study is to reveal new insights on which factors mainly trigger the formation of non-canonical amino acids and how the misincorporation rate is changing under different oscillating conditions.

4.1.3. Materials and Methods

4.1.3.1. Strain and Cultivation Conditions

_E. coli_ K12 W3110M, which carried a lacI<sup>q</sup> mutation and was transformed with the plasmid pSW3 (recombinant miniproinsulin expressed in inclusion bodies and ampicillin resistance gene) was used in all experiments. Both strain and plasmid were thankfully provided by Sanofi-Aventis Deutschland GmbH.

4.1.3.2. Media

All chemicals mentioned were purchased from either Carl Roth GmbH, Karlsruhe, Germany, or Sigma-Aldrich Chemie GmbH, Munich, Germany, if not otherwise stated. Initial cultivation was performed in LB medium composed of 10 gL<sup>-1</sup> tryptone, 5 gL<sup>-1</sup> yeast extract, 10 gL<sup>-1</sup> NaCl and 100 µgL<sup>-1</sup> ampicillin. As medium for the main preculture, EnPresso<sup>®</sup> B (BioSilta Ltd., Cambridge, UK) was applied.

The minimal medium (B. Xu, Jahic, and Enfors 1999) in the bioreactor consisted of 2 gL<sup>-1</sup> Na<sub>2</sub>SO<sub>4</sub>, 2.468 gL<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 5 gL<sup>-1</sup>NH<sub>4</sub>Cl, 14.6 gL<sup>-1</sup> K<sub>2</sub>HPO<sub>4</sub>, 3.6 gL<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub> x 2H<sub>2</sub>O, 1 gL<sup>-1</sup> (NH<sub>4</sub>)<sub>2</sub>-H-Citrat, 1 mL Antifoam Sigma 204. Per liter medium 2 mL trace element solution, 2 mL thiamin solution (50 gL<sup>-1</sup>), 2 mL MgSO<sub>4</sub>-solution (1.0 M) and 1 mL ampicillin solution (100 mgL<sup>-1</sup>) were sterile-filtered through an 0.22 µm-membrane filter into the reactor. The trace element-solution contained 0.5 gL<sup>-1</sup> CaCl<sub>2</sub> x 2H<sub>2</sub>O, 0.18 gL<sup>-1</sup> ZnSO<sub>4</sub> x 7H<sub>2</sub>O, 0.1 gL<sup>-1</sup> MnSO<sub>4</sub> x H<sub>2</sub>O, 20.1 gL<sup>-1</sup> Na-EDTA, 16.7 gL<sup>-1</sup> FeCl<sub>3</sub> x 6H<sub>2</sub>O, 0.16 gL<sup>-1</sup> CuSO<sub>4</sub> x 5H<sub>2</sub>O, 0.18 gL<sup>-1</sup> CoCl<sub>2</sub> x 6H<sub>2</sub>O. The initial batch glucose concentration was 5 gL<sup>-1</sup>.

4.1.3.3. Procedure of Precultivation

Twenty milliliters of LB medium were inoculated with 50 µL of cryostock and incubated at 37°C for six hours at 250 rpm. For the second preculture, 150 mL Enpresso<sup>®</sup> B were mixed with each 150 µL ampicillin stock and BioSilta Reagent A as well as 1.5 mL LB culture in a PreSens SFR flask and cultivated for 15.5 h at 37°C and 250 rpm. The application of a PreSens flask allowed
monitoring of the preculture via online DOT and pH measurement. The whole 150 mL were used to inoculate the bioreactor.

4.1.3.4. **Bioreactor cultivation**

A 15 L Infors Techfors-S stirred tank bioreactor (Infors AG, Switzerland) equipped with three Rushton turbines was used. For scale-down cultivations, the STR was connected to one or two PFR modules. One PFR has a working volume of 1.2 L consisting of four static mixer elements. A complete PFR module (considering the tubing from the STR and back) has a total working volume of 1.8 L. The flow rate through the PFR was set to 1.7 L\text{min}^{-1} at all cultivations, so the mean residence time in the PFRs was 68 s. The PFR modules and the setups of the two-CR and three-CR have been described in more detail previously (Junne et al. 2011; Lemoine et al. 2015).

10 L of the before described mineral salt medium were inoculated with 1.5 % (v/v) EnPresso® B preculture. After a batch phase of around 7.5 h (substrate depletion), feeding was started. The feed solution consisted of 440 gL\text{\textsuperscript{-1}} glucose x H\textsubscript{2}O solved in the same mineral salt medium described before. Differences are four times higher trace element concentrations, no MgSO\textsubscript{4}, antifoam or ampicillin. 20 mL 1.0 M MgSO\textsubscript{4}-solution was added via sterile filtration every OD\textsubscript{600} 20. The feed was connected to the top gas phase of the STR for the single-CR control cultivation and two-CR setups mimicking nutrient starvation conditions. For the scale-down cultivations involving a feeding loop configuration (two-CR and three-CR), the feeding solution was added to the inlet tube of the appropriate PFR module. To mimic aerobic conditions in the PFR module air could be supplied via an other sparger inside the PFR. The aeration rate then was 0.8 vvm to keep the DOT over 10%. An overview of the different cultivation setups is illustrated in Figure 4.1.1.
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Figure 4.1.1: Reactor setups used in this study showing applied cultivation conditions and mean residence time (τ) at the ports of the PFR modules.

The feeding phase was divided into two parts. The first part was an exponential feeding phase with a start feed rate $F_0 = 0.06 \text{ L h}^{-1}$ and an exponential increase with $\mu = 0.4$. After three hours of exponential feeding it was switched to constant with a fixed feeding rate of $F = 0.195 \text{ L h}^{-1}$ and expression of the recombinant miniproinsulin was induced via IPTG addition to a final concentration of 1 mM. The cultivation temperature was set to 35°C. The aeration rate was initially set to 0.5 vvm and increased to 1.0 vvm after feed start. The pH was first configured to $\text{pH} = 6.6$ with 25 % H$_2$SO$_4$ to match the pH of the preculture and then controlled at $\text{pH} = 7.0$ with 25 % NH$_3$ solution. The initial stirrer speed was 400 rpm and increased to 600 rpm after the DOT dropped below 30 %. At the point of feed start, the stirrer speed was increased to 1100 rpm. If needed, additional Antifoam was added to control foam formation.

4.1.3.5. Analysis

Cell growth was monitored via determination of the optical density at 600 nm (OD$_{600}$) (Novaspec III by Amersham Biosciences, Amersham, UK) and dried cell weight (DCW) analysis. Two mL fresh cell suspension were immediately transferred into dried, pre-weighted two mL microcentrifugation tubes, spun down for 10 min at 21,500g and washed once with one mL 0.9 % (w/v) NaCl solution. Following a repeated centrifugation step the tube was dried 75°C for 24 h.
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Extracellular carbon metabolite and amino acid concentrations were analyzed from supernatant samples, which were filtered through a 0.8 µm pore sized membrane filter directly at the sampling port of the STR and stored at -80°C until further analysis.

For the analysis of total free amino acid concentrations and the amino acid composition of the miniproinsulin inclusion bodies three mL of cell suspension were harvested into a syringe containing two mL pure methanol precooled and immediately stored at -80°C. Before analysis of the free amino acids, cell samples were set to a DCW concentration of 1.6 gL⁻¹ and homogenized via sonication using a sonotrode with 1 mm diameter (UP200, Dr. Hielscher, Teltow, Germany). The applied amplitude was set to 30 % for three cycles each 30s long interrupted by a 30s break. The lysed cells were centrifuged (15000g, 10 min, 4°C) and the clear supernatant used for quantification. Inclusion bodies samples were diluted to a DCW of 6 gL⁻¹. Inclusion bodies purification was done using the BugBuster® Protein Extraction Reagent as per the manufacturer’s manual (Merck, Darmstadt, Germany). SDS-PAGE has been performed as described in SOP 9.4.

Quantification of Metabolites

For identification and quantification of carbonic acids an Agilent 1200 HPLC system (Waldbronn, Germany) equipped with a HyperRez™ XP Carbohydrate H⁺ column (300 x 7.7 mm, 8 µm) (Fisher Scientific, Schwerte, Germany) and a refractive index detector was used with 5 mM H₂SO₄ as eluent at a temperature set to 15°C and a flow rate of 0.5 mLmin⁻¹. The detailed SOP is attached in chapter 8.5.

Also, glucose concentrations were determined using an enzymatic assay (Glucose Hexokinase FS* by DiaSys Diagnostic Systems GmbH, Holzheim, Germany) following the supplier’s protocol. The concentration of glucose could be calculated after measuring NADH extinction at 340 nm.

Amino acids were identified and quantified with an Agilent 5975 C GC-EI-MS equipped with a DB-5MS column. For preparation samples needed to be dried in a speed vacuum concentrator (Bachhofer, Reutlingen, Germany) and afterward derivatized with n-tert-butyldimethylsilyl-n-methyl-trifluoroacetamide. Purified inclusion bodies samples were hydrolyzed prior for 24 h in 6 M HCl at 80°C. Detailed protocols are attached as chapters 9.1 to 9.3.
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Data Fitting and Visualization

All data was fitted using TableCurve 2D v5.01 by Systat (Systat Software Inc., 2002, San Jose, CA, USA). Data plots were created with Qtiplot (Qtiplot.com).

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4.1.4.1. Comparability

Before the effects of the different scale-down conditions can be discussed, it needs to be shown that all cultivations share comparable batch phases and precultures. As the main precultivations were performed in Corning shake flasks prepared with sensors for online DOT and pH monitoring by PreSens, these cultures can be compared without the need of external sampling and analysis. In Figure 4.1.2. Monitored trends for dissolved and pH are shown for all main precultures used in this study. Except one, all are in a similar range regarding DOT and pH and so display comparability within the precultures. The outlier had the same biomass concentration like the other cultures before inoculation of the bioreactor and did not behave differently during the first phase of the bioreactor cultivation.

![Figure 4.1.2: DOT and pH trends of the precultivations. Black line represents the reference STR cultivation, blue – 2CR-Feed, red – 2CR-Air, dotted red – 2CR-Star, green – 3CR](image)

In the initial batch phase, cells were grown on five gL\(^{-1}\) glucose until depletion. Another aspect for comparability between the cultivations would be a similar trend regarding cell growth and substrate consumption. All cultivations were inoculated to an OD\(_{600}\) 0.15. All batch phases lasted 7.5 \(\pm\) 0.25 hours and ended with Biomass concentrations in between 2.2 and 2.8 gL\(^{-1}\). Only the batch approach for the 3CR cultivation was under two gL\(^{-1}\) at feed start and substrate depletion.
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Figure 4.1.3: Biomass and extracellular glucose trends during the initial batch phase. Black circles represent the reference STR cultivation, blue squares – 2CR-Feed, open red triangles – 2CR-Air, red triangles – 2CR-Star, green triangles – 3CR.

Like the similar biomass development during the initial phase also the glucose consumption decreases comparable in all cultivations and depletion could be shown for four of five batch phases. Only in the batch cultivation continued as 2CR-Star setup five mM glucose (equals 0.9 gL⁻¹) were detected although the average increase of the DOT could be seen. Both, biomass and glucose trends of these batch phases are illustrated in Figure 4.1.3.
4.1.4.2.  Growth

Figure 4.1.4: Biomass, specific growth rate, specific glucose uptake rate and specific oxygen consumption rate trends during the fed-batch phase. Black circles represent the reference STR cultivation, blue squares – 2CR-Feed, open red triangles – 2CR-Air, red triangles – 2CR-Star, green triangles – 3CR. Dotted line at 3 h marks the point of induction of protein expression.

All cultivations were inoculated to an OD$_{600}$ of 0.15. Determination of DCW started when the OD$_{600}$ reached 1. At feed start, when the PFR-extensions are plugged to the reactor, the DCW was measured in between 2.2 and 2.8 gL$^{-1}$. Only the batch approach for the 3CR cultivation was under two gL$^{-1}$ at feed start. During the exponential feeding phase until the point of induction of recombinant protein expression, all scale-down cultivations show a reduced growth compared to the STR reference. After induction, the growth rate in the STR cultivation temporarily decreased, which could not be seen in the 2CR-Starvation and 2CR-Feed cultivation. The 2CR-Star cultivation even closed the biomass gap to the STR reference cultivation, and both reached a biomass concentration around 19 gL$^{-1}$ seven h after feed start. The STR cultivation had to be stopped now due to reactor volume limits. The 2CR-Feed setup, applying the feed to the PFR to simulate a high substrate availability zone combined with oxygen limitation, resulted in a reduced growth and further cell lysis after six h of feeding and three h after induction of protein expression. Furthermore, we could observe cell lysis in the
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two reactor-setups involving the aerated starvation loop (2CR-StarAir and 3CR) 1 h after induction. Both cultivations were stabilized with additional Antifoam 204 and run until seven hours after the beginning of the feeding. In all scale-down approaches, a lower glucose uptake in comparison to the reference cultivation could be detected. Interestingly, rates for the feeding loop setups (2CR-Feed/3CR) as well as the rates for the starvation loop setups (2CR-Star/2CR-Air) were each in a similar range with the feeding loop approaches higher than the starvation loop setups. Cells in the starvation loop setups also showed an increased oxygen consumption compared to the other cultivations (Figure 4.1.4).

4.1.4.3. Carbon metabolites
Comparing the trends of some carbon metabolites certain reactor-setups trigger the production and accumulation of carbonic acids. Applying the feeding loop setup lead to lactate accumulation up to 7 µM for the 2CR and further 8.5 µM in the 3CR setup before cell lysis appeared in this cultivation. Also, lower a lactate accumulation in the cultivation applying the starvation loop with oxygen limitation till five h after feed start was seen. Lactate produced during exponential feeding was consumed during constant feeding. Further, no significant lactate production could be observed in the other cultivations.

For acetate, a steady concentration of 0.8 to 1 µM in the STR reference was seen. In the 2CR setups, similar levels for the aerated starvation loop cultivation during the exponential feeding were detected. Applying the oxygen-limited starvation loop lead to acetate levels around two µM during recombinant protein expression. Oscillating substrate availability combined with oxygen limitation in the feeding loop setup triggered acetate accumulation up to 4 µM during the exponential feeding phase. Switching to constant feeding and led to acetate resorption and a decrease to 3 µM in the medium. In the 3CR cultivation combining feeding loop an aerated starvation loop we detected even higher acetate levels up to 6 µM before cell lysis and increasing afterward. Significant formic acid levels could not be detected during the feeding phases of all cultivations except during a short duration in the cultivations using the aerated starvation loop approach after cell lysis appeared. Glucose uptake and formation rates for acetate, lactate, and formic acid are illustrated in Figure 4.1.5.
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Figure 4.1.5: Glucose uptake as well as acetate, lactate and formic acid formation rates trends during the fed-batch phase. Black circles represent the reference STR cultivation, blue squares – 2CR-Feed, open red triangles – 2CR-Air, red triangles – 2CR-Star, green triangles – 3CR. Dotted line at 3 h marks the point of induction of protein expression.

4.1.4.4. Total free amino acids

In this study, a special focus was set on the branched-chain amino acids leucine and isoleucine, and methionine as these could be falsely replaced by the non-canonical amino acids norvaline, β-methyl-norleucine and norleucine during protein translation due to misloading of tRNAs. In general, applying oscillating conditions boosted the production of branched chain amino acids. In all cultivations, except the 2CR-Starvation setup, higher concentrations of leucine, isoleucine and methionine compared to the STR reference were detected. Applying the non-aerated starvation loop led to the lower concentration of leucine and methionine in the samples. Substrate oscillations in the 2CR-Feed setup resulted in amino concentrations up to twice as high as the STR reference (Figure 4.1.7 left).

The accumulation of non-canonical amino acids started in all cultivations directly after starting the feeding. Applying oscillating conditions had an impact on the production of non-canonical amino acids as the detected concentrations were lowest in the STR-reference. The highest
norvaline concentration could be seen in the 2CR-Feed setup with 6.5 µM before cell lysis. This amount equals 20 % of the free leucine concentration at this point. In the 2CR-Starvation setup norvaline accumulated to 4 µM (also 20 % of free leucine), in the STR reference, 3.5 µM was measured equaling 10 % of free leucine in the STR reference. It seemed, based on the values detected at protein induction cultivations using an aerated starvation looped could even have a higher accumulation of free norvaline. The levels for β-methyl-norleucine were similar in all cultivations until the feed was switched to constant feeding and recombinant protein expression was induced. Here we have the fastest accumulation in the 2CR-Feed cultivation, followed by the 2CR-starvation setup and the STR reference. Also, the final concentrations of β-methyl-norleucine were closer to the levels of free isoleucine – 2CR-Feed 74 %, STR reference 52 % - or even higher - 2CR-Starvation 129 %. β-methyl-norleucine also showed the highest concentrations of the three measured non-canonical amino acids. For norleucine, the impacts of applying oscillating conditions already differentiated the observed concentration profiles during exponential feeding and led to higher concentrations of norleucine compared to the STR reference like seen for the other two non-canonical amino acids before. Comparable to norvaline substrate pulses resulted in higher accumulation than starvation conditions. Applying the 3CR or the aerated starvation loop showed highest concentrations at the point of protein induction. The final concentrations for methionine and norleucine are similar for the 2CR-Feed approach (15 – 16 µM). In the 2CR-Starvation cultivation, the norleucine concentrations were higher than methionine (8 to 5 µM). In comparison, this ratio was inverted for the STR reference, where we detected five µM norleucine compared to 8 µM methionine. All values are illustrated in Figure 4.1.7 right.
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Figure 4.1.7: Total free amino acids from crude cell extract over the cultivation time (Leucine, Norvaline, Isoleucine, β-methyl-norleucine, Methionine, Norleucine) in µM. Line at 0 h marks the start of feeding, dashed line at 3 h the point of induction. Filled circles show the STR reference, filled squares 2CR-Feed, filled triangles 2CR-Starvation. The open triangles and inverted triangles show amino acid concentrations for the 2CR-Air and 3CR cultivations.
4.1.4.5. Incorporation of non-canonical amino acids into the recombinant protein

The concentration trends of leucine, isoleucine and methionine in the IB fraction over feeding and expression time were similar independent of the cultivation conditions, so in contrast to SDS-PAGE it seemed, applying oscillating conditions has no clear impact on the productivity of the strain. Also, the incorporation of all three non-canonical amino acids was detected under all cultivation conditions. β-methyl-norleucine and norleucine concentrations in the purified inclusion bodies were higher compared to the STR. Comparable to the free amino acid trends substrate oscillations triggered a higher effect than starvation conditions. As the concentrations of the canonical increased linear, the incorporation of β-methyl-norleucine and norleucine increased exponentially over the expression time. The final exchange ratio was around 1%. Surprising was the almost non-incorporation of norvaline. All values are illustrated in Figure 4.1.9. 20% ratio of free norvaline compared to leucine were detected in the cultivations and leucine is with 15 % the most prominent amino acid in the recombinant miniproinsulin, but norvaline replaced only every 5,000 to 10,000 leucine molecule. Interestingly, the highest norvaline concentrations and exchange ratio could be detected in the STR reference.

Figure 4.1.8: Fits of non-canonical amino acids concentrations in the purified IBs in comparison to their free concentrations detected in crude cell extracts. Filled circles show the STR cultivation, squares data from 2CR-Feed and triangles the 2CR-Star cultivation data.
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Figure 4.1.9: Amino acids concentrations from purified inclusion bodies over the cultivation time (Leucine, Norvaline, Isoleucine, β-methyl-norleucine, Methionine, Norleucine) in mM for canonical and μM for non-canonical amino acids. Line at 0 h marks the start of feeding, dashed line at 3 h the point of induction. Filled circles show the STR reference, filled squares 2CR-Feed, filled triangles 2CR-Starvation. The open triangles and inverted triangles show the biomass concentrations for the 2CR-Air and 3CR cultivations.
In Figure 4.1.8 concentrations of non-canonical amino acids from the purified inclusion bodies are fitted against levels of the free amino acids in the crude cell extract. Norvaline and β-methyl-norleucine can be correlated linearly. For norvaline, the coefficient of determination is only between 70 and 90 % whereas the fits for β-methyl-norleucine are of higher quality. Remarkably, the gradients of these fits are different. For norvaline, the gradient for the STR fit is twice as high as for the starvation loop and four times as high as for the feeding loop setup, which means that significantly more produced norvaline is incorporated into the recombinant miniproinsulin under reference cultivation than under scale-down conditions. For β-methyl-norleucine the picture is similar, but here the gradient of the unaerated starvation loop has the highest slope. Nevertheless, the highest detected amounts of β-methyl-norleucine in the inclusion bodies were detected in the 2CR-Feed fraction, which has the lowest gradient. Norleucine could not be fitted linear; the relation is exponential. Here it seems, that a critical concentration exists above which incorporation of norleucine into proteins is preferred. Interestingly, this concentration is again the lowest under reference conditions. Oscillating oxygen depletion alone seems to have a higher impact on the misincorporation, than oxygen depletion combined with substrate excess.

4.1.5. Discussion

The quality of expressed recombinant proteins is a critical point to produce pharmaceutical proteins recombinantly. Mis-incorporation of non-canonical amino acids into the recombinant protein reduce the quality and yield and increase the costs of downstream processing to remove contaminated molecules. This study reveals influences of different oscillating cultivation conditions applied in a multi-compartment scale-down system on misincorporation of non-canonical amino acids into a recombinant expressed miniproinsulin in *E. coli* K12. Due to technical limitations, each scale-down approach could only be done once. To exclude possible impacts due to crucial differences in growth during precultivation or initial batch phases online monitored shake flasks were used and the cultivation protocol was timed so that all cultivations were supervised from inoculation until the end of the production phase. It was possible to show that all physiological and process related differences seen in the scale-down cultivations must be caused by the different applied oscillating cultivation conditions.
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Interestingly, there is no common trend regarding biomass development and final reached biomass concentrations comparing the various scale-down approaches. Cultivations with oscillating glucose availability show a decreased biomass yield whereas oscillating starvation conditions do not seem to influence the final biomass concentration. In contrast, applying extra oxidative stress in the starvation loop leads to massive cell lysis and process performance lost. This observation fits with results discussed in the literature. Multiple times it was shown, that an increase in the cultivation volume – and scale-down cultivations are a simulated volume increase – can lead to a crucial loss of biomass yields compared to a standard lab scale (Neubauer and Junne 2010). On the other side, no influence on the growth can be seen under oxygen oscillations. It is known, that *E. coli* cells are showing a higher cell viability under some oscillation cultivation conditions (Enfors et al. 2001). Unfortunately, here the cell viability could not be studied. A strong impact has the additional aeration of the starvation zone resulting in spontaneous cell lysis with losses of 50 % of the biomass. The group around Qiang Li has shown in experiments with *Aspergillus niger* that “excess” oxygen leads to increased nutrient consumption not for additional biomass growth but as a defense mechanism against oxidative stress as the fungi cells increase proteolytic activity to degrade oxidatively damaged proteins (Li, Harvey, and McNeil 2008). If *E. coli* should react similarly to oxidative stress, excess oxygen in combination with nutrient starvation could temporarily overwhelm the antioxidant defense and lead to cell death and lysis. Stress responses and cell lysis under scale-down conditions have been described for *E. coli* before (Enfors et al. 2001).

All scale-down cultivations show a decreased glucose uptake. Also, starvation oscillations have a higher impact on glucose uptake compared to oscillating glucose excess. One explanation could be a repeated triggered stringent response due to oscillating starvation signals, and so a decreased metabolism to save energy (Chatterji and Kumar Ojha 2001). In the feeding loop setups, acetate and lactate formation could be detected with lactate formation rates surpassing acetate production. Formation of lactate plays a major role in the regeneration of nicotinamide adenine dinucleotide (NAD+) under oxygen limitation to stabilize the intracellular redox potential (Lara, Leal, et al. 2006).
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Crucial to produce pharmaceutical recombinant proteins is the misincorporation of non-canonical amino acids. It could be seen that already under “perfect” laboratory conditions non-canonical amino acids are produced. Furthermore, oscillating cultivation conditions boosted the production of all three analyzed amino acids. It was described before, that stressful cultivation conditions increase the formation of non-canonical amino acids. Especially a feeding loop STR-PFR setup with oscillating excess glucose results in norvaline accumulation in *E. coli* (Soini, Ukkonen, and Neubauer 2011). In this study, we could show, that also oscillating oxygen limitation leads to accumulation of non-canonical amino acids.

SDS-PAGE revealed a clear impact of oscillating cultivation conditions on miniproinsulin expression (data not shown), whereas GC-MS determination of the amino acid profile in the insoluble IB fraction has shown no clear impact. It must be said, that due to the small size of the miniproinsulin (around 11 kDa) problems with staining appeared and mostly several staining approaches, as well as reruns of the SDS-PAGE, were necessary to identify the band. Therefore, conclusions based on GC-MS analysis can be considered as more stable.

Furthermore, non-canonical amino acids can be incorporated into recombinant proteins (Harris and Kilby 2014) as analogs to chemically similar canonical branched-chain amino acids. In addition to the formation of non-canonical amino acids, their misincorporation can be seen in all cultivations and is boosted by oscillating cultivation conditions. Interestingly, the incorporation of norvaline is around 90 % lower compared to the other two amino acids, although the concentrations for free norvaline is in the same range as for free norleucine. Norvaline incorporation was linked to the rarest used leucine codon (Apostol et al. 1997). If this codon is not utilized in the gene sequence for the recombinant miniproinsulin misincorporation of norvaline could be set to a non-avoidable minimum.

There is a linear relation between the formation of free non-canonical amino acids and their misincorporation for leucine/norvaline and isoleucine/β-methyl-norleucine. The relation for methionine/norleucine exchange is not linear. Surpassing a critical concentration of free norleucine, the replacement of methionine into the miniproinsulin increases with an exponential rate. This observation would fit early postulations that a critical concentration of accumulated non-canonical amino acid is needed to trigger misincorporation (Bogosian et al. 1989).
4.1.6. Outlook
To improve the robustness of large-scale pharmaceutical Recombinant *E. coli* processes, a detailed understanding of cultivation parameters triggering the production and furthermore the incorporation of non-canonical amino acids into recombinant proteins is needed. This study gives a first look into characterizing the impacts of different large-scale phenomena on strain robustness and product quality. The spectrum of utilized analysis methods needs to be enhanced. Proteome analysis and at-line studies on cell polarizability could answer the question of cell lysis. Furthermore, utilizing the possibilities of fluorescent reporters enables new opportunities on at- and on-line strain population and single cell characterization and enhances the knowledge on the behavior of recombinant *E. coli* under scale-down conditions.

4.2. Transfer of oscillating substrate availability from a Two-Compartment Scale-Down Bioreactor to pulsed feeding for studies on product quality of recombinant *Escherichia coli*

4.2.1. Abstract
Increased mixing times due to limited achievable power input cause appearing gradients in oxygen and nutrient supply in large-scale bioreactors. To investigate potential influences of these gradients on microorganisms in the lab-scale, scale-down strategies in the form of multi-compartment reactors are utilized. Unfortunately, despite the advantages these systems have - characterization of dynamic responses of microorganisms regarding oscillating conditions for efficient product-focused bioprocess development - oscillating conditions need to be transferred towards high-through-put screening to open new ways of bioprocess development focusing on product quality in addition to product yield. In this study nutrient-limited fed-batch cultivations of a recombinant *Escherichia coli* strain overexpressing a leucine-rich miniproinsulin performed in a two-compartment bioreactor, setup is compared to a single-compartment STR approach with applied pulsed feeding. The two-compartment reactor consists of a stirred tank reactor and one attachable plug-flow reactor module. In this setup, the feed inlet is connected to the PFR compartment and so a feeding zone of a top-fed large-scale bioreactor (high nutrient concentration/oxygen limitation) is mimicked. The feeding profile in the pulsed feeding approach was adapted, so glucose addition in these cultivations is comparable to the MCR cultivations.
Our research results show that the impacts on growth and physiology are comparable in both approaches. We saw a decreased biomass and increased production of metabolites deriving from pyruvate based on overflow metabolism and mixed-acid fermentation (acetate and lactate) under oscillating/pulsed conditions. Furthermore, a flux into the branched-chain amino acid pathway can be seen contributing to an increased production of branched-chain amino acids including non-canonical amino acids like norvaline. Non-canonical amino acids can be incorporated into proteins, e.g. norvaline as a substitute for leucine. We see increased incorporation of the non-canonical amino acids norvaline, norleucine, and β-methyl-norleucine when oscillating cultivation conditions are applied, influencing the quality of the recombinant product and underlining the importance of product-quality-based bioprocess development. As the results of both approaches are comparable, we see here the possibilities to mimic impacts on cell physiology using techniques that are realizable in screening-scale cultivations.

4.2.2. Introduction
The most commonly used fermentation mode is the fed-batch cultivation. By adding a highly concentrated feed solution in an amount below the maximum specific substrate consumption rate of the cultivated organism the cellular metabolism can be controlled to prevent limitations by the design of the bioreactor, e.g. cooling capacity or oxygen transfer (Larsson et al. 1996), or accumulation of undesirable by-products, e.g. acetate in E. coli-cultivations (K. Andersen and Von Meyenburg 1980). After development in small lab-scale vessels, a bioprocess needs to be scaled up to produce bioproducts for economic reasons (Bylund et al. 1998). However, the scale change is challenging. Wang et al. already showed in 1979 that in a bioprocess scale-up from 10 L to 10000 L with a fixed aeration rate it is not possible to transfer all engineering scale-up criteria and that the mixing time is increasing. One published study on the production of interferon-α1 in a recombinant E. coli shows a 33% reduction of biomass yield and an over 50% decrease in product formation after volume scale-up (Riesenber et al. 1990).
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These losses are caused by gradient formation. Increasing production scales lead to increased mixing times and significant inhomogeneities regarding substrate concentration develop in comparison to the homogeneous laboratory bioreactor. Close to the feeding point high substrate concentrations occur, which can surpass the average concentration in the reactor for about 400 times (Bylund et al. 1998) and cells are periodically passing through these areas of high substrate concentrations (Larsson et al. 1996). Several studies have shown, however, that the substrate distribution is not only dependent on the axial distance to the feeding point, but is also radial and time-dependent, as it depends on the stirrer system, feed inlet pipe and position (Enfors et al. 2001). The formation of a substrate gradient also provides other inhomogeneities, such as a dissolved oxygen tension (DOT) gradient. Inside of the feeding zone the cells consume oxygen to a great extent while they are taking up large amounts of the substrate resulting in oxygen limitations at high cell densities (Bylund et al. 1998).

This oscillating experience of cells regarding high substrate availability combined with oxygen limitation leads to several responses of *E. coli* in large scale cultivations. These reactions include the production and consumption of acetate (B. Xu, Jahic, and Enfors 1999) or the accumulation of formic acid, lactate and succinate (B. Xu et al. 1999). Similar reactions to excess of glucose have already been reported for *Saccharomyces cerevisiae* with resulting accumulation of ethanol instead of acetate, both in aerobic as in anaerobic cultures (George et al. 1998) as well as for *Corynebacterium glutamicum* (Lemoine et al. 2015).

Critical for pharmaceutical production is the influence of heterogeneities in large scale bioreactors on the amino acid metabolism. In *E. coli*, non-canonical amino acids like norvaline, norleucine, and β-methyl-norleucine are produced as by-products from the branched chain amino acid pathway normally synthesizing valine, leucine and isoleucine (Bogosian et al. 1989; Muramatsu, Misawa, and Hayashi 2003). The origin of norvaline and norleucine is closely related to leucine synthesis shown for *Serratia marcescens* (Kisumi, Sugiura, and Chibata 1976a). It is known that non-canonical amino acids can be falsely incorporated into proteins. Norvaline can replace leucine (Apostol et al. 1997), norleucine is incorporated instead of methionine (Barker and Bruton 1979) and β-methyl-norleucine acts as an analogue for isoleucine (Muramatsu et al. 2002).
Several factors can trigger the enhanced production of these unusual amino acids. The overexpression of protein rich of leucine can accelerate the synthesis of branched chain amino acids. The raised productivity of this metabolic pathway also results in the accumulation of these amino acid analogs (Bogosian et al. 1989). The accumulation of non-canonical amino acids is also closely related to oxygen supply of the culture under glucose excess (Soini et al. 2008). It is known, that the incorporation of non-canonical amino acids can change properties and activity of protein (Gilles et al. 1988). To understand the impacts of oscillating cultivation conditions on microbial cells different scale-down approaches were developed and recently reviewed and summarized (Neubauer and Junne 2016).

In scale-down systems the effects of heterogeneities have already been widely investigated for *E. coli* cultures and production systems regarding their influences on growth, production formation, physiology, transcription patterns, respiration, and organic acid formation (Enfors et al. 2001) as well as recombinant protein production (Sandoval-Basurto et al. 2005).

Due to their complexity, scale and missing parallelization these concepts are not applicable in high-through-put screening for new product quality based processes. In this study, the concept of pulsed feeding has been used in a single-compartment STR approach, which could be further minimized towards screening scale, and compared to results from a two-compartment cultivation. The pulse pattern in the STR and the residence time in the 2CR were adapted to mimic a 10 % feeding zone like in large-scale bioreactors (Bylund et al. 1999).

The focus of this study is to reveal the feasibility of oscillating substrate availability from complex scale-down simulators to easier approaches, which could be implemented into the high-through-put screening. The focus is not on scaling down the simulator but on the comparability of cellular responses like growth, organic acid production, and product quality.
4.2.3. Materials and Methods

4.2.3.1. Strain

E. coli K12 W3110M, which had a mutation causing an overexpression of the lac inhibitor and carried the plasmid pSW3 (recombinant miniproinsulin expressed as inclusion bodies and ampicillin resistance gene) was used in all cultivations. Sanofi-Aventis Deutschland GmbH thankfully provided both strain and plasmid.

4.2.3.2. Media

All chemicals were purchased from either Carl Roth GmbH, Karlsruhe, Germany, or Sigma-Aldrich Chemie GmbH, Munich, Germany, if not otherwise stated. The composition of LB medium used for first cultivation was 10 gL\(^{-1}\) tryptone, 5 gL\(^{-1}\) yeast extract, 10 gL\(^{-1}\) NaCl and 100 µgL\(^{-1}\) ampicillin. The minimal medium (B. Xu, Jahic, and Enfors 1999) in the bioreactor consisted of 2 gL\(^{-1}\) Na\(_2\)SO\(_4\), 2.468 gL\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 5 gL\(^{-1}\) NH\(_4\)Cl, 14.6 gL\(^{-1}\) K\(_2\)HPO\(_4\), 3.6 gL\(^{-1}\) NaH\(_2\)PO\(_4\) x 2H\(_2\)O, 1 gL\(^{-1}\) (NH\(_4\))\(_2\)-H-Citrat, 1 mL Antifoam Sigma 204. Per liter medium 2 mL trace elements, 2 mL thiamin solution (50 gL\(^{-1}\)), 2 mL MgSO\(_4\)-solution (1.0 M) and 1 mL ampicillin solution (100 mgL\(^{-1}\)) were sterile-filtered through an 0.22 µm-membrane filter into the reactor. The trace element solution contained 0.5 gL\(^{-1}\) CaCl\(_2\) x 2H\(_2\)O, 0.18 gL\(^{-1}\) ZnSO\(_4\) x 7H\(_2\)O, 0.1 gL\(^{-1}\) MnSO\(_4\) x H\(_2\)O, 20.1 gL\(^{-1}\) Na-EDTA, 16.7 gL\(^{-1}\) FeCl\(_3\) x 6H\(_2\)O, 0.16 gL\(^{-1}\) CuSO\(_4\) x 5H\(_2\)O, 0.18 gL\(^{-1}\) CoCl\(_2\) x 6H\(_2\)O. The initial batch glucose concentration was 5 gL\(^{-1}\).

4.2.3.3. Procedure of Precultivation

Twenty-five milliliters of LB medium was inoculated with 50 µL of cryostock and incubated at 37°C for three h at 250 rpm in a 125 mL Ultra-Yield flask. For the second preculture, 100 mL (pulsed-fed) or 500 mL (2CR) cultivation medium was inoculated with one mL / five mL LB-preculture normalized to OD\(_{600}=1\) in suitable UltraYield flasks and cultivated like before. At an OD\(_{600}\) of 0.3 total pre-culture volumes were used to inoculate the bioreactors to a final OD\(_{600}\) of 0.015.
Bioreactor cultivation
For multi-compartment cultivations, a 15 L Biostat E stirred tank bioreactor (Sartorius Stedim Biotech GmbH, Göttingen, Germany) equipped with three Rushton turbines was used. For scale-down cultivations, the STR was connected to one PFR module. The PFR has a working volume of 1.2 L consisting of four static mixer elements. A complete PFR module (considering the tubing from the STR and back) has a total working volume of 1.8 L. The flow rate through the PFR was set to 1.7 Lmin⁻¹ at all cultivations, so the mean residence time in the PFRs was 68 s. The PFR modules and the setups of a two-CR and possible three-CR have been described in more detail previously (Junne et al. 2011; Lemoine et al. 2015).

The pulsed-fed cultivation was done in a KLF 2000 with a maximum volume of 3.7 L from the Bioengineering AG (Wald, CH).

10 L or 2 L of the before described mineral salt medium were inoculated with 5 % (v/v) preculture. After a batch phase until first substrate depletion feeding was started. The feed solution consisted of 440 gL⁻¹ glucose x H₂O solved in the mineral salt medium described before. Differences are four times higher trace element concentration, no MgSO₄, antifoam or ampicillin. 20 mL or 4 mL 1.0 M MgSO₄-solution was added via sterile filtration every OD600 20. The feed was connected to the top gas phase of the STR for the single-CR control cultivation and pulsed-feeding setups. For the scale-down cultivations involving a feeding loop configuration (two-CR), the feeding solution was added to the inlet tube of the PFR module. An overview of the different cultivation setups is illustrated in Figure 4.2.1.

The feeding phase could be divided into two parts. The first part was an exponential feeding phase with a start feed rate $F_0 = 0.06$ Lh⁻¹ (10 L scale) or $F_0 = 0.0125$ Lh⁻¹ (2 L scale) and an exponential increase of $\mu = 0.3$. After three hours of exponential feeding expression of the recombinant protein was induced via the addition of IPTG to a final concentration of 1 mM and the feeding rate is switched to constant feeding at $F = 0.160$ Lh⁻¹ (10 L) or $F = 0.032$ Lh⁻¹ (2 L). In pulsed-fed cultivations, a feeding cycle of 10 minutes was applied feeding the whole glucose in the first minute of a cycle followed by a 9-min recovery. The specifically fed glucose per time is in all approaches identical. The cultivation temperature was set to 35°C. The aeration rate was initially set to 0.5 vvm and increased to 1.0 vvm after feed start. The pH was set to pH = 7.0 controlled with 25 % NH₃ solution.
Figure 4.2.1: Reactor setups used in this study showing the mean residence time (τ) at the ports of the PFR modules. It is also describing which cultivation conditions occur in the PFR module.

4.2.3.5. Analysis

Optical density measurements monitored cell growth at a wavelength of 600 nm (OD$_{600}$) (Novaspec III by Amersham Biosciences, Amersham, UK) and dried cell weight (DCW) determination. To measure DCW 2 mL of cell suspension were transferred into a dried, pre-weighted two mL microcentrifugation tube. After centrifugation for 10 min at 21,500 xg, the supernatant was discarded, and the cell was resuspended in 1 mL 0.9 % (w/v) NaCl solution. Following a repeated centrifugation step the tube was dried 75°C for 24 h.

Supernatant samples for analysis of extracellular metabolites and amino acids concentrations were directly filtered through a 0.8 µm pore sized membrane filter directly at the sampling port of the STR and stored in 1.5 mL tubes at -80°C.
Samples for analysis of the total free amino acid concentration and the amino acid composition of the inclusion bodies protein three mL of cell suspension were harvested into a syringe containing two mL methanol precooled and immediately stored at -80°C. Before the analysis of the free amino acids, cell samples were diluted to a DCW concentration of 1.6 gL⁻¹ and homogenized via sonication using a sonotrode (UP2000, Dr. Hielscher, Teltow, Germany) with a diameter of 1 mm. The applied amplitude was set to 30 % for three cycles each 30 s long interrupted by a 30 s break. The homogenized cells were centrifuged (15000g, 10 min, 4°C) and the supernatant used for quantification. For the concentration measurements of the inclusion bodies protein samples were diluted to a DCW of 6 gL⁻¹. Inclusion bodies purification was done using the BugBuster® Protein Extraction Reagent (Merck, Darmstadt, Germany).

**Quantification of Metabolites**

Quantification of organic acids and Glucose was performed using an Agilent 1200 HPLC system (Waldbrohn, Germany) equipped with a HyperRez™ XP Carbohydrate H+ column (300 x 7.7 mm, 8 µm) (Fisher Scientific, Schwerte, Germany) and a refractive index detector. As eluent, five mM H₂SO₄ was applied at a temperature of 15°C with a flow rate of 0.5 mLmin⁻¹.

Amounts of amino acids were quantified with an Agilent 5975 C GC-EI-MS equipped with a DB-5MS column after drying the samples in a speed vacuum concentrator (Bachhofer, Reutlingen, Germany) and derivatization with n-tert-butyldimethylsilyl-n-methyl-trifluoroacetamide. Purified inclusion bodies samples were hydrolyzed prior for 24 h in 6 M HCl at 80°C. Detailed analysis protocols are attached in chapter 9.1, 9.2 and 9.3.

**Data Fitting and Visualization**

All data was fitted using TableCurve 2D v5.01 by Systat (Systat Software Inc., 2002, San Jose, CA, USA). Data plots were created with Qtiplot (Qtiplot.com).
4.2.4. Results

To point out, the STR vessel itself has no impact on the cultivation results. Data from reference cultivations in both vessels without oscillating conditions proved comparable results regarding growth, metabolic behavior, amino acid concentrations (canonical and non-canonical) as well as productivity and quality of the produced miniproinsulin (partly shown in Figure 4.3.2).

![Graphs showing growth of biomass, oxygen uptake rate over cultivation time, glucose consumption rate and leucine content in the IB protein fraction. Line at 0h marks the start of feeding, dashed line at 3 h the point of induction. Filled circle show the 10 L cultivation, the open circles the 2 L scale cultivation data.](image)

**Figure 4.3.2**: Growth of biomass, oxygen uptake rate over, glucose consumption rate and leucine content in the IB protein fraction the cultivation time. Line at 0h marks the start of feeding, dashed line at 3 h the point of induction. Filled circle show the 10 L cultivation, the open circles the 2 L scale cultivation data.
4.2.4.1. **Growth**

Both cultivations show a similar growth profile with exponential growth during the exponential feeding and linear growth after switching to constant feeding. What can be seen is a higher specific growth rate and an increased biomass yield in the 2CR cultivation compared to pulsed feeding (final DCW 19 gL\(^{-1}\) compared to 13 gL\(^{-1}\)), although the same specific glucose feeding rate is applied. Also, more Antifoam was needed for the pulsed fed culture to control a higher production of foam. A look at the respiratory activity reveals slightly increased oxygen uptake in the pulsed setup during exponential feeding. After switching to constant feeding the specific oxygen uptake is constant at around 5.1 mmol/gh in the STR department of the 2CR configuration, but decreasing in the pulsed fed setup (Figure 4.3.3).

![Figure 4.3.3](image-url): Growth of biomass and oxygen uptake rate over the cultivation time. Line at 0h marks the start of feeding, dashed line at 3 h the point of induction. Filled triangles show the 2CR cultivation, the open triangles the pulsed-fed cultivation data.
Monitored during the constant feeding phase (protein production) phase the glucose uptake is increased in the pulsed-fed setup compared to the 2CR cultivation. During this phase, it was seen that glucose pulses, which delivered the same amount of glucose compared to ten minutes 2CR cultivation time, was used up before the ten-minute interval was over indicated by a steep increase in DOT 60 to 90 seconds before the next glucose pulse. Although the glucose uptake is higher under pulsed-fed conditions a slightly lower acetate production can be observed including acetate consumption near the end of the cultivation time. Interestingly, almost no lactate production can be seen under pulsed-fed conditions, although a phase of formation and consumption can be observed in the 2CR setup. The reason is a phase of glucose accumulation in the 2CR system during exponential feeding triggering overflow metabolism until glucose limitation is reached (Figure 4.2.4).

**Figure 4.2.4:** Glucose uptake and production rates for lactate and acetate from the point of induction. Filled triangles show the 2CR cultivation, the open triangles the pulsed-fed cultivation data.
4.2.4.3. Production of and Incorporation of non-canonical amino acids into the recombinant protein

Comparing the exchange rates of essential non-canonical amino acids to their chemical similar canonical counterparts only one real difference between the systems can be seen (Figure 4.2.5). In both approaches, the exchange of Leucine to Norvaline in the total amino acid pool and the Inclusion bodies fraction can be detected. Up to 4% of the cellular Isoleucine is exchanged by β-Methyl-Norleucine. This ratio is increasing over the time of insulin expression and is comparable to both systems. The exchange rate in the IB fraction is not changing over time (around 0.3%) and is not influenced by the type of oscillating conditions. The highest exchange rate in the IB fraction is observed by Norleucine replacing Methionine at around 1.8%. Crucial is here the high exchange rate (13%) of Methionine by Norleucine in the total cellular fraction under 2CR conditions this rate is 3.5% in the pulsed fed setup while at the same time. It must be mentioned that the concentration of Norleucine in both cultivations was comparable. The higher exchange rate is a result of lower methionine concentrations under 2CR conditions.

Figure 4.2.5: Ratio of non-canonical amino acids in comparison to their chemical similar canonical counterpart. Filled circles show the 2CR cultivation, the open circles the pulsed-fed cultivation data.
4.2.5. Discussion

This study compares the comparability of two scale-down approaches. As both techniques vary in the basic setup (multi-compartment vs. single compartment) different sets of information can be gained from these experiments. As the focus was set on basic growth and the misincorporation of non-canonical amino acids into a recombinant produced miniproinsulin required samples could be taken in both experimental setups. Due to technical limitations, each of both scale-down approaches had to be done in a different scale (MCR: 10 L stainless steel vs. SCR: 2 L glass). To exclude possible impacts due to the reactor materials on comparability reference cultivations in both vessels proved comparable cultivation results on all relevant and monitored biological and process factors if a scale-adapted cultivation protocol is applied. All physiological and process related differences seen in the scale-down cultivations must be caused by the altered application of oscillating cultivation conditions.

Interestingly, the trend of biomass development and final reached biomass concentrations is similar in between the scale-down approaches compared to their reference cultivations. In general, scaled up cultivations lead to a significant decrease in biomass yield compared to a typical lab scale cultivation (Onyeaka, Nienow, and Hewitt 2003). This decrease cannot be seen in the shown scale-down approaches. One reason could be, that the residence time in the PFR and so the pulse profile in the STR were not inducing a stress level comparable to large-scale cultivations. A more rapid pulse pattern has been shown to result in biomass yields reduced by 40 % (Ying Lin and Neubauer 2000). On the other side, it has been reported that E. coli cells experiencing oscillating cultivation conditions have a higher cell viability compared to lab scale cultivations (Enfors et al. 2001).

Besides, a clear difference in growth behavior can be detected comparing the STR-PFR setup compared to the pulsed STR approach. As in the MCR cultivation, the final biomass yield even surpassed the biomass concentrations gained in the reference cultivation the final biomass concentration in the pulsed fed setup is 33 % lower, although this cultivation shows a slightly higher glucose uptake rate and at the same time lower production rates for lactate and acetate. In previous scale-down studies with E. coli, it has been shown oscillating cultivation conditions lead to increased expression of stress-induced genes (Enfors et al. 2001) or even increased cell lysis (Bylund et al. 2000). The higher need of Antifoam in the pulsed culture could be a hint of higher stress in the cultivation. A major difference in the pulsed fed approach
is the reduced stirrer speed during the pulse. This leads periodically to a decreased gas transfer and oscillating CO₂ accumulation in the cultivation medium. It has been reported that CO₂ oscillations can influence growth rate and biomass yield in recombinant E. coli cultivations (Baez et al. 2009).

The second main difference between both cultivation modes is the differing oxygen consumption rate. This observation could be explained by the oscillating changes in gas transfer due to changing stirrer speeds during the glucose pulses. Furthermore, due to the reduced glucose uptake rate a short phase of glucose accumulation during the exponential feed in the two-CR setup could be detected, although (adapted to the scale difference) the same amount of glucose was fed. This accumulated glucose did not lead to an increased formation of acetate compared to the pulsed-fed cultivation but in a temporary accumulation of lactate. Formation of lactate is an important part to obtain the redox potential by regenerating nicotinamide adenine dinucleotide (NAD+) under oxygen limitation (Lara, Leal, et al. 2006). It is metabolized, as soon the cultivation reached glucose limitation again. As the pulsed-fed cultivation is under glucose limitation during the complete feeding phase, the reason for no detection of lactate could be immediate consumption.

Crucial for the future application of this study is the comparability of misincorporated non-canonical amino acids. Although a lower biomass yield was reached in the pulsed fed setup, recombinant expression of the miniproinsulin was not affected by the scale-down approach. Furthermore, ratios of misincorporation of the analyzed non-canonical amino acids norvaline, norleucine and β-methyl-norleucine are almost identical. It is known, that stressful cultivation conditions lead to increased production of non-canonical amino acids in E. coli cells (Soini, Ukkonen, and Neubauer 2011). These non-canonical amino acids can be incorporated into recombinant proteins (Apostol et al. 1997; Fenton et al. 1997; Muramatsu, Misawa, and Hayashi 2003). This study also shows, that quality impacting misincorporations can be triggered in easy to apply experimental setups on a comparable level and a complex scale-down simulator is not essentially needed to gain information on possible production scale behavior of the selected production host.
Interestingly, the “contamination” rate of norleucine is increased by 10 % in the total proteome pool of the two-compartment setup compared to the single vessel approached, although the impurification rate in the miniproinsulin is almost identical. Here, it should be noted, that the total concentration of norleucine is on a similar level in both cultivations, but a significantly lower concentration of methionine could be detected in the TCR cultivation leading to this higher rate. The reason for lower methionine synthesis is unknown and not discussed in literature before.

4.2.6. Outlook
To improve the robustness of large-scale recombinant E. coli processes, a detailed understanding of cultivation parameters triggering the production and furthermore the incorporation of non-canonical amino acids into recombinant proteins is needed already at the stage of process development. Here we could show that simplifying the scale-down procedure delivers comparable results at the cost of dynamic process characterization. This possible drawback is compensated by the possibility to transfer the pulsed-feeding protocol to even smaller STR vessels up to the mL-scale and so enables parallelized development runs screening for best production under non-optimum conditions. Furthermore, utilizing the possibilities of fluorescent reporters enables new opportunities on at- and on-line strain population and single cell characterization and enhances the knowledge on the behavior of recombinant E. coli under scale-down conditions.
4.3. Impacts of oxygen oscillations on product quality in recombinant *E. coli* cultivated in multi-well plates

4.3.1. Abstract
Gradient formation regarding oxygen availability and nutrient supply cannot be prevented in industrial-scale bioreactors due to limited power input causing insufficient mixing. To understand microbial responses to these gradients, scale-down systems based on connected multiple compartments have been established. Unfortunately, the complexity of these approaches targeting on detailed characterization of changes in microbial physiology disqualifies their use in high-throughput screening. In this study, the fed-batch simulating EnBase® technology is used to research the impact of oxygen oscillations on the quality of a miniproinsulin expressed in recombinant *E. coli* with different feeding rates and inducer concentrations using 24-low-well plates equipped with fluorescent oxygen and pH sensors provided by the company PreSens.

Our research results show that norvaline, norleucine, and ß-methyl-norleucine are produced and incorporated into the recombinant miniproinsulin under oscillating as well as under reference conditions. Interestingly, the misincorporation can be boosted under reference conditions with increasing feeding rate and stronger induction of protein expression. Misincorporation rates of all three non-canonical amino acids are significantly increased under oxygen oscillations. Also, the effect of oxygen oscillations is so crucial that no additional effects of feeding rate or inducer concentration on the incorporation of norvaline, norleucine, and ß-methyl-norleucine can be detected.

4.3.2. Introduction
The scale-up of bioprocesses is crucial due to economic reasons (Bylund et al. 1998) and changes the environmental conditions cells experience unavoidable. Physical factors defining these conditions such as the shear rate, volumetric oxygen transfer rate, volumetric power input, and mixing time are usually optimized in the laboratory scale and cannot be moved as whole to a new scale. Even with a similar reactor geometry and an equivalent aeration rate, each physical parameter would need different stirrer speeds to maintain constant during scale-up. Especially the stirrer speed to keep a similar mixing time increases significantly with higher volumes as in larger scale processes the cultivation broth needs to be moved faster to
be spread throughout the reactor (D. Wang 1979) resulting in the formation of heterogeneities (Delvigne, Destain, and Thonart 2006).
Considering increased mixing times during scale up, the addition of a highly concentrated and viscous substrate solution from the top of the reactor causes the formation of a gradient regarding substrate distribution. The added feed solution is not immediately dispersed evenly across the whole bioreactor. Thus, an area close to the substrate inlet can be detected and characterized by a significant higher substrate concentration than the average concentration in the remaining bulk reactor. In literature, this area is known as feeding zone. With increasing distance to the feed inlet, the substrate concentration decreases up to depletion, as it is consumed faster than it spreads. For a large-scale bioprocess, the substrate concentration is not only dependent on the distance to the point of feed addition, but also differs regarding the radial distance, time, stirrer type and the position of the substrate inlet (Enfors et al. 2001).
Also, the described substrate gradient supports the formation of an oxygen gradient. If the large-scale process is aerated from below, the dissolved oxygen pressure at the bottom of the reactor is high due to the nearness of the aeration system. This area is called aeration zone. With increasing distance to the aeration system, the oxygen partial pressure decreases owing to the insufficient mixing. Furthermore, with the increase in substrate concentration, the consumption of oxygen by the cells increases and supports the formation of an oxygen gradient (Bylund et al. 1999). These effects of a process scale-up can crucially impact the productivity of recombinant microorganisms. It was shown for recombinant *E. coli* cells that a volume increase from 30 to 450 L (16.7x) resulted in 1.5x lower biomass and 2.75x lower product yield during the cultivation (Riesenbergen et al. 1990).
At high substrate concentrations overflow metabolism is triggered in *E. coli* due to a substrate flow through glycolysis which surpasses the maximum capacity of the TCA cycle leading to pyruvate accumulation. This pyruvate is converted to acetate, which is assimilated under substrate limitation conditions (B. Xu, Jahic, and Enfors 1999) and so serves as kind of energy storage, but also inhibits growth (Lee 1996). Acetate is also a product of the mixed acid fermentation pathway along formic acid, lactate, and succinate under substrate excess conditions combined with oxygen limitation (as it occurs in a large-scale reactor feeding zone) (B. Xu et al. 1999). Further, the branched chain amino acid biosynthesis pathway is upregulated under conditions which induce pyruvate accumulation. This increased activity leads to the synthesis of valine, leucine, and isoleucine, but also non-canonical amino acids.
like norvaline (Kisumi, Sugiura, and Chibata 1976b), norleucine (Bogosian et al. 1989), and β-methyl-norleucine (Muramatsu, Misawa, and Hayashi 2003). Overexpression of a leucine-rich recombinant protein in combination with oxygen limitation or oxygen oscillations and glucose excess have been reported to trigger the synthesis of these non-canonical amino acids (Veeravalli et al. 2015). It also has been reported, that these non-canonical amino acids can become incorporated into recombinant proteins, like norvaline for leucine (Apostol et al. 1997), norleucine instead of methionine (Van Hest, Kiick, and Tirrell 2000), or β-methyl-norleucine for isoleucine (Muramatsu, Misawa, and Hayashi 2003).

It is assumed that only a minor fraction of the synthesized non-canonical amino acids is incorporated into native proteins of the organism because native proteins are only a small fraction of the newly synthesized proteins at the time of induction and overexpression of the recombinant protein (Bogosian et al. 1989).

Previously, several systems have been used to simulate oscillating cultivation conditions like they appear in large-scale bioreactors, such as the two-compartment reactor. A two-compartment reactor can be implemented through the combination of two stirred tank reactors (STR-STR) (Sandoval-Basurto et al. 2005) or a stirred tank reactor coupled to a plug flow reactor (STR-PFR). The PFR has the possibility to add a feeding solution or to be aerated (Enfors et al. 2001). These techniques allow the simulation of the passage of the cells through different “zones” of the reactor, and so to investigate the effects of different gradients.

A used alternative to multi-compartment setups is the application of oscillations inside a single cultivation vessel via variation of aeration, pH, mixing and the pulse addition of a feeding solution. This approach can be combined with automatization, parallelisation, and miniaturization of the system to reach a higher throughput of numbers of experiments (Neubauer and Junne 2010). Several investigations on the response of E. coli to these conditions have been performed while applying these scale-down devices. Influences on growth, product formation, physiology, transcription pattern, stress responses, respiration and organic acid production were analysed (Enfors et al. 2001).
However, the results of these investigations have shown several deviations between homogenous lab scale cultivations and cultivations performed under simulated large-scale conditions. For the recombinant production of human growth hormone (Bylund et al. 2000), pre-proinsulin (Sandoval-Basurto et al. 2005), green fluorescent protein (Lara, Vazquez-Limón, et al. 2006), and α-glycosidase (Ying Lin and Neubauer 2000) was observed that the product yield and concentration decreased under scale down conditions. At the same time, these conditions caused a 10% increase in correctly folded proteins for human growth hormone and a decrease of proteolysis (Bylund et al. 2000). Additionally, the viability of wild-type *E. coli* W3110 increased (Onyeaka, Nienow, and Hewitt 2003), but heterogenic conditions lead to the formation of subpopulations which had lost their capability for the general stress responses regulated by the σ^S–factor (Delvigne et al. 2009). For *E. coli* the plasmid stability increased under oscillating conditions (Ying Lin and Neubauer 2000).

In this study, the impact of oxygen oscillations on the quality (amount of misincorporated non-canonical amino acids) of a recombinant miniproinsulin is observed in an easy to set up approach. 24-low-well plates equipped with fluorescent oxygen and pH sensors and provided by the company PreSens are used in combination with the fed-batch simulating EnBase® technology to cultivate the recombinant *E. coli* with different feeding rates and inducer concentrations within one cultivation.
4.3.3. Material and Methods
The main focus of these experiments was to investigate the influences of oscillating oxygen supply on the misincorporation of non-canonical amino acids into a recombinant miniproinsulin at different feeding rates and inducer concentrations in a high-throughput compatible setup. The experiments were performed using an on/off shaking approach in comparison to a continuously agitated culture within 24-well plates enabled for online pH (HydroDish®HD24) and dissolved oxygen tension (DOT) (OxoDish®HD24) measurements manufactured by PreSens GmbH (Regensburg, GER). The dissolved oxygen concentration oscillations were applied in ten-minute cycles consisting of nine minutes shaking followed by a shaker stop for one minute. These oscillations lead to recurring oxygen limitation for 10 % of the expression time. E. coli K12 W3110M, which had a mutation causing an overexpression of the lac inhibitor and carried the plasmid pSW3 (recombinant miniproinsulin expressed as inclusion bodies and ampicillin resistance gene) was used in all cultivations. Sanofi-Aventis Deutschland GmbH thankfully provided both strain and plasmid.

4.3.3.1. Preculture
20 mL LB containing 0.1 g L⁻¹ ampicillin in a 100 mL Erlenmeyer flask was inoculated with 50 µL cryo stock and cultivated for six hours at 37 °C and 250 rpm. A final optical density (OD₆₀₀) of 5.4 was reached.

4.3.3.2. Cultivation Conditions
In both cultivations, the Enpresso® B medium by Biosilta was used to apply fed-batch conditions. Before induction, cells were cultivated in 75 mL medium in a 500mL pre-calibrated Corning®-Erlenmeyer flask equipped with online measurement probes for dissolved oxygen and pH (PreSenS GmbH, Regensburg, Germany). 3 UL⁻¹ of reagent A were used as initial feeding rate. The medium was inoculated to an OD₆₀₀ of 0.054 with the LB preculture. The flask was set on the PreSens Shake Flask Reader system (PreSens GmbH) to monitor DOT and pH online and cultivated overnight at 35°C and 250 rpm up to an OD600 of approximately 11.5.

Then the culture was distributed on HydroDish®HD24 and OxoDish®HD24 multi-well plates (PreSens GmbH). In each of the 24 wells per plate one mL culture was filled. Another 0.1 gL⁻¹ ampicillin were added into each well as well as IPTG (for induction of miniproinsulin expression) and reagent An (adjustment of feeding rate) to match the concentrations pictured
in Table 4.3.1. Purified water was used to equalize the volumes of added substances. Both plates were cultivated under the same cultivation conditions with the HydroDish®HD24 for pH monitoring and the OxoDish®HD24 for measuring the DOT.

**Table 4.3.1:** Schematic illustration of the applied cultivation conditions regarding feeding rate (amylase concentration) and induction intensity (IPTG concentration) for the HydroDish®HD24 and the OxoDish®HD24 during the oscillating and the homogenous cultivation.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Well</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amylase [U/L]</td>
<td>A</td>
<td>3.0</td>
<td>4.5</td>
<td>6.0</td>
<td>7.5</td>
<td>9.0</td>
<td>10.5</td>
</tr>
<tr>
<td>IPTG [µM]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Amylase [U/L]</td>
<td>B</td>
<td>3.0</td>
<td>4.5</td>
<td>6.0</td>
<td>7.5</td>
<td>9.0</td>
<td>10.5</td>
</tr>
<tr>
<td>IPTG [µM]</td>
<td>50</td>
<td>50</td>
<td>50</td>
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<td>50</td>
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<td>50</td>
</tr>
<tr>
<td>Amylase [U/L]</td>
<td>C</td>
<td>3.0</td>
<td>4.5</td>
<td>6.0</td>
<td>7.5</td>
<td>9.0</td>
<td>10.5</td>
</tr>
<tr>
<td>IPTG [µM]</td>
<td>250</td>
<td>250</td>
<td>250</td>
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<td>250</td>
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</tr>
<tr>
<td>Amylase [U/L]</td>
<td>D</td>
<td>3.0</td>
<td>4.5</td>
<td>6.0</td>
<td>7.5</td>
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<tr>
<td>IPTG [µM]</td>
<td>1000</td>
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The plates were cultivated on the associated SDR Sensor Dish Reader system (PreSenS GmbH). This expression phase lasted five hours at 35 °C. For the homogenous culture 200 rpm were applied continuously, as for the oscillating shaking cultivation 9 min with 200 rpm were followed by one min of shaking stop.

### 4.3.3.3. Analytical Methods

**Optical Density at 600 nm**

Optical densities for both plates (data from HydroDish®HD24 not shown) were determined as endpoint measurements in duplicate diluted 1:100 in 0.9 % NaCl solution. The measurements were performed using a Synergy™ Mx Microplate Reader (BioTek Instruments, Winooski, USA).
Results

Analysis of amino acid composition of inclusion bodies proteins

800µL of cultivation broth sample drawn from each well of the OxoDish®HD24 at the end of the cultivation time were immediately centrifuged for 10 min at 16000 rpm and 4°C. The supernatant was discarded and the cell pellets were stored at -20°C until further analysis.

For IB purification the cells were resuspended in purified water to an OD$_{600}$ of 18 (around 6 g L$^{-1}$ DCW). 200 µL were taken for inclusion bodies purification using the BugBuster® Protein Extraction Reagent (Merck, Darmstadt, Germany) and then hydrolyzed and analyzed like described in SOP 2 (Chapter 9.2).

4.3.4. Results

4.3.4.1. Environmental conditions

The initial batch phase of both cultivation approaches was performed in a shake flask before splitting the culture into multi-well plates at the point of induction. A comparison of the online monitored parameters pH and DOT of both cultures is illustrated in Figure 4.3.1. Both cultivations show the same trend towards DOT and pH development, and so it can be assumed that both cultivations are in the same physiological state at the point of distribution and all upcoming differences are based on further applied cultivation conditions.

Based on the DOT trend, both cultivations show an exponential growth rate for the first three hours followed by glucose-limited growth. Dissolved oxygen levels never decreased under 60% indicating always efficient supply of the cells with oxygen. For both cultures, the pH slightly decreases during the cultivation time from around 7.2 to 6.6. This reduction displays the consumption of ammonium from the medium as a nitrogen source, which leads to a pH decrease as no pH controlling reagent as nitrogen source has been fed.
Results

Christian Reitz

After the initial fed-batch phase each of the two cultures has been distributed into two 24 well dishes allowing either the online measurements of oxygen or pH (HydroDish®HD24 and OxoDish®HD24). While the reference cultivation was performed with constant shaking the oscillating cultivation suffered periodical oxygen limitation triggered via switching off the shaking of the incubator. Furthermore, different feeding rates and inducer concentrations were applied to each well. The comparison of the online measurements per well for both cultures is shown in Figure 4.3.2 and 4.3.3.

Figure 4.3.1: Online measurement of the dissolved oxygen tension and the pH for the initial fed-batch phase ahead of induction performed in a shake flask equipped with online measurement probes. Feeding started at 0h.
Observing oxygen level spikes (Figure 4.3.2) due to non-continuous shaking can clearly be detected in the early phase of the cultivation. Switching off shaking leads to a sudden decrease in DOT and an increase in DOT can be seen with continued shaking. With increasing cell density, especially under higher feeding rates these spikes disappear and minimum oxygen levels of 8.3 % are measured in wells of both the continuous and non-continuous cultivations. It seems doubtful that stopping the shaking stops to have an influence on the culture after reaching a certain biomass. Furthermore, the combination of filling volume and stirrer speed could have led to a non-optimum liquid level above the sensor spot and so resulted in a limited measurement range at low oxygen concentrations. In general, trends in DOT levels are comparable for both experiments. Oscillating oxygen availability does not largely influence the overall oxygen uptake. Under all cultivation conditions, a linear decrease in available DOT is detectable until the detection mentioned above limit is reached. With increasing feeding rate this point is reached earlier.
Results

Christian Reitz

Figure 4.3.2: Online monitoring of DOT per well performed with OxoDish®HD24. Different addition of amylase and IPTG per well. Induction started at 0 h.
Figure 4.3.3: online measurements of pH per well performed with HydroDish™ HD24. Different addition of amylase and IPTG per well. Induction started at 0 h.
In the oxygen oscillating cultures, an increasing feeding leads to lower oxygen concentrations during the shaking breaks. Furthermore, with increasing concentrations of IPTG and so the strength of recombinant protein production, the oxygen consumption is raising at low feeding rates. This effect is compensated by the overall increased demand for oxygen with increasing feeding rate.

Oscillating oxygen availability has no effect on the pH level (Figure 4.3.3) as nearly no difference can be detected between both cultivations. While pH values showed almost no change at the lowest feeding rate adjusted with 3 UL\(^{-1}\) reagent A, higher feeding rates led to a pH decrease accelerated with increasing growth. IPTG concentration does not seem to have an impact on the pH trend.

4.3.4.2. Cell growth
Due to limited cultivation volume, cell growth was monitored via optical density measurements only at three-time points: end of preculture, at the point of induction, and at the end of the experiment. A comparison of ODs for both cultivation modes and all applied cultivation conditions is shown in Figure 4.3.4.

Comparable ODs could be detected at the end of the preculture and the point of protein induction, which means at the point of splitting the culture into the multi-well plates. Both cultivations are in a comparable metabolic state, and all differences seen at the end of the multi-well phase of the experiments are based on effects due to cultivation conditions.

As the total cultivation volume was limited to 2x 1mL the ODs at the endpoint were measured in a Synergy™ Mx Microplate Reader (Biotek, USA) using a sample volume of 150µL.
Figure 4.3.4: Optical density measurements. Before induction performed for the shake flask cultures with an Ultrospec 2100 pro Photometer. After induction (0 h) performed with a Synergy™ Mx Microplate Reader for each well. Different addition of amylase and IPTG per well.
Unfortunately, these endpoint measurements showed a higher deviation compared to the earlier cuvette measurements in some cases. As these data points were the basis for the normalization of biomass concentration for the analysis of the inclusion bodies, this might cause a problem regarding wrongly calculated amino acid concentrations at the analysis of the recombinant protein. Therefore, in cases of a high standard deviation outliers differing significantly from other optical density measurements were not included in the following normalization calculation.

In general, optical densities under oscillating oxygen cultivation conditions are lower than for the reference cultivation. An increasing feeding rate at first seems to result in higher cell densities due to more available substrate released over the cultivation time. There is a trend towards lower ODs using reagent A concentrations greater than 7.5 \text{UL}^{-1}, which could be based on the accumulation of growth inhibitory side products. There is no obvious influence of the induction intensity on the cell growth.

4.3.4.3. **Amino Acid composition**

After purification and hydrolysis, the amino acid composition of the inclusion bodies fraction has been analyzed using GC-MS to reveal the influences of periodically applied oxygen limitation on the incorporation of the non-canonical amino acids norvaline, norleucine, and $\beta$-methyl-norleucine into the leucine-rich recombinant miniproinsulin. Before the grade of purity was checked on an SDS-PAGE gel stained with Coomassie Blu. No contaminations with other cellular proteins could be detected on the gel (data not shown).

Norvaline can be incorporated into proteins instead of leucine (Apostol et al. 1997) and is such an important marker for the quality of expressed recombinant proteins with a higher leucine amount especially in the pharmaceutical industry. Looking at the non-induced cultivations (Figure 4.3.5) four base results can be seen:

1. There is a base level of leaky expression detectable.
2. No norvaline incorporation is measured under any feeding rate.
3. There is no remarkable difference in the leucine amount analyzed triggered by the cultivation mode (in average 6.2 $\mu$mol/OD for the reference and 5.8 $\mu$mol/OD for the oscillating culture).
4. Increasing the substrate availability does not increase the protein amount in the inclusion bodies fraction per cell (similar leucine concentrations over all feeding rates).
At 50 μM IPTG norvaline inside of the inclusion bodies for both cultivation modes can be detected. Overall, the amount of leucine inside of the inclusion bodies is not influenced by the increasing feeding rate (in average 16.5µmol/OD for the reference and 15.8 µmol/OD for the oscillating culture), indicating that an increase in feeding does not increase the product yield, which was also seen on SDS-PAGE (data not shown). At the same time, norvaline concentrations in the inclusion bodies per cell increase with increasing feeding rate and the associated oxygen limitation for both cultivation modes. Furthermore, applied oscillating availability of oxygen enhances the amount of misincorporated norvaline into the recombinant miniproinsulin. Interestingly, the normalized ratio of norvaline/leucine is rising stronger with increasing feeding rate under normal conditions. Mis-incorporation triggered by oscillating oxygen availability seems to overlay this effect and has a stronger impact than the applied feeding rate. These observations are also true for cultures induced with 250µM or 1000µM IPTG (Figure 4.3.5A and 4.3.5B).

When comparing the different intensities of induction, only a slight increase in leucine concentrations in the inclusion bodies fraction per cell can be seen, leading to only a modest increase in protein synthesis due to increased inducer concentrations. For the cultures induced with 250 μM IPTG, there is an average leucine IB concentration per cell of 16.6 µmol/OD for the reference and 16.2 µmol/OD for the oscillating cultivation. Cultures induced with 1000µM IPTG the average leucine IB concentration per cell is of 17.1 µmol/OD for the reference and 15.8 µmol/OD for the oscillating culture. At lower feeding rates a higher IPTG concentrations result in stronger misincorporation of norvaline. This effect diminishes with increasing feeding rates. This result indicates that overproduction of a recombinant protein, under strong substrate limitation, increases incorporation of norvaline, whereas the strength of induction has no impact on sufficient substrate supply.
Figure 4.3.5: (A) Concentrations of the canonical amino acid leucine and the corresponding non-canonical amino acid norvaline for the purified inclusion bodies of the homogenous reference cultivation and the oscillating oxygen cultivation. Samples were drawn 5h after induction and normalized to the OD<sub>600</sub>. Break between 0.01 and 1 µmol/OD. (B) Ration of incorporated norvaline to leucine normalized to biomass.
Regarding methionine and its corresponding non-canonical amino acid norleucine (Figure 4.3.6), it is interesting to see already norleucine misincorporation in non-induced cultures. In the cultivations induced with 50 µM IPTG norleucine like norvaline can be detected in both cultivation modes. For the reference cultivations, the norleucine proportion in the inclusion bodies slightly increases with rising feeding rates. This increase is less steep than it was for norvaline (Figure 4.3.5). For the oscillating cultivations, the incorporation of norleucine increases compared to the homogenous reference plate, but the amount of incorporated norleucine to methionine per cell decreases with increasing feeding rate (Figure 4.3.6).

For higher inducer concentrations, the same slight increase of the norleucine misincorporation was monitored with increasing feeding rate for the reference cultivations, also oxygen oscillations triggered norleucine incorporation. For the reference cultivations, there is no significant impact of increasing induction intensity on the amount of incorporated norleucine, and the oxygen oscillating cultures show no common trend.

The normalized concentrations of isoleucine and β-methyl-norleucine inside of the inclusion bodies are shown in Figure 4.3.7. The concentration of leucine is around five times higher than the one of isoleucine and almost matching the leucine/isoleucine ratio of the recombinant miniproinsulin of 1:3. For the non-induced cultures, the non-canonical amino acid (β-methyl-norleucine) is already detected at higher levels than norleucine.

Regarding the culture induced with 50 µM IPTG, β-methyl-norleucine is detected for both cultivation modes. Like described earlier, the periodical exposure of the culture to oxygen oscillations caused an increased misincorporation of the non-canonical amino acid.

The ratio of incorporated β-methyl-norleucine compared to isoleucine increased with increasing feeding rate in the reference cultivation plate. For the oscillating cultivations, the amount of incorporated β-methyl-norleucine in the recombinant protein remained almost constant with increasing feeding rate indicating that oscillating oxygen availability has a stronger influence on the incorporation of β-methyl-norleucine than the feeding rate. Same observations can be seen for the cultures induced with 250 µM and 1000 µM IPTG. β-methyl-norleucine incorporation is not influenced by the concentration of applied IPTG.
Figure 4.3.6: (A) Concentrations of the canonical amino acid methionine and the corresponding non-canonical amino acid norleucine for the purified inclusion bodies of the homogenous reference cultivation and the oscillating oxygen cultivation. Samples were drawn 5h after induction and normalized to the OD$_{600}$. Break between 0.1 and 0.1 µmol/OD. (B) Ration of incorporated norleucine to methionine normalized to biomass.
Comparing detected concentrations for norvaline with norleucine it can be seen that, although it is a leucine rich protein with an around 4 to 5 times higher measured concentrations of leucine in the inclusion bodies fractions than for methionine, norleucine is incorporated in a significantly higher amount than norvaline. For the induced cultivations, the concentrations of measured norleucine surpass norvaline concentrations from six up to eleven times, depending on the cultivation conditions.

Also, the leucine concentrations are also about five times higher than the detected isoleucine amounts. Comparing the measurements of norvaline to β-methyl-norleucine for the induced cultures reveals a 5 to 11x higher incorporation of β-methyl-norleucine about norvaline depending on the applied cultivation conditions.

While the concentrations of isoleucine and methionine are measured approximately in the same range in the inclusion bodies fractions of the induced cultivations, analyzed amounts of norleucine are twice as high as the concentrations of β-methyl-norleucine for both cultivation modes.

The analysis of the canonical amino acids valine, alanine, and glycine (Figure 4.3.8) reveals no significant difference in expressed recombinant protein amount triggered by the cultivation mode. There is no clear relation between feeding rate or inducer concentration to the final productivity of the recombinant miniproinsulin in comparison to the effects on product quality due to misincorporation of non-canonical amino acids.
Figure 4.3.7: (A) Concentrations of the canonical amino acid isoleucine and the corresponding non-canonical amino acid β-methyl-norleucine for the purified inclusion bodies of the homogenous reference cultivation and the oscillating oxygen cultivation. Samples were drawn 5h after induction and normalized to the OD600. Break between 0.1 and 0.1 µmol/OD. (B) Ratio of incorporated β-methyl-norleucine to isoleucine normalized to biomass.
Results

Discussion

The results of this study reveal a connection between different applied feeding rates, inducer concentration (expression strength) and the oxygen oscillations on the incorporation of non-canonical amino acids like norvaline, norleucine, and β-methyl-norleucine into a leucine-rich recombinant expressed miniproinsulin.

Figure 4.3.8: Concentrations of the canonical amino acid valine alanine and glycine for the purified inclusion bodies of the homogenous reference cultivation and the oscillating oxygen cultivation. Samples were drawn 5h after induction and normalized to the biomass.
Although due to culture volume limitations a complex analysis during the cultivation time was not possible, it can be assumed that both cultivations are comparable. Based on the online measurement of dissolved oxygen tension and pH also during the initial fed-batch phase in combination with the offline determination of ODs before and after the initial fed-batch phase the cells in both experiments were in the same physiological state at the point of transfer into the multi-well plates. Therefore, differences detected at the end of the experiments can be related to effects happened due to different cultivation conditions and oxygen oscillations.

To our knowledge, this is the first study researching on the impacts of oxygen oscillations, substrate availability, and induction intensity focussing on the incorporation of non-canonical amino acids into a recombinant protein and its quality in a screening scale setup instead of the final protein yield.

In principle, we could detect the incorporation of the non-canonical amino acids at all feeding rates, induction intensities, and both cultivation modes. It has been previously reported that the overexpression of a recombinant protein enhances non-canonical amino acid synthesis (Veeravalli et al. 2015). Therefore, we did not only test non-induced and induced cultivation conditions but also varied the inducer concentrations to apply different induction intensities to see, whether this influences the incorporation of these amino acid analogs.

It is assumed that the overexpression of e.g. a leucine-rich protein generates an enhanced demand for this amino acid and therefore results in increasing the productivity of the branched chain amino acid pathway. This correlation has been shown to lead to the accumulation of non-canonical amino acids (Bogosian et al. 1989; Apostol et al. 1997) and also resulting in increased incorporation (Harris and Kilby 2014).

Norvaline incorporation as only analyzed non-canonical amino acid could not be proven for non-induced cultures. Apostol and Bogosian postulated that a minimum concentration limit needs to be reached before a non-canonical amino acid is incorporated (Bogosian et al. 1989; Apostol et al. 1997). This observation could explain why no norvaline was incorporated under leaky expression conditions in comparison to induced expression. Accumulation of norvaline triggered by induction of expression to levels higher than needed to cause misincorporation. As norleucine and β-methyl-norleucine are already incorporated into the recombinant miniproinsulin without induction, their synthesis seems to be favored compared to norvaline.
Furthermore, the combination of oxygen limitation or oxygen oscillations with a steady supply of substrate has been shown to result in norvaline accumulation, linking the synthesis to a glucose overflow reaction (Soini, Ukkonen, and Neubauer 2011). Oxygen limitation is the second influence parameter for non-canonical amino acid accumulation and was implemented in two different ways for this experimental study:

(1) The increase of feeding rate resulting in a stable and persisting oxygen limitation at higher feeding rates.

(2) Oscillating oxygen availability mimicking conditions cells experience in large-scale processes (Enfors et al. 2001).

Previously it has been reported from Soini and colleagues that after a sudden and permeant oxygen downshift combined with sufficient substrate supply leads to an increase in norvaline formation (Soini et al. 2008). Regarding the influence of feeding rate and the resulting stable oxygen limitation in the reference cultivations (without oxygen oscillations) an increasing incorporation of norvaline, norleucine and β-methyl-norleucine could be detected.

Soini also observed norvaline accumulation for oscillating oxygen profiles under oscillating substrate supply (Soini, Ukkonen, and Neubauer 2011). The screening scale experiment in this study was performed with oxygen oscillation and a constant feeding and has shown that exposure of cells to periodical oxygen limitation increases the incorporation of all three amino acid analogs into the recombinant protein. Similar results were observed before with oscillating oxygen and glucose concentrations within a two-compartment scale-down reactor (Chapter 4.2.1).

Leucine is the most abundant amino acid in the sequence of the recombinant protein, followed by both methionine and isoleucine which are almost equal (three and five residues per peptide molecule). Nevertheless, norvaline is the least incorporated non-canonical amino acid of the three examined non-canonical amino acids, followed by β-methyl-norleucine and subsequently followed by norleucine. This result indicates that the frequency of the canonical amino acid in the peptide sequence is not the main factor responsible for misincorporated amino acid analogs. As incorporation on non-canonical amino acid occurs via misaminoacylation of tRNAs, increasing accumulation of the non-canonical amino acid...
influences the incorporation rate (Barker and Bruton 1979; Apostol et al. 1997; Muramatsu, Misawa, and Hayashi 2003). The comparable low incorporation of norvaline, despite the significant portion of leucine in of the recombinant protein, could lead to the assumption that norvaline might be produced in lesser amounts than norleucine and β-methylnorleucine, which has been seen in similar experiments in bioreactors (Chapter 4.1 and 4.2).

The optical density measurements indicate a reduced growth behavior under oxygen oscillations, which also has been previously reported for other scale-down studies and large-scale bioprocesses. Xu and colleagues reported a biomass loss of 8.4 % for their two-compartment system with oxygen and substrate oscillations while Oneyaka described a 10.45 % loss for a 30 m³ bioreactor compared to the expected biomass (B. Xu et al. 1999; Onyeaka, Nienow, and Hewitt 2003). Further groups could observe this effect comparing large scale reactors with lab scale processes (Bylund et al. 1998) or for a pulsed feeding scale down device (Ying Lin and Neubauer 2000). The reason for this decrease could be an accumulation of growth inhibiting by-products. Furthermore, Bylund reported an increased rate of cell lysis for a recombinant E. coli W3110 strain experiencing glucose and oxygen oscillations in a two-compartment reactor (Bylund et al. 2000).

4.3.6. Outlook
This study shows that effects based on cultivation environments seen in production-scale bioreactors influencing the quality of produced recombinant proteins can already be applied in screening experiments without the need of complicated experimental setups. It is a proof-of-principle that already in the early stages of bioprocess development valuable data can be gained for efficient choosing of suitable production hosts and cultivation protocol development with product quality as the focus.
4.4. At-line monitoring of inclusion bodies formation in recombinant *E. coli* cultivations using the fluorescent dye Thioflavin-S

4.4.1. Abstract

Until today *Escherichia coli* is a preferred microbial host to produce recombinant proteins in industrial and pharmaceutical bioprocesses. Due to its limited capabilities of posttranslational modifications compared to eukaryotic cells many heterologous proteins expressed in *E. coli* are agglomerating to wrongly folded and inactive but almost pure bacterial inclusion bodies. Recent studies could show that inclusion bodies in bacterial cells share mechanical and structural properties with amyloid plaques, which are linked to several severe diseases like Alzheimer’s, type II diabetes, Parkinson’s or Rheumatoid arthritis. These shared structural features turned inclusion bodies formation in microorganisms into a valuable tool for characterization of amyloid agglomeration and screening for agglomeration decreasing drugs. Assays to identify and to quantify amyloid plaques in mammalian cell cultures and tissues have been adapted to bacterial systems like the application of the fluorescent dye Thioflavin-S.

This study shows the application of Thioflavin-S staining to monitor recombinant protein expression at-line during a bioreactor cultivation. We could detect a linear correlation between concentrations of formed inclusion bodies and detected fluorescence signals from intact *Escherichia coli* cells. As Thioflavin-S staining is fast, easy to use, and inexpensive, this opens possibilities for new and innovative approaches in productivity based high throughput screenings and bioprocess development or to use the recombinant protein production rate for productivity based process control.

4.4.2. Introduction

*Escherichia coli* belongs to the most widely applied industrial host strains for the expression of recombinant proteins in the commercial scale (Festel 2010). Several advantages are known, which turn *E. coli* into a valued host for the commercial production of heterologous proteins for pharmaceutical and industrial purposes. These include established genetic engineering tool boxes as well as inexpensive cultivation of cells and high expression of proteins resulting in the straightforward and fast production of sufficient concentrations of product (L. Hewitt and McDonnell 2004). Nevertheless, *E. coli* as a host for heterologous protein production has drawbacks. *E. coli* is not known for efficient secretion of proteins to the cultivation medium. Furthermore, fast and strong induction of heterologous protein expression in *E. coli* cells often
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results in agglomeration of the target protein into almost pure intracellular inclusion bodies isolated against post-translational modifications (Fahnert, Lilie, and Neubauer 2004). However, the production of recombinant proteins as inclusion bodies in *E. coli* shows a sustained demand until today as favored production technique and is widely applied for heterologous protein production for commercial purposes regardless of complicated downstream processing (Panda 2003). Inclusion bodies formation can be seen in the cytoplasmic as well as the periplasmic areas of *E. coli* cells. They are usually described as non-native insoluble aggregates showing no biochemical activity.

This view has changed significantly over the last years as it could be demonstrated that inclusion bodies can consist of highly ordered structures comparable to amyloid aggregates (Dasari et al. 2011). Further, it was discussed if these observations are limited to aggregations of amyloidogenic proteins. It could be proven that formed inclusion bodies for several non-amyloidogenic polypeptides constitute amyloid representing structures turning bacterial inclusion bodies formation into a powerful tool for analyzing amyloid aggregation (L. Wang et al. 2008). Numerous techniques have been described to detect protein agglomeration in microorganisms, e.g. using fluorescent protein fusion tags (e.g. GFP) or fluorescent dyes specifically binding to amyloid-like structures (e.g. Thioflavin-S) (Ami et al. 2013).

Fusion tags load an additional burden on the cellular translation machinery. For small recombinant proteins like insulin or Interleukin-2 the size of the fusion partner even would surpass the size of the desired product. Also, protein aggregation could lead to misfolding of the GFP and so inhibit fluorescence (Villar-Piqué et al. 2012). It could be shown, that the amyloid binding fluorescent dye Thioflavin-S can penetrate *E. coli* cells and stain bacterial inclusion bodies (Espargaró et al. 2016). Three amyloid-binding dyes are used in literature: Congo-Red, Thioflavin-T, and Thioflavin-S. Thioflavin-S is the preferred applied dye for staining intracellular inclusion bodies in bacteria in-vivo as it can easily pass and penetrate cell membranes and has no impact on inclusion bodies formation. Thioflavin-T has been shown to be inferior regarding membrane penetration (Darghal, Garnier-Suillerot, and Salerno 2006), and Congo-Red is known for possible decreased agglomeration (Spólnik et al. 2007). Also, Thioflavin-S shows a conformational change after binding to amyloid structures leading to a
shift in the fluorescence wavelength spectrum and increasing fluorescent intensity (LeVine 1999).

It would be a significant advancement for recombinant bioprocess development, optimization, and control if the product protein synthesis rate would be easily detectable. A drawback here is that usually the product synthesis rate can be calculated after sampling, purification and quantification of the target compound.

This study shows the adaptation of an approach based on intracellular staining of inclusion bodies formed in E. coli cells for monitoring recombinant product formation during bioreactor cultivations. This method, firstly described by Espargaró, Sabate, and Ventura in 2012, was originally designed to screen for amyloid aggregation inhibitors. Here, we illustrate that the assay is sensitive enough to describe inclusion bodies aggregation during a bioprocess at-line and almost immediate, simple to use and inexpensive. Also, no additional reporter proteins or peptides need to be used preventing the added burden of the cellular protein synthesis or possible problems regarding the detection of the reporter signal.

4.4.3. Material and methods
Staining of amyloid protein plaques in mammalian cells using Thioflavin-S is a standard technique. As described before bacterial inclusion bodies display similar protein characteristics like amyloid plaques and Thioflavin-S was already positively applied for comparative quantification of inclusion bodies under varying cultivation conditions. Now, the published protocols were adapted to record the formation of inclusion bodies of a miniproinsulin, interleukin-2, and an alcohol dehydrogenase in E. coli W3110M and RB791. This approach is separated into three parts:

1. Test of the available method with two recombinant E. coli strains producing a miniproinsulin, human interleukin-2 and an alcohol dehydrogenase on agar plates.

2. Monitoring of a test cultivation using E. coli W3110M expressing the miniproinsulin.

3. At-line monitoring of three recombinant E. coli shake flask cultivations using global fluorescence and flow-cytometry.
Results

4.4.3.1. Strain

*E. coli* K12 W3110M with a mutation triggering the overexpression of the lac inhibitor and its recombinant derivatives W3110M pSW3 (plasmid encoding for recombinant miniproinsulin expressed as inclusion bodies and ampicillin resistance gene) and W3110M pCTUT7-IL2 (plasmid encoding for recombinant human interleukin-2 expressed as inclusion bodies and chloramphenicol resistance gene) were used in almost all experiments. *E. coli* RB791 is like W3110M and carries the lacI\(^Q\) mutation. It was transformed with pADH (plasmid encoding for alcohol dehydrogenase and ampicillin resistance gene). Strain W3110M and plasmid pSW3 were thankfully provided by Sanofi-Aventis Deutschland GmbH. The strain RB791 and the plasmids pCTUT7-IL2 and pADH were obtained from the laboratory strain and plasmid collection. The backbone of pCTUT7-IL2 is part of the plasmid library described in Kraft et al. (2007).

4.4.3.2. Media

All chemicals mentioned were acquired from either Carl Roth GmbH, Karlsruhe, Germany, or Sigma-Aldrich Chemie GmbH, Munich, Germany, if not otherwise specified. The composition of standard LB medium used for initial precultivations was 10 gL\(^{-1}\) tryptone, 5 gL\(^{-1}\) yeast extract, 10 gL\(^{-1}\) NaCl and appropriate antibiotics (100 µgL\(^{-1}\) ampicillin, 34 µgL\(^{-1}\) chloramphenicol). For the first test of Thioflavin-S staining on plates, 15 gL\(^{-1}\) agar-agar and if needed antibiotics like mentioned before as well as IPTG to a final concentration of 1 mM were added to LB medium before pouring.

The bioreactor cultivation to test monitoring as well as to produce biomass for parameter optimization was done in a 2 L-scale bioreactor. Biomass was harvested as 20 mL aliquots in 50 mL centrifugation tubes, centrifuged at 4°C and 15000 g for 10 min, and stored at -20°C until further use. The used minimal medium (B. Xu, Jahic, and Enfors 1999) in the bioreactor contained 2 gL\(^{-1}\) Na\(_2\)SO\(_4\), 2.468 gL\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), 5 gL\(^{-1}\), NH\(_4\)Cl, 14.6 gL\(^{-1}\) K\(_2\)HPO\(_4\), 3.6 gL\(^{-1}\) NaH\(_2\)PO\(_4\) x 2H\(_2\)O, 1 gL\(^{-1}\) (NH\(_4\))\(_2\)-H-Citrat, 1 mL Antifoam Sigma 204. Per liter medium 2 mL trace elements, 2 mL MgSO\(_4\)-solution (1.0 M), 2 mL thiamine solution (50 gL\(^{-1}\)), and 1 mL ampicillin solution (100 mgL\(^{-1}\)) were sterile-filtered through an 0.22 µm-membrane filter into the reactor after sterilization. The trace element-solution consisted of 0.5 gL\(^{-1}\) CaCl\(_2\) x 2H\(_2\)O, 0.18 gL\(^{-1}\) ZnSO\(_4\) x 7H\(_2\)O, 0.1 gL\(^{-1}\) MnSO\(_4\) x H\(_2\)O, 20.1 gL\(^{-1}\) Na-EDTA, 16.7 gL\(^{-1}\) FeCl\(_3\) x 6H\(_2\)O, 0.16 gL\(^{-1}\) CuSO\(_4\) x 5H\(_2\)O, 0.18 gL\(^{-1}\) CoCl\(_2\) x 6H\(_2\)O. The starting batch glucose concentration was 5 gL\(^{-1}\).
EnPresso® B (BioSilta Ltd., Cambridge, UK) was used in the final characterization experiments in shake-flasks.

4.4.3.3. Testing Thioflavin-S staining on recombinant model systems
Twenty milliliters of LB medium were inoculated with 50 µL of cryostock and incubated at 37°C for five hours at 200 rpm in a 100 mL Erlenmeyer flask. 200 µL were spread on freshly prepared LB-agar plates containing the appropriate antibiotic and IPTG for recombinant strains and incubated overnight at 37°C. Then the cells were washed from the plates with one mL PBS (8.0 gL⁻¹ NaCl, 0.2 gL⁻¹ KCl, 1.42 gL⁻¹ Na₂HPO₄, 0.27 gL⁻¹ KH₂PO₄) and adjusted to an OD₆₀₀ of 1.

Thioflavin-S staining was done based on the protocol published by Espargaró, Sabate, and Ventura (2012) with the modifications from Aguilera et al. (2016). Cells were washed twice with one mL fresh PBS with centrifugation steps at 5000 g and 4 min. The published 1100 g as centrifuge setting resulted in too soft cell pellets and biomass loss during sample preparation. After washing, the cell pellet was resuspended in 500 µL Thioflavin-S solution (5 % (w/v) in 12.5 % ethanol) and incubated for 60 minutes at room temperature. After incubation, the stained cell pellet was washed three times with fresh PBS. Finally, the pellet was again resuspended in 1 mL fresh PBS, and 4 * 200 µL were transferred into wells of a UV-transparent multi-well plate (UV-Star® 96-Well Microplates, Greiner Bio-One, Kremsmünster, Austria). Fluorescence (excitation: 375 nm, emission: 455 nm) and OD₆₀₀ were measured in a Synergy™ Mx Microplate Reader from BioTek Instruments. The internal detector sensitivity was set to 100.

4.4.3.4. Precultivation for the bioreactor cultivation
Twenty milliliters of sterilized LB medium were inoculated with 50 µL cryostock of the desired strain and incubated for five hours at 37°C and 200 rpm in a 100 mL glass Erlenmeyer flask. For the second precultivation, 100 mL mineral salt medium were inoculated with one mL LB-preculture standardized to OD₆₀₀ 1 in a 500 mL Erlenmeyer flask and cultivated like before. As the OD₆₀₀ reached 0.3, the total broth volume was taken and transferred into the bioreactor for a final OD₆₀₀ of 0.015.
4.4.3.5. **Cultivation conditions for the bioreactor cultivation**

The bioreactor cultivation was performed as pulsed-fed fed-batch experiments using *E. coli* W3110M pSW3 in a KLF 2000 with a total volume of 3.7 L from Bioengineering AG (Wald, CH). 2 L of the earlier defined mineral salt medium in the reactor vessel were inoculated with 5 % (v/v) preculture broth. After the batch phase feeding was started at substrate depletion. The feeding solution contained 440 g\(\text{L}^{-1}\) dextrose solved in fresh cultivation medium. Changes were a four times increased trace element concentration to avoid limitations at high cell densities as well as no MgSO\(_4\), antifoam or ampicillin. Extra 4 mL 1.0 M MgSO\(_4\)-solution were added every OD\(_{600}\) 20. The feed inlet was connected to the top gas phase of the STR.

The feeding phase was divided into two parts. The first part was performed as exponential feeding phase with an initial feeding rate \(F_0 = 0.0125 \text{Lh}^{-1}\) and an exponential growth of \(\mu = 0.3\). After three hours of feeding, recombinant protein synthesis was induced by addition of IPTG to a final concentration of 1 mM. From now on, the feeding rate was no longer increased. In this pulsed-fed cultivation, a nutrient oscillation cycle of 10 minutes was used feeding the whole glucose of one cycle in the first minute followed by a 9-min recovery. After 6 hours of additional feeding, pulsed-feeding was stopped and switched to constant feeding with a rate of \(F = 0.016 \text{Lh}^{-1}\). The cultivation temperature was regulated at 35°C during the whole process. The aeration rate was set to 0.5 vvm in the beginning and raised to 1.0 vvm after feed start. The pH was controlled at pH = 7.0 with 25 % NH\(_4\)+ solution.

4.4.3.6. **Analytical Methods**

Optical density measurements monitored cell growth at a wavelength of 600 nm (OD\(_{600}\)) (Novaspec III by Amersham Biosciences, Amersham, UK) in addition to dried cell weight (DCW) determination. To measure DCW 2 mL of cell suspension were transferred into a dried, pre-weighted two mL microcentrifugation tube. After centrifugation for 10 min at 21,500g, the supernatant was discarded, and the cell was resuspended in 1 mL 0.9 % (w/v) NaCl solution. Following a repeated centrifugation step the tube was dried 75°C for 24 h.

For the weight measurements of inclusion bodies protein fractions, samples were purified using the BugBuster® Protein Extraction Reagent (Merck, Darmstadt, Germany).

Data plots were created with Qtiplot (Qtiplot.com), MODDE 10 (MKS Data Analytics Solution, Malmö, Sweden) and Excel 2016 (Microsoft, Redmond, USA).
4.4.3.7. *Shake-flask cultivations using EnPresso® B*

Twenty milliliters of fresh LB medium were prepared and mixed with 50 µL of *E. coli* cryostock and incubated for eight hours at 200 rpm and 37°C. For the main cultivation, 25 mL Enpresso® B medium was completed with each 25 µL ampicillin or chloramphenicol stock and BioSilta Reagent A along with 250 µL LB culture and cultivated for around 14 h at 37°C and 250 rpm in a PreSens SFR flask mounted on the SFR platform. Using PreSens flasks enabled on-line monitoring of DOT and pH levels. Then, protein expression was induced in recombinant cultures by addition of IPTG to a final concentration of 1 mM. In addition, the feeding rate was doubled with extra 25 µL Reagent A per flask.

4.4.3.8. *SDS-PAGE*

Insoluble protein expression was checked via SDS-PAGE analysis. Cell samples were collected and normalized to an OD$_{600}$ 18 and treated with the BugBuster® Protein Extraction Reagent (Merck, Darmstadt, Germany). Each 10µL of the soluble and insoluble protein fractions were mixed with 20µL demineralized water and 30µL of 2x loading buffer (100 mM Tris-Cl (pH 6.8), 20% glycerine 4 % SDS, 0.2 % bromphenol blue, 200 mM DTT). Mixed samples were incubated for 5 min at 95°C. After cooling down to room temperature, polyacrylamide gels (5 % stacking, 12 % separation) were loaded either with 10 µL of sample, 5 µL of Roti®-Mark TRICOLOR size marker or 10 µL of 2x loading buffer (empty pockets). The electrophoresis was run at 64 V for 30 min followed by around 90 min at 120 V.

Afterwards, the gels were washed to remove residual SDS and stained with Coomassie solution (60 – 80 g Coomassie® Brilliantblue G250 solved in 1L demineralized water and stirred for 2 – 3 hours followed by addition of 35 mM HCl) overnight. After 18 h the Coomassie solution was discarded and the gels were washed in demineralized water to remove the remaining dye and finally photographed (for a detailed protocol see SOP 9.3).

4.4.3.9. *Fluorescence Microscopy*

Microscopic observations were done with a DMI6000 B (Leica, Wetzlar, Germany) inverse microscope equipped with a 63x/NA 1.40 oil immersion objective. Thioflavin-S fluorescence was recorded using a GFP filter exciting from a range from 450 – 490 nm. Emission was detected in a range from 500 – 550 nm. Digital images were recorded with Leica LAS X. Stained cell samples were prepared as described in 4.4.3.3.
4.4.3.10. Flow-Cytometry
Thioflavin-S staining for flow cytometry analysis was done as described in 4.4.3.3. Flow cytometry measurements were performed using a Miltenyi MacsQuant flow cytometer. Before and after staining cells were at first analyzed by forward (FSC) and side scatter (SCC) signals recorded at 561/10 nm, and then characterized for Thioflavin-S fluorescence by exciting at 405 nm and registering the emission at 450/50 nm.

4.4.4. Results
4.4.4.1. Testing Thioflavin-S staining on recombinant model systems
The aim of this first test using cells washed from LB-plates was to prove binding of Thioflavin-S to intracellular inclusion bodies consisting of a miniproinsulin (pSW3) or a human interleukin-2 (pCTUT7-IL2). Figure 4.4.1 shows a comparison between the detected fluorescence of all three strains.

![Figure 4.4.1](image-url)

**Figure 4.4.1:** Fluorescence comparison between an empty *E. coli* host (W3110M), a recombinant strain producing a miniproinsulin (pSW3) and a recombinant strain expressing human interleukin-2 (pCTUT7-IL2) in relative Fluorescence units and % based on the cell fluorescence of W3110M.
A 28 % higher fluorescence could be seen for the interleukin-2 strain. The fluorescence of W3110M pSW3 was increased by 62 % compared to the empty host. Both types of inclusion bodies can be detected using Thioflavin-S. Figure 4.4.2 shows an example of how stained cell pellets change their color and a stained Interleukin-2 producing cell under a fluorescence microscope.

**Figure 4.4.2:** Left: Difference between unstained (left tube) and (stained cells) after sample preparation. A clear yellowish change can be seen caused by Thioflavin-S. Right: Recombinant cells stained with Thioflavin-S under a fluorescence microscope. It can be seen, that fluorescence is clearly caused by specific binding on inclusion bodies.

4.4.4.2. **At-line monitoring of inclusion bodies protein concentration along a cultivation**

Based on the results gained from an optimization approach using cells stored at -20°C (data not shown), it seems the assay is very sensitive to changed physiological conditions of the cells and damaged or dead cells cannot be analyzed with this method. To see, if the assay is limited to fresh samples, a bioreactor cultivation using *E. coli* W3110M pSW3 producing a miniproinsulin was monitored via hourly sampling. Also, aliquots were stored overnight at 4°C in PBS and at -20°C in PBS + 10 % glycerol and analyzed the next day (Figure 4.4.3.).

Observing the fresh samples an increase in fluorescence can be seen for the first three hours after induction of miniproinsulin expression followed by a decline in between three to five hours. From five hours after induction on the signal is stable. Interestingly, also three hours after induction the feeding profile was changed from pulsed feeding to reduced constant feeding. The trends for the stored samples are not comparable to fresh samples. Analyzing the aliquots from the fridge showed reduced signals compared to fresh samples and a slight fluorescence increase over time. As already stated above, frozen cells had like described before a very high background noise overlaying the signal values measured with fresh
samples. It can be said, that application of Thioflavin-S monitoring of inclusion bodies formation is only suitable for recently taken samples without extended storage.

**Figure 4.4.3:** Specific fluorescence trends during a bioreactor cultivation after induction of protein expression. Closed circles: Fresh samples, open squares: samples stored at 4°C, open diamonds: samples stored at -20°C. Storage has a crucial impact on the fluorescence signal. Thioflavin-S staining can only be applied directly after sampling.

First it was thought, that the decrease in fluorescence was caused by physiological changes of the cells due to the changed feeding profile and is so again showing the limits of the methodology. Inclusion bodies purification on samples taken between 0 and 5 hours after induction revealed, that the Thioflavin-S signals followed the protein concentration trend. It was seen that also after 3 hours the intracellular inclusion bodies concentration was declining. The reason is unknown as physiological changes would have influenced detected fluorescence signals. Specific fluorescence signals and inclusion bodies concentrations during this 5 h production phase are compared in Figure 4.4.4.
Figure 4.4.4: Specific fluorescence trend of and purified inclusion bodies concentrations during a bioreactor cultivation after induction of protein expression. Green circles: Specific fluorescence, orange circles: specific purified inclusion bodies concentrations. Both parameters are normalized to dried biomass concentration.

As both parameters follow the same trend, it is reasonable to suggest a correlation between both factors. Figure 4.4.5 illustrates the linear correlation between volumetric fluorescence and purified inclusion bodies protein with a coefficient of determination around 0.97. As long the cells are in an undamaged physiological state it is possible to monitor inclusion bodies formation at-line with an easy and uncomplicated methodology and get a good guess on the actual protein concentration without the need for complex sample analysis.

Figure 4.4.5: Linear correlation between relative fluorescence against purified inclusion bodies concentrations. Thioflavin-S staining can be used to approximate recombinant product concentrations at-line during the cultivation time without the need of downstream processing and complex analytical methods.
4.4.4.3. **Optimization of staining time and population characterization on different recombinant model strains**

This experiment had the aim to test the applicability of the Thioflavin-S assay on three different recombinant proteins as well as a non-recombinant strain as negative control. All four strains were cultivated in EnPresso B medium with a Start-OD\(_{600}\) 0.05 under identical cultivation conditions. At the same time, two staining times were tested (15 and 60 min) to see, if the staining procedure can be shortened. Figure 4.4.6. illustrates biomass growth starting at 13.5 h, at which the protein production was induced. Further monitored DOT and pH trends can be seen. Besides the Interleukin-2 expressing strain, all strains behave similar regarding biomass growth. The OD\(_{600}\) at induction was around 9 and increased to around 12 in the next five hours. The interleukin-2 strain had an OD\(_{600}\) around 6, which did not change significantly during the production phase.

Looking at the DOT levels, the Interleukin-2 strain reached glucose limitation around 1.5 hours after the other strains. The wild-type and the ADH strain showed an almost identical oxygen consumption profile. Interestingly, the miniproinsulin expressing strain changed from exponential into limited growth 1.5 hours earlier than the other two

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**Figure 4.4.6:** Biomass, DOT, and pH trends of all four model strains during the cultivation. Dashed line marks the point of protein induction.
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strains. Besides sampling, the DOT level was always above 20% in all cultivations, which excludes oxygen limitations.

The Interleukin-2 strain also had the lowest drop of pH in the medium (7.1 to 6.8), matching biomass and DOT development. The other strains performed again similarly showing a drop pH 6.45 for the two recombinant and to 6.3 for the wild-type strain.

Unfortunately, there were problems to stain the SDS-PAGE gels properly (Figure 4.4.7), but the essential data could be gained. For Interleukin-2, a stable protein band could be detected for each point starting already at induction, which suggests strong leaky expression and would explain the deficits in growth compared to the other strains.

Interestingly, IPTG addition did not enhance the amount of expressed protein. The insulin concentration increases and declines again towards the end of the cultivation. Alcohol dehydrogenase starts to aggregate four hours after protein induction.

In addition to flow cytometry, we had also the possibility to analyze one sample under a fluorescence microscope. Figure 4.4.8. shows recorded pictures of cell prepared of samples taken four hours after induction. It could be seen that aggregation of each recombinant protein is quite different. The interleukin-2 strain shows the strongest illumination. In addition, a large inclusion bodies were observed, which clearly focus the fluorescence.
**Figure 4.4.8:** Recombinant cells stained with Thioflavin-S under a fluorescence microscope. Samples for these pictures were taken four hours after protein induction and stained with Thioflavin-S for 60 min.
Furthermore, these cells were larger than all other strains. Cells expressing the miniproinsulin did not have these large inclusion bodies. Moreover, the fluorescence was scattered over the whole cell suggesting the formation of numerous small aggregates distributed in the cells. Unfortunately, the aggregation of the alcohol dehydrogenase just began around this sampling point. The observed cell sizes matched sizes of cells producing miniproinsulin, but it seemed, the aggregation is restricted to a lower number of inclusion bodies like seen for the interleukin-2. Wild-type cells had only a faint background fluorescence.

The observations concerning cell sizes were confirmed in the flow cytometry analysis. Figure 4.4.9. illustrates comparisons in cell size and granularity for each strain at each sampling point for unstained and stained cells. The most obvious result was the comparability between stained and unstained cells. Treating cells with Thioflavin-S had no impact on the cell physiology. In addition, except for the Interleukin-2 strain all samples were very similar regarding cell size and granularity. Interleukin-2 containing cells were in average bigger and had a slightly increased granularity.

Gating unstained and stained cells, a relative fluorescence above 100 was defined as stained. Cell sizes and fluorescence intensity are compared in Figure 4.4.10. It could be seen, that in samples producing Inteleukin-2 and the alcohol dehydrogenase the fluorescence signal is detected stronger in larger cells of the samples, which would match our observation of only a few but bigger inclusion bodies in these cells. There seems to be no influence of cell size on the distribution of stained cells in the samples of miniproinsulin producing cells. Further, the ratio of stained cells in Interleukin-2 samples is always around 50 %, but does not surpass 20 % in samples of the other two recombinant strains. Also, the high ratio of stained wild-type cells, which is drastically decreasing during the experiment, needs to be mentioned. These values cannot be explained, as there were no insoluble proteins on the SDS-PAGE gel (data not shown) as well as other recorded hints for an unexpected physiological behavior.
Figure 4.4.9: Comparison of cell size and granularity of stained and unstained cells at 0, 2, 4, 6, 8, and 26 hours after induction. From top to bottom: W3110M, W3110M pCTUT7-IL2, RB791 pADH, W3110M pSW3. It can be seen, that the staining procedure has not influence on the cell physiology. Grey = stained, Blue = unstained. No unstained data is available at 0 h.
Figure 4.4.10: Comparison of cell size and fluorescence of stained and unstained cells at 0, 2, 4, 6, 8, and 26 hours after induction. From top to bottom: W3110M, W3110M pCTUT7-IL2, RB791 pADH, W3110M pSW3. It can be seen, that the stained population is not larger in cell size compared to the total sample. Grey = stained, Blue = unstained. No unstained data is available at 0 h.
Figure 4.4.11: Comparison of granularity and fluorescence of stained and unstained cells at 0, 2, 4, 6, 8, and 26 hours after induction. From top to bottom: W3110M, W3110M pCTUT7-IL2, RB791 pADH, W3110M pSW3. It can be seen, that the stained population does not show increased granularity compared to the total sample. Grey = stained, Blue = unstained. No unstained data is available at 0 h.
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Figure 4.4.12: Histogram of stained and unstained cells showing the ratio of positive stained cells in comparison to the total sample at 2, 4, 6, 8, and 26 hours after induction. From top to bottom: W3110M, W3110M pCTUT7-IL2, RB791 pADH, W3110M pSW3. Grey = stained, Blue = unstained.
Comparing granularity and fluorescence strength, the observations made for cell sizes can be transferred to cell granularity. Thioflavin-S positive cells are mostly the cells with higher granularity in all samples (Figure 4.4.11.). Figure 4.4.12. illustrates the development of the ratio of clearly stained cells during the analyzed production phase. As mentioned before, the high ratio of stained wild-type cells is interesting and unexpected, but decreases below 10% during the day. Around every second cell in interleukin-2 samples was detected as stained. These values were fluctuating below 20% for the other two recombinant strains. Figure 4.4.13. displays this trend development over time.

**Figure 4.4.13:** Ratios of fluorescing cells in stained samples over the time of protein production.

Besides cellular characterization, the at-line monitoring of inclusion bodies formation in all strains was a major task in this experiment. Also, it was tried to reduce the preparation time by staining samples for 15 min in addition to 60 min.
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Figure 4.4.14: Comparison of culture fluorescence after 15 min and 60 min staining with Thioflavin-S. Only slightly increased total values can be seen with the longer staining time. Furthermore, an increased noise can be discussed. As the fluorescence strain indicating ADH agglomeration (blue) follows more the profile seen on SDS-PAGE, 15 min staining with Thioflavin-S is more recommend for monitoring purposes.

Figure 4.4.14. illustrates fluorescence trends throughout the production phase. Fluorescence of the interleukin-2 samples fluctuated in-between 100,000 and 150,000 relative Units after 15 min of staining and was not significantly influenced by longer staining times. Accumulation of alcohol dehydrogenase could be observed beginning at four hours after induction, matching the results of SDS-PAGE. Longer staining reversed the trend of fluorescence, with higher values in the beginning which declined during the monitored time. The values for miniproinsulin producing cells were always around the level for wild-type samples, independent of staining times. Consequently, staining longer than 15 min is not increasing the total fluorescence signals significantly. Furthermore, signals recorded after 15 min staining matched observations via other analytical methods far better than longer staining times. So, these were the values used to try the linear correlation between fluorescence and protein concentration.

Figure 4.4.15. shows the fluorescence levels recorded after 15 min of incubation with Thioflavin-S, fluorescence levels after normalization concerning the wild-type data and linear correlations between weighed inclusion bodies amounts and their corresponding fluorescence signals. We could fit data for interleukin-2 and miniproinsulin samples with a coefficient of determination around 0.95.
Interestingly, the slope of the fit for the miniproinsulin is three times higher than for interleukin-2. We have seen under the microscope that interleukin-2 aggregated in large inclusion bodies whereas the miniproinsulin seemed to form more but smaller inclusion bodies. The inclusion bodies surface at the same protein concentration would be higher in miniproinsulin producing cells compared to interleukin-2 cells, so more Thioflavin-S could bind in miniproinsulin containing cells leading to higher fluorescence at comparable protein concentrations. The correlation coefficient for the alcohol dehydrogenase was lower at 0.78. It must be mentioned, that the alcohol dehydrogenase is usually expressed as native and soluble protein. The strong induction forces the aggregation of the protein over time. Finally, the Thioflavin-S procedure can be used to for a quick overview on protein concentration for both tested insoluble produced recombinant proteins.

Figure 4.4.15: Fluorescence of culture samples measured after 15 min staining with Thioflavin-S. The second Figure shows the difference to the reference strain. The lowest Figure illustrates linear fits of Fluorescence against amounts of purified inclusion bodies.
4.4.5. Discussion

Inclusion bodies detection using Thioflavin-S staining is a fast, simple, and inexpensive method. Characterization of amyloid plaques via fluorescence detection after preparation with Thioflavin-S in mammalian cell cultures is an established standard methodology. In 2012, the range of application was enhanced towards bacteria and continuously developed further (Espargaró et al. 2016). Now, Thioflavin-S staining of amyloid forming E. coli cells is a reputable screening system for anti-aggregation drugs. In our study, Thioflavin-S staining should be validated as monitoring platform for proteins, which show no amyloid properties but are expressed as inclusion bodies in E. coli. As model proteins, the known miniproinsulin and a human interleukin-2 as well as an alcohol dehydrogenase were chosen. Increased fluorescence could be seen for all recombinant strains in comparison to the non-recombinant host. This result corresponds to published data proving that bacterial inclusion bodies share structural properties with amyloid plaques independent from the agglomerating protein (Carrió et al. 2005).

This is the only known study, where Thioflavin-S staining was applied to monitor and quantify product formation not focusing on drug screening at cultivation endpoints. We could see, that even without further optimization the published protocols for this method are sensitive enough to detect agglomeration kinetics during a production phase. Further, fluorescence and inclusion bodies concentrations could be linearly correlated turning this assay into a possible process analytical tool.

Unfortunately, in our experiments, Thioflavin-S staining can only be applied to fresh intact cells. The cells, which were used for the attempted parameter optimization, were stored at in a freezer at -20°C and thawed for the staining experiments. Freeze-thawing is known to damage the cell membrane (Souzu 1980). It seems that damaged cell membranes of E. coli cells caused background noise on a level, which overlays every inclusion bodies signal. Interestingly, similar results could be seen for recombinant and wild-type E. coli cells grown for 24 h in shaken LB cultures (data not shown). Further, we could prove that a short-time storage at 4°C also negatively influences Fluorescence. Also, freezing cells in PBS containing 10 % glycerol to stabilize the cell membrane (Souzu 1980) was not successful. Without a high-throughput approach using a liquid handling system to reduce screening time significantly a parameter optimization is not possible.
4.4.6. Outlook
This study shows that the application of the fluorescent dye Thioflavin-S is not limited to final point determinations like they are described in the literature. We could prove its usability for monitoring a production phase during a bioreactor cultivation to detect increasing and decreasing protein amounts inside the cells. Also, a linear correlation between fluorescence and inclusion bodies concentrations could be derived from the data, turning Thioflavin-S staining into an easy and fast at-line technique for initial protein quantification. This new insight could support parallelized high-throughput screenings for bioprocess development approaches to detect suitable production clones in an uncomplicated way. Furthermore, for model-based automatized bioprocess development screenings Thioflavin-S staining turns the product formation rate into an at-line determinable process parameter, which opens the door for innovative protein expression controlled cultivation procedures using *Escherichia coli* as a host if the product is expressed in inclusion bodies.
5. Discussion
Several interesting observations were made regarding oscillating cultivation conditions and their influences on growth, metabolism, and the formation of non-canonical amino acids as well as their incorporation into recombinant proteins in *Escherichia coli* within the scope of this thesis. In the following chapters, these results will be further discussed in detail.

5.1. Alterations in growth behavior caused by oscillating cultivation conditions
Cell growth was differently influenced via oscillating cultivation conditions. It was seen that glucose oscillations have an inhibitory effect on growth. Applying pulse-based feeding had a higher influence than an STR-PFR setup. Oxygen oscillations on the other side had almost no impact on the final biomass yield. Oscillating oxygen excess in combination with substrate limitation triggered spontaneous cell lysis resulting in canceled cultivations. In earlier characterization studies of *E. coli* in scale-down systems with a simulated feeding zone (oscillating substrate excess in combination with oxygen limitation) revealed, that oscillating oxygen availability is the main reason for increased side-product accumulation and reduced biomass yield and not glucose excess (Enfors et al. 2001). Nevertheless, the substrate excess is indirectly linked to this observation, because increased glucose concentrations are the reason for oxygen limitation due to increased metabolic activity. The results shown in this thesis accord to these observations. Focusing on the three hours long fed-batch phase in scale-down cultivations before induction of protein expression we can see a reduced growth rate under substrate and oxygen oscillating conditions as well as oxygen oscillations under substrate limitation and increased the formation of acetate and lactate. These observations indicate that the loss of biomass is linked to an inefficient catabolic utilization of carbon under oxygen limitation via fermentation and down-regulated aeration pathways.

Numerous studies have shown decreased biomass yields between 10 to 35 % comparing 3 and 5 L lab-scale vessels to scale-down systems or large scale cultivations with volumes between 12 to 30 m³ (Bylund et al. 1998; B. Xu et al. 1999; Bylund et al. 2000; Onyeaka, Nienow, and Hewitt 2003). Losses of biomass yields during fed-batch phases without protein production in cultivations done for this thesis were in the same range. Even small dissolved oxygen gradients and exposure times of just a few seconds have been proven to trigger the transcription of anaerobic metabolism genes as a response in *E. coli* cells (T. Schweder et al. 1999). This
response leads to a metabolic detour of carbon into fermentative products like acetate, formic acid, and lactate as well as to a reduced yield of biomass (T. Schweder et al. 1999).

Remarkably, protein induction does not influence growth under oscillating cultivation conditions whereas a temporary decrease in growth can be seen in reference experiments. It has been described before, that this observed growth inhibition is common and caused by reprogramming effects of the host cell metabolism owing to recombinant protein formation (Kurland and Dong 1996). Further, overexpression of a heterologous protein, which has no function or is even harmful to the host's metabolism, could significantly influence the final biomass yield due to disturbance of proliferation or cell maintenance (Dong et al. 1995). It is discussed if this inhibited growth is based on the pure competition for the synthesis of cellular proteins and the recombinant product (Ying Lin and Neubauer 2000). After induction, a physiological adaption towards recombinant protein production is forced by the additional claim for polymerases and ribosomes for product formation. Interestingly, these adaptation effects cannot be seen in oscillating or pulse-based scale-down cultivations. This observation is not discussed in the literature and further analysis towards cellular physiology is needed to get a detailed look at the reasons. In 1997, Bhattacharya and Dubey showed an increased oxygen uptake in recombinant E. coli after during heterologous protein expression of a soluble active enzyme (Bhattacharya and Dubey 1997). In none of our cultivations, an increase in oxygen consumption could be seen during protein production, which could be explained with altered metabolic activity due to the changed stronger limiting glucose feeding.
5.2. Impacts of oscillating cultivation conditions on the central metabolic carbon flux
As described before, *Escherichia coli* cells can utilize carbon substrates mainly via aerobic respiration or anaerobic fermentation. There are anaerobic respiration pathways in *E. coli*, but fermentation of sugars or derivative molecules is the preferred method to produce energy under oxygen-limited conditions (Peekhaus and Conway 1998). Anaerobic mixed-acid fermentation of sugars results in a spectrum of organic acids (acetate, formic acid, lactate, succinate) and ethanol (see Figure 2.2) (Clark 1989). Under aerobic conditions, *E. coli* can re-metabolize all fermentation products except ethanol and succinate (B. Xu et al. 1999). The pyruvate dehydrogenase is catalyzing the chemical transformation of pyruvate into Acetyl-CoA which is further metabolized in the TCA cycle or converted into acetate as a product of overflow metabolism. Pyruvate dehydrogenase is only active during oxygen presence. Pyruvate metabolism under oxygen limited conditions is mainly controlled by the pyruvate formic acid lyase and following lactate dehydrogenase. Both enzymes are inhibited by feedback repression or presence of oxygen (Böck and Sawers 1996). Acetate and formic acid are produced in detectable amounts already after two seconds if glucose pulsed are applied during oxygen limitation (Lara et al. 2009). Transcription of genes linked to anaerobic metabolism is seen within seconds under temporary oxygen limitation (T. Schweder et al. 1999). This fast response proves the capability of *E. coli* to recognize changed environmental conditions and to adapt its central metabolism within short timeframes (T. Schweder et al. 1999; Sandoval-Basurto et al. 2005). If *E. coli* cells are shifted from aerobic to oxygen-limited conditions their aerobic respiration is shut down, and the pyruvate dehydrogenase is inhibited. At the same time, expression of genes linked to mixed acid fermentation is immediately upregulated to redirect energy generation to fermentation reactions.

Expression of the pyruvate formic acid lyase is described as the most sensitive response to changing oxygen availability in *E. coli* cultivations (T. Schweder et al. 1999). Remarkably, in no cultivation done for this thesis, formic acid accumulation cold be detected in the STR compartment, which means the formed formic acid in the PFR compartment is immediately metabolized. Defined cultivation media can be optimized to suppress formic acid accumulation by adding the trace elements nickel, molybdenum, and selenium (Soini, Ukkonen, and Neubauer 2008). These trace elements were not added in our cultivations and still formic acid produced in the PFR compartment is not seen in the STR.
The lactate dehydrogenase catalyzes the conversion of pyruvate into lactate under oxygen limited cultivation conditions. At the same time, one NAD is recovered per pyruvate converted (Tarmy and Kaplan 1968). The expression rate of this enzyme is increased tenfold in *E. coli* cultivations under anaerobic conditions combined with an acidic pH (Clark 1989). Except one, the FNR regulatory protein controls the expression of all genes linked to anaerobic fermentation and respiration. Its intracellular concentration increases immediately under oxygen limitation (Tolla and Savageau 2010). The main purpose of the FNR protein is the downregulation of respiration and upregulation of transcription of fermentation enzymes under oxygen limited environmental conditions (Unden and Schirawski 1997). Interestingly, the lactate dehydrogenase is the only enzyme in anaerobic metabolic pathways, which is not regulated by FNR (Clark, Nikolova, and Jiang 2001). It is however discussed that the lactate dehydrogenase should be the first enzyme transcribed and translated in *E. coli* cells under oxygen limitation and lactate formation should also be a fast response to oxygen oscillations (Lara, Leal, et al. 2006). We could see lactate accumulation during the exponential phase in our scale-down cultivations, which was re-metabolized in the protein production phase under stronger glucose limitation. Highest formation rates for lactate were detected in setups using a feeding loop configuration (2CR-Feed, 3CR) or pulse-based feeding, but also under oxygen oscillations without glucose excess minor temporary lactate accumulation and re-consumption can be seen to support growth and cellular maintenance under glucose-limited environmental conditions. Our results regarding lactate match observations described before (B. Xu, Jahic, and Enfors 1999).

Acetate is an additional product of the mixed acid fermentation pathway and further the key product of overflow metabolism under aerobic conditions in *E. coli* (B. Xu et al. 1999). Though, it is known to influence growth and to decrease biomass yields in cultivations (B. Xu, Jahic, and Enfors 1999). During the initial batch phases, glucose pulses, or in the initial fed-batch phases near the maximum specific growth rate in our cultivations unlimited or near maximum glucose uptake and metabolization leading to acetate accumulation could be expected and is seen in the cultivations. During the protein production phases at strict glucose limitation, acetate consumption can be detected.
5.3. Effects of oscillating cultivation conditions on the branched-chain amino acids synthesis

α-ketobutyrate is the common precursor molecule for the synthesis of isoleucine as well as the non-canonical amino acids norvaline, isoleucine, and β-methyl-norleucine. Its formation is usually formed by deamination of threonine catalyzed by ilvA. Remarkably, knocking out the threonine deaminase does not result in prevention of accumulation of norvaline and norleucine (Sycheva et al. 2007). Alternative synthesis routes for α-ketobutyrate by other metabolic reactions are discussed with the most prominent one defining pyruvate as key metabolite catalyzed by the enzymes of the leuABCD operon (Soini et al. 2008). It is identical to the proposed biosynthesis pathway for the non-canonical amino acids in S. marcescens (Kisumi, Sugiura, and Chibata 1976a). This other pathway to produce α-ketobutyrate directly from pyruvate for threonine-independent isoleucine formation is exploited by some microorganisms (Howell, Xu, and White 1999; H. Xu et al. 2004). Formation of non-canonical amino acids is increased under conditions where enzymes of the leuABCD operon are highly expressed, and it is known that accumulation of non-canonical amino acids can be prevented by feeding canonical branched-chain amino acids (Sycheva et al. 2007). It is supposed, that overexpression of heterologous proteins with a leucine ratio higher than the cellular average of 8 % results in formation and accumulation of norvaline and norleucine in E. coli (Apostol et al. 1997). The excessive demand for leucine in protein synthesis could lead to the higher expression of the enzymes responsible for leucine synthesis and deregulation of the leucine controlled biosynthetic pathway. Thus, the non-canonical amino acids norvaline, norleucine, and β-methyl-norleucine have a higher production rate (Bogosian et al. 1989; Apostol et al. 1997). Also, Soini et al. (2008) have shown an accumulation of pyruvate and increased the formation of pyruvate-derived products like mixed-acid fermentation products or branched-chain amino acids including non-canonical amino acids in E. coli W3110 after an oxygen downshift. It was also the first study to prove norvaline accumulation in a non-recombinant strain.

Interestingly, there is no pyruvate accumulation detectable in any experimental setup. This quite remarkable as several scale-down studies mark pyruvate accumulation under glucose excess or oxygen limitation as a reason for increased formation of organic and amino acids including non-canonical amino acids (Soini, Ukkonen, and Neubauer 2011). In all our
cultivations (bioreactor and multi-well plate scale), accumulation of norvaline, norleucine and β-methyl-norleucine can be detected, which is boosted under every kind of oscillating cultivation conditions. Under oscillating conditions, norleucine and β-methyl-norleucine accumulated at higher levels than norvaline. It seems that the formation of these two amino acids is preferred in the used E. coli K-12 W3110M under the non-optimum conditions. Also interesting is the intense accumulation of β-methyl-norleucine after the induction of miniproinsulin expression. As described before, the synthesis of norleucine and β-methyl-norleucine is closely related as their formation is catalyzed by the same enzymatic reactions performed on different intermediates (Sugiura, Kisumi, and Chibata 1981b). Enzymes of the leuABCD operon are responsible for norleucine formation whereas β-methyl-norleucine production is catalyzed by enzymes of the ilv family. It seems that the upregulation of the branched-chain amino acid pathway is not globally affected by the induction of the miniproinsulin expression and has a stronger impact on the ilv family activity than on the leuABCD operon. Interestingly, the amination of α-ketobutyrate to norvaline is also catalyzed by IlvE, but no comparable accumulation of norvaline can be seen.

In our results of both fermentations, accumulation of these three non-canonical amino acids started immediately after the PFR circulation. This fast formation is probably caused by the oxygen limitation in the PFR module which has been earlier indicated to trigger norvaline formation by Soini (Soini, Ukkonen, and Neubauer 2011). The conditions in the two-compartment scale-down reactor caused remarkable accumulation of norleucine and β-methyl-norleucine, which are formed as the side products of the isoleucine biosynthesis pathway with the farther distance than for norvaline formation as postulated by previous studies. This indicates probably that the accumulation of norleucine and β-methyl-norleucine might be favored in E. coli K-12 recombinant strains under so stressed oscillating starvation cultivation. In the case of our experimental set-up, the drastically accumulating β-methyl-norleucine is interesting after the induction of the recombinant product in comparison of norvaline and norleucine accumulation. It may be assumed, that oscillation conditions might strongly induce the stress genes after induction of a recombinant leucine-rich protein responding to β-methyl-norleucine formation.
5.4. Expression of a leucine-rich protein under oscillating cultivation conditions

The central focus of this study was to reveal and enhance understanding of connections between different types of oscillating conditions on the expression of a recombinant miniproinsulin. During all stages of the research *E. coli*, W3110M pSW3 was the production system and cultivated in different scales using varying scale-down approaches. The focus laid on the misincorporation of the three non-canonical amino acid norvaline, norleucine, and β-methyl-norleucine, which are side-products of isoleucine biosynthesis, into the recombinant protein spoiling its quality. The produced miniproinsulin consisted of 96 amino acid residues with a combined molecular weight of 11 kDa. Important for the discussion on misincorporated non-canonical amino acids the number of leucine, isoleucine, and methionine residues is necessary. The expressed recombinant protein had 14 leucine positions (15 %), five isoleucine residues (5 %) and three molecules methionine incorporated per peptide molecule (3 %) and in its composition pretty similar to interleukin-2. The average leucine ratio of native cellular proteins is around 8 % (Neidhardt and Umbarger 1996). Consequently, the used miniproinsulin can be defined as leucine-rich in comparison to native proteins in *E. coli* cells. It is interesting that misincorporation of non-canonical amino acids into recombinant proteins is not intensively discussed in the literature although it is a crucial topic for recombinant protein production in large-scale cultivations. Historically, the research focus was more set on the synthesis mechanism of non-canonical amino acids in different bacterial species like *B. subtilis* (Nandi and Sen 1953) or the already mentioned *S. marcescens* (Sugiura, Kisumi, and Chibata 1981a). Sycheva et al. studied the formation of non-canonical amino acids in ilvA knocked-out *E. coli* (Sycheva et al. 2007). In the last years, Soini and coworkers revealed connections between the accumulation of norvaline and oxygen limited cultivation conditions (Soini, Ukkonen, and Neubauer 2011).

Well-known studies regarding recombinant *E. coli* focused on synthesis and misincorporation of norleucine during the production phase of a recombinant protein (Bogosian et al. 1989) or the formation and misincorporation of norvaline into recombinant proteins under aerobic lab-scale conditions (Apostol et al. 1997). Furthermore, Biermann et al. could show, that supplementing the trace elements nickel, molybdenum, and selenium during recombinant protein expression in pulse-based feeding approach could significantly reduce the accumulation of non-canonical amino acids. Unfortunately, they had not analyzed how these
reduced concentrations influence misincorporation into the produced recombinant protein (Biermann et al. 2013). The central aim in this study was set on the misincorporation under large-scale simulating conditions and adds new insights to the scientific discussion.

Overall, leucine synthesis and in connection the branched-chain amino acid pathway is feedback-controlled by the intracellular concentration of free leucine. Though, with increasing concentrations of synthesis pathway enzymes, leucine inhibition is becoming ineffective, which was shown for mutants of *S. typhimurium* with de-repressed *leu* operons (Calvo, Margolin, and Umbarger 1969). Overexpression of a leucine-rich recombinant protein can also deregulate the branched chain amino acid pathway (Bogosian et al. 1989). Expression of heterologous proteins can deplete intracellular leucine pools which trigger an increased activity of the enzymes encoded by *leuABC* operon to match the risen leucine demand (Burns et al. 1966). Consequently, a deregulated branched-chain biosynthesis could also boost production of pathway-linked side-products like non-canonical amino acids (Sycheva et al. 2007). Also, a highly active α-isopropylmalate synthase triggers keto-acid chain elongation reactions with α-ketobutyrate or pyruvate enabling the formation of non-canonical amino acids.

In our scale-down approaches, base levels of non-canonical amino acids can be detected during the cultivations. These concentrations are significantly increasing after induction of the miniproinsulin expression. In contrast to the literature, we could not see a decrease in the free leucine pool caused by induction of protein expression in all cultivations. Quite the contrary, we detected increasing concentrations of free leucine as well as free isoleucine and non-canonical amino acids. Increasing non-canonical amino acid levels during the expression of heterologous proteins is consistent with results gained by Sycheva et al. (2007) and explained with higher activity and expression of the *leuABC* enzymes. On the other side, leucine depletion during recombinant protein production was seen by Apostol et al. (1997), but it must be considered that a different protein (human hemoglobin) was produced in their study and possible effects due to protein size and composition need to be considered.

Concluding, metabolic changes regarding the carbon flux due to protein induction leads to increased formation of non-canonical amino acids. Under oscillating conditions, simulating large-scale bioprocesses synthesis of these amino acids is boosted. Remarkably, we have seen
the highest probability of misincorporation of non-canonical into the recombinant miniproinsulin under reference lab-scale conditions although levels of free non-canonical amino acids were the lowest. The formation of β-methyl-norleucine is preferred in comparison to norvaline and norleucine concentrations under oscillating cultivation conditions.

5.5. Impacts on product quality caused by process perturbations
Heterologous proteins containing leucine, isoleucine, and methionine in their amino acid sequence and are overexpressed in E. coli can be “contaminated” by wrongly incorporated molecules of norvaline, norleucine or β-methyl-norleucine. Numerous studies revealed that these amino acids behave like isostructural analogs to canonical amino acids. They can be misincorporated due to mischarged tRNAs. The mechanism is explained and summarized in chapter 22.4 and Table 2.3. Norvaline is known to replace leucine if certain rare codons are used (Apostol et al. 1997). Norleucine is an analogue for methionine (Sunasara et al. 1999) and isoleucine can be replaced by β-methyl-norleucine (Muramatsu, Misawa, and Hayashi 2003).

Under large-scale conditions, recombinant protein production is accompanied by additional stresses due to stress responses to oscillating environmental changes. Interestingly, oscillating stress has no predictable influence on the productivity of the host strain regarding recombinant protein formation due to altered metabolic fluxes. A loss of product yield is described under oxygen oscillating conditions in a scale-down system (Sandoval-Basurto et al. 2005) whereas a 10 % increase in productivity was seen in another study if oscillations for substrate and oxygen were applied (Bylund et al. 2000). The comparison of expressed miniproinsulin in our studies via SDS-PAGE was technically difficult as staining efficiency was varying. Concentrations of leucine, isoleucine, and methionine from the purified inclusion bodies fractions were on a similar level with comparable trends in all cultivations indicating that there is no effect on the productivity of miniproinsulin expression under any cultivation condition applied. Therefore, influences of stress conditions on the produced amount of heterologous proteins dependent on the host strain and the desired product. As productivity is not influenced, the quality of the expressed recombinant protein is the essential parameter to evaluate effects of applied oscillating cultivation conditions. We have seen significantly raised concentrations for norleucine and β-methyl-norleucine in the purified inclusion bodies
fractions under oscillating oxygen availability. If oxygen limitation is combined with substrate perturbations misincorporation for both amino acids is even more increased. Remarkably, there is no relevant norvaline incorporation detected in the recombinant miniproinsulin under any cultivation conditions. Norvaline exchange is favored for certain rare codons in the DNA sequence (Apostol et al. 1997). Unfortunately, the gene sequence for the used miniproinsulin is unknown, so the codon-based limitation of norvaline incorporation cannot be discussed.

Apostol et al. (1997) could prove that the ratio of free norvaline in comparison to leucine is an essential factor for triggered misincorporation of norvaline instead of leucine. The proportion of substitutions in the expressed hemoglobin was correlating to the ratio of free norvaline to leucine detected in the cultivation medium. Our results are consistent with these observations. We can see linear correlations between free and incorporated norvaline and similar trends for β-methyl-norleucine. Interestingly, norvaline incorporation is triggered earlier under reference condition in comparison to oscillating conditions. Glucose excess influences the correlation regarding β-methyl-norleucine. There is no linear correlation between free and incorporated norleucine. Here, misincorporation increases exponentially with accumulating free norleucine.

In conclusion, oscillating oxygen availability is the main factor triggering the accumulation of non-canonical amino acids in high levels. Substrate oscillations further increase the formation. Higher levels of produced non-canonical amino acids lead to a reduced quality of the desired recombinant protein due “contamination” of the protein with misincorporated residues. We could show that misincorporation of non-canonical amino acids depends on higher concentrations of free amino acids under oscillating cultivation conditions. Therefore, a reduced formation of non-canonical amino acids, e.g. in an engineered strain optimized for this purpose, should increase product quality especially under process perturbations and lead to more robustness regarding against misincorporation of wrong amino acid residues.
6. Conclusions and Outlook
This thesis enhances the view on more than one topic regarding oscillating cultivation conditions and formation as well as misincorporation of non-canonical amino acids into recombinant proteins. It could be seen, that not only one gradient is triggering the formation of these amino acids, but several conditions found in industrial scale bioprocesses have an influence on this undesired behavior. For efficient and robust bioprocess development, oscillating cultivation conditions need to be included in the early stages of bioprocess development.

As multi-compartment scale-down simulators, designed for physiological characterization studies, are complex systems and not a desirable approach for high-throughput or even automatized and parallelized screening this study could also prove that simplified approaches down to the mL-scale in multi-well plates are suitable for the first screening against the oscillating substrate or oxygen availability. In combination with the possibility to monitor the formation of insoluble recombinant proteins at-line using a fluorescent dye, this thesis builds a base for follow-up studies on recombinant E. coli physiology under stress conditions. Furthermore, it inspires new and innovative approaches in the areas of automatized bioprocess development and strain engineering for new and more robust host strains and more efficient cultivation strategies for recombinant protein processes.
7. Theses

7.1. Oscillating substrate availability decreases the biomass yield by up to 20% in multi-compartment reactor cultivations whereas oscillating oxygen availability has no significant impact on growth of *E. coli*.

7.2. Lactate is the preferred formed mixed acid fermentation product under oscillating conditions.

7.3. Environmental oscillations in the multi-compartment reactor as well as under pulsed feeding or in the multi-well plate experiments have no significant influence on the produced amount of the recombinant miniproinsulin per cell. Efficiency of miniproinsulin production is defined by the gained biomass yield under each cultivation condition.

7.4. Oscillating excess oxygen supply combined with substrate starvation results in extensive cell lysis and cultivation failure.

7.5. Application of oscillations can be simplified with a comparable level regarding influences on recombinant protein quality.

7.6. Linear correlations between the formation of Norvaline and β-methyl-norleucine and the misincorporation into the recombinant miniproinsulin are seen in STR as well as multi-compartment reactor cultivations. The correlation between formed in incorporated Norleucine is exponential. Interestingly, the highest incorporation probability of NCAAs can be seen under reference cultivation conditions at the lowest NCAA formation rates.

7.7. Exchanges of canonical amino acids with similar analogs is not linked to the number of individual positions in the protein sequence. Norvaline is the at least misincorporated non-canonical amino acids under all applied cultivation conditions, although the leucine amount in the recombinant miniproinsulin is 3 – 5 times higher compared to methionine or isoleucine.

7.8. Single-use labware equipped with on-line monitored sensors and fed-batch simulating media allow a new quality of screening experiments regarding the misincorporation of NCAAs into recombinant proteins produced in *E. coli* focusing on growth rate, inducing strength as well as oxygen availability.

7.9. At-line monitoring of product formation without the need of reporter proteins using the fluorescent dye Thioflavin-S enables the development of new process controlling approaches.
8. References


References

Christian Reitz


References


References

Christian Reitz


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9. Appendix

9.1. SOP 1: GC-MS Short Manual

SOP
GC-MS Short manual

Authors: Robert Spann, Sergej Trippel

Date: June 2013

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General Information
This SOP is a summary of the most important things regarding GC-MS. Detailed information you can find in the following documents. It is recommended to read them before using GC-MS the first time. Both double underlined documents are highly recommended!

Literature Software

Literature Hardware

Devices
- GC/MS-System (Agilent Technologies, Waldbronn, Germany), consisting of:
  - Auto sampler AS G26 14A
  - Injector 76 83B
  - Gas chromatograph GC 7890A
  - Mass spectrometry detector MSD 5975G
  - Software G 1701 EA
- Carrier Gas: Helium
- Isopropanol
- Contact for utilization (persons in charge): SJ, EB, BB
GC-MS Run
In this chapter, it is explained how to start a GC-MS run. Therefore it is worth to know how to load the required method, how to program the auto sampler (write the sequence) and what to check before starting the run.

i. Open GCMSD (on desktop).

Method
First you must load the method you want to use. Later in the sample log table it is possible to refer different methods to distinct samples.

i. Select Method - Load Method in the menu to load your method of choice.

ii. All methods are stored at the data path D:\MSDCHEM1\METHODS

iii. After column change the program might warn you that the columns/max. temperature of the method are not consistent with the GC Configuration. Then Method Resolution and Method Resolution Report dialog boxes appear.

   a. To check that the right column is installed choose the CFT Settings (oven icon) tap in the Method resolution dialog box. (“installed” means that the software is aware of the newly installed column)

   b. To check the maximum oven temperature setting see section Column changes.

   c. Click OK to close the Method Resolution dialog box. Close the Method Resolution Report as well.

   d. Save your method.

   e. Check the temperature profile. Especially by using the DB5-ms column check the maximum oven temperature.

Sequence
Second you must program the auto sampler.

i. In the menu select Sequence - Edit Sequence... to open Sample log table dialog.

ii. Click Browse next to the text box Data Path and choose the Data Path where all results should be saved after GC-MS run.

iii. Click Browse next to the text box Method Path and choose the folder where your methods for GC-MS run are saved (e.g. D:\MSDCHEM1\METHODS\DFG_Norvalin)
iv. Name your samples in the column *Data File*.

v. Enter the corresponding number of the sample position in the auto sampler tray in the column *Vial*.

vi. To choose the Method in the column *Method/Keyword* select the menu item *Browse for Method...* from the right click context menu. Important: In one sample log table, you can only choose methods which are in the folder you have chosen for *Method Path*, not in subfolders!

vii. Choose *Sample* in the *TYPE* drop-down menu for all samples.

viii. Enter any number in the column *Sample* (typically 1, 2, 3, …).

ix. Click *OK*.

x. To save the Sequence select *Sequence - Save Sequence (As...) in the menu.

xi. Important: Save the Method even if you have not changed it! *Method - Save Method*.

xii. To check whether the sequence programming is right select *Sequence - Simulate Sequence...* in the menu.

**Daily checks**
Before you can start the run, you should do some daily checks:

i. check vials in the solvent supply of the injector
   
   a. Check the Isopropanol level in flask A and B.
   
   b. Positions 2-5 'WB' (=Waste) must be occupied with empty glass vials

ii. A green light should be seen at the icon with a GC column and a thermometer under the section *Instrument*. Otherwise not all adjustments are reached and you must wait until you can go on.

iii. Before the first run an *Air and water check* must be performed every day:
   
   a. Select *View - Tune and Vacuum Control…, Tune - Air and Water Check* in the menu. Answer with no when asked: SELECT: YES … NO to use current values? Save the pdf in the desktop folder *Shortcut to Air and Water Checks*. The system should reach H₂O, N₂ and O₂ <1 %! Otherwise repeat the air and
water check some minutes later. If a value exceeds 2% a MSD Tune should be performed (see G1701-90070 p. 17ff.).

b. Select View – Instrument Control in menu to close GCMSD Tune dialog box. Proceed with Yes.

c. Fill out the columns in the GC-MS Logbook (red book beside the PC). The High vacuum pressure and Foreline pressure can be noted at the GCMSD Enhanced dialog box. H₂O, N₂ und O₂ are listed in the created Air and Water Check pdf file. High vacuum pressure should be in the range of 1*10⁻⁷ and 1*10⁻⁵.

iv. The injections liner must be replaced after 100 – 150 injections.

a. To check the counter, select Instrument – EMF Utilities in the menu. Choose Set Counters and proceed with OK. The values in the Enter counter values for EMF parameters dialog should be lower than 150. Otherwise ask a technical assistant to replace the injection liner.

b. To close the dialogs, click OK first and Done finally.

Run

Finally, you can start the run.

i. Select Sequence - Run Sequence in the menu to start the run.
Evaluation
It will be explained how you can identify unknown substances, how to quantify substances and how to determine the isotopic ratio.

i. Open GCMS Data Analysis (on desktop)

ii. Load the correct method. This is only necessary for quantification and determination of the isotopic ratio. If you want to identify substances just open the method, you used for GC-MS run.

iii. Open your sample:
   a. if you are asked:
      1. DataFile last Quantititated using … Load? select No!
      2. Select Quant Results file: select the method which was used by GC-MS run.

Identification
Notice that identification by using the data base is only possible by using a SCAN mode run. If you have done a SIM mode run, the known retention time and the specific fragments distribution can be only used for manual identification.

i. Double click a peak in the upper dialog [2] TIC with the right mouse button.

ii. Double click in the lower dialog [1] with the right mouse button anywhere.

iii. NIST MS Search 2.0 is loaded.
   a. Lib. Search tab under the left area shows you the database matches.
      1. When you choose one match you see in the right area the overlaid spectrum of your sample and the database spectrum

Quantification
When a calibration exists an automated and manual integration for quantification purposes is possible.

i. If you quantify this sample the first time: Click either Calc Quant Report icon or select Quantitate - Calculate in the menu. This is an automated integration which overwrites your manual integration if you did it before!
ii. To check and correct the automated integration select View - QEdit Quant Results in the menu to open Quick Qedit dialog box and Window #6, [1] and [7] in the background.

   a. Double click one metabolite in the Quick Qedit dialog.

      1. Window #6 shows you the current integration. In [7] you can see the response and the calculated concentration.

   b. In Window #6 you can zoom with the left mouse button (double click is zoom minus) and integrate with the right mouse button.

iii. Check the integration of the IS.

iv. Check the integration of all metabolites. You can integrate manually if you are not satisfied. (Only the main ion is responsible for the concentration.) If you want to delete the integration of a metabolites at all, select the metabolite and click QDel in the Quick Qedit dialog.

v. Click Exit: Save changes made to quantitation results? Press Yes if you want to save them!

vi. You can export the results as *.txt and *.pdf:

   a. Best way: *.txt: Select Quantitate->Summary Report with Compound Groups... in the menu. To close the Enter Names for Quant Summary Report dialog click Ok. Save the opened text file!

   b. *.pdf: Select Quantitate - Generate Report in the menu. Style: Summary, Destination Printer. Select the data pathway and press Save (pdf type you can convert to excel: copy the pdf text into excel and select Daten->Text in Spalten: the rows are separated by space!)

vii. NOTE: If you select anything at Export Reports in the menu your manual integration will be overwritten!!!

Isotopic ratio
To determine the isotopic ratio, it is important to subtract the background. Finally, you can calculate the isotopic ratio of your chosen fragment.
i. Select View - Analyze Multiple Data Files in the menu to open Select Data Files (max 9) dialog box.

ii. Select the files you want to analyze and click the ---> icon to move them to the Files Selected for Processing area. You can change folder when you click on Change Path.

iii. Click Process. Dialog box Enter m/z values (1-1050 amu; TIC=0) appears.

iv. Enter M/Z values you want to see for this sample and click OK. Repeat this for all samples or use the same fragments for all samples.

v. Zoom in with the left mouse button to see the whole peak.

vi. Push the right mouse button and move the mouse so that the rectangle borders the peak and release the button.

vii. Push right mouse button again and do the same procedure with basis line close to the left side of the peak. consider only 0.1 min of basis line.

viii. Select Spectrum - Subtract in the menu to substract basis line noise from peak spectrum.

ix. Select Spectrum - Tabulate to open Tabulate dialog.

x. Click Copy.

xi. Paste data into excel file.

xii. Repeat the procedure for every peak of your interest and for every chromatogram.
Column and liner changes

Column and injection liner changes are performed by a technical assistant.

Column change

i. After column change at the GC hardware, you must change the column in the software settings.
   a. Open GCMSD/Enhanced dialog box (online mode).
   b. Take the already opened method, favorable TEST01-NO-COOLING.M.
   c. Select Instrument – GC Edit Configuration... to open Agilent 7890GC dialog box.
   d. Choose the tap Columns in the tap Configuration.
   e. Click Inventory to open Install Column 1 dialog box.
   f. Select the newly installed column. If you cannot find the appropriate column you can add it: Select Add Column to Local Inventory ...
   g. Click Install Selected Column.
   h. To close select OK.
   i. Save Method As dialog box appears. You can overwrite TEST01-NO-COOLING.M.
   k. If you choose the CFT Settings (oven icon) tap you see that the column is changed.
   l. Click OK to close the Method Resolution dialog box. Close the Method Resolution Report as well.
   m. A warning beeping indicates a negative FRONT INLET pressure. In that case increase the oven temperature to 100 °C by activating the oven heater: Select Instrument – GC Edit Parameters and choose the Oven tap. Tick the Oven Temp On checkbox.
   n. IMPORTANT: If you install the DB-5ms column:
      1. Close all dialogs down to GCMSD/Enhanced and Instrument Control dialog box.
      2. At GC Keypad press the Oven button.
      3. In the software select Instrument – GC Edit Configurations to open Agilent 7890GC dialog box. Choose the tap Miscellaneous in the tap Configuration. Enter 290 °C in the Maximum Oven Temperature text
box. Tick the *Slow Fan* checkbox. Remove the tick in the *Slow Fan*
checkbox! Close the dialog box and save the method.

**Injection liner change**

ii. After injection liner exchange reset the counters.
   a. Select *Instrument – EMF Utilities…* to open *Select EMF action* dialog box. Choose *Reset Counters* and proceed with *OK*. Tick all desired checkboxes and confirm with *OK*.

**Set up a Calibration**

It is recommended to perform a calibration curve in SIM mode to quantify most accurately. Therefore you should inject each compound separately first in SCAN mode with the desired temperature profile to determine the retention time. When you have collected all retention times and appropriate fragments, write the SIM mode. Perform the calibration runs with mixtures of all compounds. Prepare different concentrations and run them with your programmed SIM mode. In the sequence table choose in the column TYPE *Sample NOT Calibration*. In the following part, it is described how to create a calibration curves using these runs.

i. Open the chromatogram of the sample with the lowest concentration.

ii. Select *Set up quantitation* form main menu *Calibrate*.
   a. *Calibration title*: enter the calibration title e.g. Amino acids.
   b. *ISTD concentration*: enter the internal standard concentration.
   c. *Curve Fit*: Choose the curve fit algorithm e.g. linear regression, force 0.0.
   d. Accept with *ok*.

iii. *Edit compounds* dialog box appears. Here the new components are inserted.
   a. To insert the compounds, proceed with *Insert above*.

iv. *Quant setup* dialog box appears. Refer the retention time and most abundant fragments to each compound.
   a. Enter the name of the first compound (or Internal standard).

1. In case of IS tick the *ISTD* checkbox.
b. Zoom to the correct peak in the TIC dialog.

c. Double click the right mouse button at the middle of the peak to define the retention time and to see the MS Spectrum.

d. *Tgt*: Target Ion: Choose one ion in the MS Spectrum: In most cases the target ion is the base ion. This Ion will be used for quantification. Therefor be sure that this ion is meaningful for this compound.

1. To select the ion, click with the crosshair on the ion with the left and the right mouse button successively.

e. Proceed with Q1, Q2 and Q3: These are ions to identify the compound correctly. In most cases it is favorable to take the molecule peak for Q3.

f. When all fragments are chosen proceed with Save.

v. Repeat iii. and iv. for all compounds.

vi. Finish with Exit.

vii. *Edit compound* dialog box with all compounds appears.

viii. Close with Exit.

ix. *Update Calibration* dialog box appears.

a. Close with Exit.

x. Before you create the calibration curve the integration of all calibration samples must be corrected.

a. Open a calibration sample.

b. Open QEdit Quant Results from menu View.

1. Check the integration of all compounds.

2. Correct wrong integrations manually, note these manual responses, because the system will not take your manually integration by creating the calibration curve. Instead you should put in the responses afterwards manually.
xi. Open *Update Calibration* window by selecting *Update... - Update one level* from menu *Calibrate*.

a. If you want to overwrite any existing calibration in this method answer with *yes*, when asked: *File has previously been quantitated. Requantitate now?*

b. Select *Add Level*

1. *Compound Concentration*: Insert the concentration of the compounds of the first sample

2. Control *ISTD Concentration*.

3. Enter new level ID: e.g. 1 (for 1 µM).

4. Select *Do Update*.

5. *Edit Compounds* dialog box appears.

   1. Insert manual responses if necessary.

6. Exit and confirm with *Ok*.

xii. Repeat x. and xi. with all calibration samples.

Retention time shifts
It happens that due to column change the retention time shifts. If you use SIM mode, this influences your analysis dramatically because peaks can move out of their time window. Therefor it is explained how to correct SIM mode and quantification settings.

i. Run a standard sample containing all compounds and the internal standard in SCAN mode (take all other settings from the SIM mode, e.g. temperature profile).

   a. Duplicate the SIM method and change the *Acq. Mode* to Scan:

      1. Select *Instrument - MS SIM/Scan Parameters* to open MS SIM/Scan Parameters dialog box. Choose from the *Acq. Mode* drop-down menu *Scan*.

      2. Save the new method. Do not overwrite the SIM method!

ii. Note the retention times of all compounds.

iii. Adjust the SIM mode settings in GCMCD/Enhanced dialog box (or use offline mode):

   a. Load the method
b. Select Instrument - MS SIM/Scan Parameters. Click SIM Parameters to open Edit SIM Parameters dialog box.
   
   1. Adjust the start time of each Group.

c. Click Close, OK and save the method.

iv. Adjust the quantification settings in Enhanced Data Analysis:
   
a. To open Edit compounds dialog box select Calibrate - Edit Compounds… in the menu.

   b. For each compound change the value in the Ret Time text box to adjust the retention time.

v. Perform a control run to check the new settings before analyzing many samples!
9.2. SOP 2: GC-MS Sample preparation for analysis of amino acids by acidic hydrolysis

SOP

GC-MS Sample preparation for analysis of amino acids by acidic hydrolysis

Authors: Dennis Runge, Sergej Trippel, Robert Spann
Date: June 2013

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Devices

- Pipettes 10 µL, 200 µL, 1000 µL
- Pasteur pipettes
- Heating block (Block heater H250, Fa. Roth, in lab 265c next to the heat sterilizer)
- Speed Vac (Fa. Bachofer, in lab 265c)
- GC-MS (Agilent 5975 C) (see Appendix for GC Settings)
- Nitrogen (O₂<2 ppm-mol)
- Centrifuge

Chemicals

- Deionized water (DI H₂O) (Barnstead Easypure II RF)
- Acetonitrile (conc. H₂O < 30 ppm) (VWR, Art. 83639.320)
- MTBSTFA (N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide) (Sigma-Aldrich, Art. 394882) derivatisation grade ≥97 %
- Purified water for chromatography (VWR, Art. 23595.328)
- HCl p.A. 37 % (Sigma-Aldrich, Art. 339253)
  - HCL must be diluted to 6 M: Prepare 49,9 mL DI H₂O and add carefully and slowly 50,1 mL HCl from the stock
- 1-Butanol (Fluka, Prod. 19430) >98 %
- α-aminobutyric acid (Sigma Aldrich, Art. 162663)
  - α-aminobutyric acid is used as internal standard. Prepare a stock solution of 18 mM α-aminobutyric acid, dissolved in 4 mM sodiumdihydrogenphosphate-dihydrate (buffer A).
  - Dilute α-aminobutyric acid to 0,225 mM (dilution 1:80) before usage with buffer A.
  - IS stock solution must be prepared 2 weeks before usage. Then it can be used for 2 weeks. This is due to degradation!
- Molecular sieve 5Å (Roth, Art. 8475.1)

Equipment

- most can be found in lab 265b, in the cupboard next to GC-MS

Vials for Speed Vac and Derivatization

- Brown glass vials 1,5 mL (Fisherbrand, Art. 1008-0952) ø 9mm for heating block
- Blue screw caps with integrated septum (Fisherbrand, Art. 1052-0443) ø 9mm for heating block
Vials for GC-MS Autosampler

- Brown glass vials 1.5 mL (Fa. Fisherbrand, Art. 1072-8684) ø 8mm for injection of samples into gas chromatograph
- Black screw caps (reused; wash with VE H₂O)
- Septum (Fisherbrand, Art. 3146116) ø 8mm
- Microinlets 0.05 mL (Fisherbrand, Art. 1024-4612)
- Springs (Federn) for microinlets 36 x 5 mm (Fisherbrand, Art. 3205576) (reused; wash with spoiled Ethanol 70% and VE H₂O)

Sample Preparation

Preparatory work

Work on ice during the preparatory work. Original samples are always cooled on ice and exposed to room temperature as short as possible.

Supernatant samples

1. Thaw cultivation sample on ice and vortex it afterwards.
2. Transfer 150 µL supernatant into a 1.5 mL Eppendorf tube.
3. Centrifuge the 150 µL for 10 min, 15 000 x g and 4°C.
4. Freeze the rest of your sample at -80°C again.
5. Go on with Processing

MeOH Quenching samples

1. Prepare the sample: Appendix SOP 140 modified for GC-MS analysis of amino acids by acidic hydrolysis. (OD₆₀₀ = 1)
2. Centrifuge the 500 µL for 10 min, 15 000 x g and 4°C.
3. Go on with Processing.

Processing

Acidic Hydrolysis and Speed Vac

- Give 750 µL of 6 M HCL into the glass vials (use the vials and screw caps which fit into the heating block! It means 9 mm neck and blue cap).
- Add 125 µL of your sample and 125 µL of internal standard (0.225 mM α-aminobutyric acid).
- Put the vials closed into the heating block for 24 hours at 80°C.
- Store the vials opened in Speed Vac. Speed Vac should be switched on 30 min before the run! For further details check the manual placed next to the Speed Vac. Vials are dried in centrifuge for 3 hours at 30°C temperature.
Derivatization

Amino acids are derivatized with MTBSTFA. This reagent is sensitive to oxygen. So, the opened MTBSTFA-vial must be continuously flushed with nitrogen. For this, set up a pipe connection from nitrogen bottle to MTBSTFA-vial. Nitrogen should be flushed into the head space of the MTBSTFA-vial. It is recommended to use a gas flow meter for better adjustment of the gas flow (2 L / min).

- Take dried samples from centrifuge.
- Add 50 µL acetonitrile.
- Add 50 µL MTBSTFA (don’t forget to flush with nitrogen/argon all the time!).
- Add 5 µL butanol. Butanol must be previously dewatered with molecular sieve (200 µL per MS).
- Close vials and put them in the heating block for 60 min at 60° C.

Transferring into inlets for GC-MS injection

- Prepare new vials and caps (ø 8mm) for the injection of samples into GC-MS:
  - insert springs into vials
  - insert microinlets into springs
- Use Pasteur pipettes to transfer derivatized samples from their vials into the micro inlets: Turn the vial of the derivatized samples in this way that the liquid phase is away from the solid phase. Therefor you avoid transferring solid particles into the GC-MS.
- Closed vials with inlet are now ready for injection.

GC-MS run

Start GC-MS analysis as described in SOP GC-MS short manual.

Use the following methods for GC-MS run:

a) to determine the concentration:
   Method: D:\MSDCHEM\1\METHODS\DFG_Norvalin\AMINO-ACIDS_CONC_RUN_SIM.M
b) to determine the isotopic ratio
   Method: D:\MSDCHEM\1\METHODS\DFG_Norvalin\AMINO-ACIDS_ISOTOPEN_RUN_SIM.M
GC-MS evaluation

For detailed explanations about fragmentation of amino acids see SOP GC-MS Sample preparation for analysis of free amino acids.

Use the following method for evaluation: for concentration and isotopic ratio run:
D:\MSDCHEM\METHODS\DFG_Norvalin\Amino-Acids_SIM_Quantification.M

Keep in mind that hydrolysis partly destroys some amino acids. This leads to the loss of 10-40% of Serine, Threonine and Methionine and 50-100% of Cysteine and Tryptophane. Asparagine and Glutamine are completely desaminated to Aspartate and Glutamate.¹

SOP 140 modified for GC-MS analysis of amino acids by acidic hydrolysis

**Number:** 140  **Version:** 1.0  **Last Modified:** Sun Aug 05 15:02:18 EEST 2012  **Status:** Official  **Author:** Klingner, Arne

**Name:** Protocol for Sonication of *E. coli* cells  **Description:** This short protocol shows how to disrupt *E. coli* cells via being sonicated.

**Related SOPs:**  **Categories:** laboratory  **Keywords:** sonication, *E. coli* W3110, cell disruption

**Comments:** 0 ([Place comment](#)) SOPs can be improved by posting constructive comments.

---

**Sonification of *E. coli* cells**

**Used strain:** *Escherichia coli* W3110

**Sonication device:** Hielscher UP200S with 2 mm sonotrode

**Sonication protocol:**

- measure the OD at 600 nm of the taken sample
- dilute the sample to an \( \text{OD}_{600} \text{ of } 1 \) for disruption with buffer A 1:10.
- transfer the diluted sample into 1.5 mL Eppendorf tubes (0.5 mL per tube)
- adjust the amplitude of the sonication device to 30%
- sonicate the cells using 5 cycles, each cycle 30 seconds long (30 seconds break between two cycles)
- use iced water to cool the sample during sonication!
- vortex each sample shortly just before it is sonicated.

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### GC Settings

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SOP
GC-MS Sample preparation for analysis of free amino acids

Authors: Sergej Trippel, Robert Spann, Dennis Runge
Date: June 2013

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Devices
- Pipettes 10 µL, 200 µL, 1000 µL
- Pasteur pipettes
- Heating block (Block heater H250, Fa. Roth, in lab 265c next to the heat sterilizer)
- Speed Vac (Fa. Bachofer, in lab 265c)
- GC-MS (Agilent 5975 C) (see Appendix for GC Settings)
- Nitrogen (O₂<2 ppm-mol)
- Centrifuge

Chemicals
- Deionized water (DI H₂O) (Barnstead Easypure II RF)
- Acetonitrile (conc. H₂O < 30 ppm) (VWR, Art. 83639.320)
- MTBSTFA (N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide) (Sigma-Aldrich, Art. 394882) derivatisation grade ≥97 %
- HCl p.A. 37 % (Sigma-Aldrich, Art. 339253)
  - HCL must be diluted to 0.1 M → 1. Prepare 29.9 mL DI H₂O and add slowly and carefully 50.1 mL HCl from the stock (→ 6 M); 2. Prepare 59 mL DI H₂O and add 1 mL 6 M HCl (→ 0.1 M)
- 1-Butanol (Fluka, Prod. 19430) >98 %
- α-aminobutyric acid (Sigma Aldrich, Art. 162663)
  - α-aminobutyric acid is used as internal standard. Prepare a stock solution of 18 mM α-aminobutyric acid, dissolved in 4 mM sodiumdihydrogenphosphate-dihydrate (buffer A).
  - Dilute α-aminobutyric acid to 0.225 mM (dilution 1:80) before usage with buffer A.
  - IS stock solution must be prepared 2 weeks before usage. Then it can be used for 2 weeks. This is due to degradation!
- Molecular sieve 5Å (Roth, Art. 8475.1)

Equipment
- most can be found in lab 265b, in the cupboard next to GC-MS

Vials for Speed Vac and Derivatization
- Brown glass vials 1,5 mL (Fisherbrand, Art. 1008-0952) ø 9mm for heating block
- Blue screw caps ø 9mm for heating block (reused; wash with VE H₂O)
- Septum (VWR, Cat. 548-3324) ø 9mm for blue screw caps
Vials for GC-MS Autosampler

- Brown glass vials 1,5 mL (Fa. Fisherbrand, Art. 1072-8684) ø 8mm for injection of samples into gas chromatograph
- Black screw caps (reused; wash with VE H₂O)
- Septum (Fisherbrand, Art. 3146116) ø 8mm
- Microinlets 0,05 mL (Fisherbrand, Art. 1024-4612)
- Springs (Federn) for microinlets 36 x 5 mm (Fisherbrand, Art. 3205576) (reused; wash with spoiled Ethanol 70% and VE H₂O)

Sample Preparation

Preparatory work

Work on ice during the preparatory work. Original samples are always cooled on ice and exposed to room temperature as short as possible.

Supernatant samples

- Thaw cultivation sample on ice and vortex it afterwards.
- Transfer 150 µL supernatant into a 1.5 mL Eppendorf tube.
- Centrifuge the 150 µL for 10 min, 15 000 x g and 4°C.
- Freeze the rest of your sample at -80°C again.
- Go on with Processing

MeOH Quenching samples

- Prepare the sample: Appendix SOP 140 modified for GC-MS analysis of amino acids by acidic hydrolysis. (OD₆₀₀ = 5)
- Centrifuge the 500 µL for 10 min, 15 000 x g and 4°C.
- Go on with Processing.

Processing

Speed Vac

- Give 750 µL of 0.1 M HCL into the glass vials (use the vials and screw caps which fit into the heating block! It means 9 mm neck and blue cap).
- Add 125 µL of your sample and 125 µL of internal standard (0,225 mM α-aminobutyric acid).
- Store the vials opened in Speed Vac. Speed Vac should be switched on 30 min before the run! For further details, check the manual placed next to the Speed Vac. Vials are dried in centrifuge for 3 hours at 30°C temperature.
Derivatization

Amino acids are derivatized with MTBSTFA. This reagent is sensitive to oxygen. So, the opened MTBSTFA-vial must be continuously flushed with nitrogen. For this, set up a pipe connection from a nitrogen bottle to the MTBSTFA-vial. Nitrogen should be flushed into the head space of the MTBSTFA-vial. It is recommended to use a gas flow meter for better adjustment of the gas flow (2 L / min).

- Take dried samples from centrifuge.
- Add 50 µL acetonitrile.
- Add 50 µL MTBSTFA (don’t forget to flush with nitrogen/argon all the time!).
- Add 5 µL butanol. Butanol must be previously dewatered with molecular sieve (200 µL per MS).
- Close vials and put them in the heating block for 60 min at 60° C.

Transferring into inlets for GC-MS injection

- Prepare new vials and caps (Ø 8mm) for the injection of samples into GC-MS:
  - insert springs into vials
  - insert microinlets into springs
- Use pasteur pipettes to transfer derivatized samples from their vials into the microinlets:
  Turn the vial of the derivatized samples in this way that the liquid phase is away from the solid phase. Therefore you avoid transferring solid particles into the GC-MS.
- Closed vials with inlet are now ready for injection.

GC-MS run

Start GC-MS analysis as described in SOP GC-MS short manual.

Use the following methods for GC-MS run:

c) to determine the concentration:
   Method: D:\MSDCHEM\1\METHODS\DFG_Norvalin\AMINO-ACIDS_CONC_RUN_SIM.M

d) to determine the isotopic ratio
   Method: D:\MSDCHEM\1\METHODS\DFG_Norvalin\AMINO-ACIDS_ISOTOPEN_RUN_SIM.M

GC-MS evaluation

Use the following method for evaluation: for concentration and isotopic ratio run:
D:\MSDCHEM\1\METHODS\DFG_Norvalin\Amino-Acids_SIM_Quantification.M
Appendix

SOP 140 modified for GC-MS analysis of free amino acids

Number: 140  Version: 1.0  
Last Modified: Sun Aug 05 15:02:18 EEST 2012  
Status: Official  
Author: Klingner, Arne

Name: Protocol for Sonication of E. coli cells  
Description: This short protocol shows how to disrupt E. coli cells with a sonication device.

Related SOPs: Categories: laboratory

Keywords: sonication, E. coli W3110, cell disruption

Comments: 0 (Place comment) SOPs can be improved by posting constructive comments.

Sonification of E. coli cells

Used strain: Escherichia coli W3110

Sonication device: Hielscher UP200S with 2 mm sonotrode

Sonication protocol:

- measure the OD at 600 nm of the taken sample
- dilute the sample to an \( \text{OD}_{600} \) of 5 for disruption with buffer A 1:10.
- transfer the diluted sample into 1.5 mL Eppendorf tubes (0.5 mL per tube)
- adjust the amplitude of the sonication device to 30%
- sonicate the cells using 5 cycles, each cycle 30 seconds long (30 seconds break between two cycles)
- use iced water to cool the sample during sonication!
- vortex each sample shortly just before it is sonicated.

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Information about MTBSTFA Fragmentation

**Derivatisierungsreagenz:**
MTBSTFA (N-tert-Butyldimethylsilyl-N-methyltriflouracetamid)

MTBSTFA reagiert mit wasserstoffgebundenen Heteroatomen unter Abspaltung eines Wasserstoffatoms zu unpolaren tert-Butyldimethylsilyl-(TBDMS-)derivaten

\[
\text{R-} \text{OH} + \text{MTBSTFA} \rightarrow \text{R-} \text{SiTBDMS}
\]

Substituiert werden –OH, -SH, -NHR, -COOH, -CONH₂; bei Reaktion mit Aminosäuren werden sowohl Carboxy- als auch Aminogruppe einfach derivatisiert, ggf. erfolgt eine weitere Derivatisierung der Seitenkette

TBDMS besitzt eine molare Masse von 115 g/mol, nach Anlagerung an eine Aminosäure unter Abspaltung eines H-Atoms erhöht sich die molare Masse des Aminosäurederivats um 114 g/mol.

Typische Fragmentierungsmuster der Derivate ergeben sich durch folgende Abspaltungen innerhalb von TBDMS:

- Methylgruppe (MW=15g/mol)
- Tert-Butylgruppe (MW=57g/mol)
- Tert-Butylgruppe + C=O (MW=85g/mol)
- TBDMS-COO (MW=159g/mol)

**Abbildung 1 S.476**

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<td>Aux 2</td>
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<td>MS Source</td>
<td>230°C</td>
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<tr>
<td>Column</td>
<td>DB-5MS-column (5% Phenyl – 95% Methylpolysiloxan, 30m x 250µm x 0,25µm)</td>
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<td>Temperature profile</td>
<td>150°C hold for 2 min</td>
</tr>
<tr>
<td></td>
<td>3°C/min to 210°C</td>
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<tr>
<td></td>
<td>5°C/min to 280°C hold for 10 min</td>
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<tr>
<td>Pressure</td>
<td>91.924 kPa</td>
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<tr>
<td>Flow</td>
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<tr>
<td>Average Velocity</td>
<td>38.051 cm/sec</td>
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<tr>
<td>Holdup time</td>
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<td>Group name</td>
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<td>-----</td>
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<tr>
<td>1</td>
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<tr>
<td>2</td>
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<tr>
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<td>Leu, Ile, Norl, Pro</td>
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<td>6</td>
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<td>Arg</td>
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<td>Tryptophan</td>
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# 9.4. SOP 4: SDS-PAGE Gel Electrophoresis

**Title:** SDS- PAGE Gel Electrophoresis  
**Responsible Person:** Petra Grunzel, Florian Glauche  
**Facilities:**  
1.)  
2.)  
**SOP- Number:** 001  
**Working Field:** Analytics  
**Status:** under construction  
**Date:** 11.08.2014

### Theoretical Background:

During preparation, a protein containing sample is mixed with SDS (sodium dodecyl sulfate), a strongly anionic detergent (Figure 1 A), combined with a reducing agent (here DTT, dithiothreitol). By these the sample preparation occurs under conditions that ensure dissociation of proteins. Per each gram of denatured protein up to 1.4 grams of SDS are bound. This results in an overlay of the protein own charge which leads to completely negative charged molecules (Figure 1 B). By this the electrophoresis only occurs via protein size.

![Figure 1: structure and function of SDS.](image)

Prepared samples are placed in the pockets of a double layered gel. Each gel consists of a stacking gel, containing Tris- HCl (pH 6.8) and responsible for protein focussing, and a separation or resolving gel, containing Tris- HCl (pH 8.8) and responsible for separation by protein size. All system components contain 0.1 % of SDS.

The samples and the stacking gel also contain chloride ions forming the leading edge and glycerol molecules forming the trailing edge of the moving boundary. In combination with the high porosity of the stacking gel the denatured proteins are enriched in a very thin zone on the surface of the resolving gel.

Per the higher pH the glycerol molecules of the trailing edge are ionised and the so formed glycerol ions migrate through the stacked proteins and now directly following the leading chloride ions. Independent from the ions the SDS- loaded proteins are separated by size due to the molecular sieve effect.

The determination of protein bands can be done by staining with coomassie staining solution. Here the dye is bound to cationic and non-polar, hydrophobic side chains of the proteins after washing with de-ionized water. After de-staining to eliminate the background the proteins are visible as deep blue bands. Also, a determination using silver staining is possible (see extra protocol). [1; 3- 5]

### Material & Preparation:

**Loading Buffer (2x):**

1. DTT (stored at -20 °C)  
2. loading buffer

**5% Stacking Gel (1 ml):**

1. 0.68 ml H₂O  
2. 0.17 ml Rotiphorese® Gel 30 (in the fridge)  
3. 0.13 ml 1 M Tris/HCl; pH 6.8  
4. 0.01 ml 10% SDS  
5. 0.01 ml 10% APS (needs to be prepared fresh by addition of 1 ml H₂O)  
6. 0.002 ml TEMED (in the fridge)
### 12% Separation Gel (5 ml):

1. 1.6 ml H₂O  
2. 2.0 ml Rotiphorese® Gel 30 (in the fridge)  
3. 1.3 ml 1.5 M Tris/HCl; ph 8.8  
4. 0.05 ml 10% SDS  
5. 0.05 ml 10% APS (needs to be prepared fresh, see stacking gel)  
6. 0.002 ml TEMED (in the fridge)

### 1x Running Buffer w/o Urea:

1. 10 ml SDS  
2. 100 ml 10x Tris/ Glycin  
3. fill up to 1l with H₂O

### Comassie Staining Solution:

1. dissolve 60 – 80 mg of G250 Comassie Brilliant Blue in 1 l bidest H₂O  
2. stirring for 2 – 3 h  
3. add HCl until a concentration of 35 mM is reached (about 3 ml/l)

*if not mentioned otherwise all buffers are stored in the closet below the SDS-PAGE machines*

### Process:

#### Sample preparation:

1. mix 10 µl sample with 10 µl loading buffer (2x)  
2. incubate 5 min at 95 °C  
3. store samples overnight in the fridge or freeze at -20 °C  
4. after longer storage repeat step (2) before use

#### Preparation of electrophoresis chamber:

1. cleaning of all parts of the electrophoresis chamber with de- ionized water  
2. build together all parts (see Figure 2.1- 2.2)  
3. fill with 5 ml de- ionized water for leak testing (remove afterwards by decantation)
Preparation of 12% separation gel:

1. for each gel 5 ml solution are necessary (recipe see above)
2. mix all components except of TEMED and APS
3. add needed volume of TEMED and APS; working under the hood!!!
4. fill 5 ml in each chamber, mark the upper edge with a small dot on the glass (Figure 2.3)
5. cover each gel with 1 ml de-ionized water
6. wait 10 to 15 min for complete polymerization

Preparation of 5% stacking gel:

1. for each gel 1.5 ml solution are necessary (recipe see above)
2. mix all components except of TEMED and APS
3. removal of de-ionized water from separation gel surface by decantation or soaking with tissue paper
4. add of TEMED and APS, working under the hood!!!
5. fill 1.5 ml in each chamber (Figure 2.3)
6. stacking of combs of preferred size
7. wait up to 15 min for polymerization, mark the pockets with small lines on the glass
8. places gel carrier in tank (Figure 2.4)

Addition of samples and running electrophoresis:

1. add 10 µl prepared sample or 7.5 µl protein- marker using specific loading tips (also stored in the closet)
2. add 10 µl loading buffer (2x) in each unused pocket
3. run the gel for 20-30 min at 64 V (until the surface of the separation gel is reached)
4. switch to 120 V until the leading edge of the loading buffer reaches the lower edge of the separation gel (takes about 1.5 h)
Staining with coomassie solution:

1. wash the gels with 30-40 ml de-ionized H₂O for 5 min
2. place the gel for 10 s in the microwave; 600 W
3. repeat the washing twice
4. addition of 30-40 ml staining solution
5. place the gel for 10 s in the microwave; 600 W
6. Incubation for 2 to 4 h with agitation (also overnight possible)
7. wash with 30-40 ml de-ionized H₂O for 5 min
8. repeat washing twice
9. place a small folded piece of paper in one corner and de-stain for 2-4 h (de-stain until the background is colorless)
10. place gels between 2 overhead transparencies and scan (pdf and jpeg possible), send via email

Reagent-Overview:

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Ordering information</th>
<th>CAS-Number</th>
<th>Safety instructions</th>
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</thead>
<tbody>
<tr>
<td>DTT</td>
<td>ROTH</td>
<td>6908.x</td>
<td>3483-12-3</td>
<td>irritating to skin, eyes and respiratory system, harmful to health</td>
</tr>
<tr>
<td>Rotiphorese® Gel 30</td>
<td>ROTH</td>
<td>3029.x</td>
<td>—</td>
<td>contains acryl amide ➔ harmful to health, risk of cancer and genetic mutations, only working with coat and protective cloves</td>
</tr>
<tr>
<td>SDS</td>
<td>ROTH</td>
<td>0183.x</td>
<td>151-21-3</td>
<td>flammable, harmful to eyes and respiratory system</td>
</tr>
<tr>
<td>APS</td>
<td>ROTH</td>
<td>9592.x</td>
<td>7727-54-0</td>
<td>flammable, harmful to eyes and respiratory system; working under the hood is recommended, only working with coat and protective cloves</td>
</tr>
<tr>
<td>TEMED</td>
<td>ROTH</td>
<td>2367.x</td>
<td>110-18-9</td>
<td>flammable, causes serve corrosion to skin, harmful to eyes and respiratory system; working under the hood is recommended; only working with coat and protective cloves</td>
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</tbody>
</table>

Literature:

[3] Lottspeich
[4] Seifert
[5] Taschenatlas Biochemie
9.5. SOP 5: Analysis of sugars, alcohols and acids by HPLC-RID

Number: 120  Version: 0.8  Last Modified: Sun Aug 05 15:02:17 EEST 2012  Status: Validation  Author: Stosch, Henry

Name: Sugars_alcohol_acids_HPLC-RID

Description: The method describes the analysis of sugars, alcohols and acids by HPLC-RID from supernatant.

Related SOPs: Categories: laboratory

Keywords: HPLC, acid, alcohol, RID, sugar

Comments: 0 SOPs can be improved by posting constructive comments.

1. Chemicals, reagents and consumable parts

1.1 For eluents

- Ultrapurified water out of purification device in lab 265c
- H2SO4 95-98% Rotipuran® (Carl Roth GmbH), #X944.1

1.2 For sample preparation

- 0.2 µm Nylon filters(CarlRoth GmbH), #5822.1
- alternatively, regenerated cellulose filters also work well (Carl Roth GmbH), #5992.1

1.3 Standards

- D(+) glucose anhydrous (Carl Roth GmbH), #HN06.1
- Formic acid Rotipuran®pur. >99.8% (Carl Roth GmbH), #4724.3
- Acetic acid (Merck KgaA), #1.00063.1000
- Ethanol pur.>99.8% (Carl Roth GmbH), #5054.1
- Pyruvic acid>98% (Carl Roth GmbH), #8788.1
- Lactic acid (Merck KgaA), #366.0500
1.4 Sample vials at HPLC

- White glass vials for samples (Carl Roth GmbH), #159.1
- Septum (Carl Roth GmbH), #164.1
- Caps (Carl Roth GmbH), #161.1
- Inlets 100 µl for 1.5 ml vials (CarlRoth GmbH), #C516.1

2. Equipment

HPLC System: AGILENT TECHNOLOGIES 1200 SERIES (Agilent Technologies, Waldbronn, Germany)

(Contact for utilization: SJ, JG)

Column: HyperRez XP Carbohydrate H+, 300x7.7mm, 8µm

(Fisher Scientific Inc.), # 69008-307780, batch no. 026/H/012, column no. 190

3. Sample preparation for HPLC analysis of the supernatant

1. Centrifuge 1.5 mL culture broth for 10 min at 9.000 x g.

2. Transfer 1 mL of cell-free supernatant of the top phase into a new Eppendorf and store at -20°C until analysis.

   (Hint: at feed-cultures, samples need to be filtered directly for fast removal of active cells (step 3))

3. Filter samples using an 0.2 µm Nylon filter (alternatively use regenerated cellulose filter).

   (Hint: If this step was done already directly after sampling, you might have to repeat it, when samples were stored several weeks.)
4. Transfer all supernatant into a lc vial. If you have not enough sample volume to completely fill the vial, use inlets to prevent evaporation.

4. Analysis of the compartment of the supernatant with HLPC-RID

4.1 Methods and settings

The method is stored in the file ANALYSIS.M in the directory C:\methods.

(Hint: For dirty samples, it is recommended to apply a sample of ultra-purified water at every fourth run. Also, the method PURGE.M (solely 20 min post time) can be included to clean the column.)

Set up pump

Control
Flow 0.5 [mL/min]
Stop Time 65.00 [min]
Post Time Off

Solvent A 100 % 0.005 M H₂SO₄

Pressure limit
Max 60 [bar]
Min 0 [bar]
Set up injector

Injection
Injection with Needle Wash
Injection Volume 20.0 [µL]
Wash Vial 1
Optimization 1. none

Auxiliary
Draw Speed 200 [µL/min]
Eject Speed 200 [µL/min]
Draw Position 0.3 [mm]

Time:
Stop time 65.00 [min] as Pump
Post time Off

Column thermostat method

Temperature 15 [°C]

Time
Stop Time 65.00 [min] as Pump
Post Time Off
Temperature (right)
Temperature (right) Same as left

Store
Temperature left

Enable analysis
With any temperature

**RIDSignals**

Optical unit temperature 20.0 °C
Polarity Positive
Automatic recycling after analysis Off

**Time**
Stop Time 65.00 [min] as Pump
Post Time Off

Peak width: (Response Ttime) >0.2 min (4s standard)

**Analog output**
Zero offset 5%
Attenuation $500 \times 10^3$ [nRIU]

Automatic zero before analysis: On
Automatic purge

Purge Time 1.00 [min]

Wait Time 1.00 [min]

**Signal details**

Available signals RID1 A

**4.2 Calibrations**

Calibrations are stored in the directory:

D:\Dokumente und Einstellungen\AllUsers\RID\Calibrations

(Hint: Samples should be stored in the same subdirectory in the folder ‘Samples’.)

Attention: Always store the column in 0.005 M H2SO4. Flush the column prior to analysis until a stable baseline is received. This can take some hours.

© Sun Aug 05 15:02:17 EEST 2012 Bioprocess Technology Technische Universitat Berlin, Department of Biotechnology

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SOPAS 0.4.2, June 1, 2013

© 2012 - 2013 Triacle Biocomputing
9.6. SOP 6: Thioflavin-S staining of inclusion bodies containing *E. coli* cells

**Title:**
Thioflavin-S staining of recombinant *Escherichia coli* cells

<table>
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<th>Facilities:</th>
<th>SOP- Number:</th>
<th>Working Field:</th>
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<td>1.) Plate Reader</td>
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**Theoretical Background:** (~ 10 sentences)
Many heterologous proteins expressed in *E. coli* cells cannot achieve their native form and activity due to limited posttranslational modification capacities. These proteins in their mis-folded state will form protein and peptide agglomerations called bacterial inclusion bodies. In recent studies, it was shown that inclusion bodies in bacterial cells share structural and mechanical features of amyloid plaques, which are linked to several human disorders like Alzheimer’s or type-II diabetes. This discovery turned inclusion bodies formation in bacterial cells into a valuable tool to characterize amyloid aggregation.

![Figure 1: Thioflavin S is a homogenous mixture of compounds that results from the methylation of dehydrothiotoluidine with sulfonic acid (Sharp et al. 2009).](image)

Thioflavin-S (Figure 1) is a fluorescent dye able to penetrate bacterial cell membranes without harming the cells and to bind to amyloid plaques and bacterial inclusion bodies without influencing their formation. In addition, its wave length spectrum and fluorescence intensity change if bound to inclusion bodies. Cells prepared using this protocol can further be analyzed with flow-cytometry for population studies.

**Material & Preparation:**

**PBS:**
- One Liter buffer consists of:
  - 8.0 g NaCl, 0.2 g KCl,
  - 1.42 g Na$_2$HPO$_4$ or 1.78 g Na$_2$HPO$_4$ x 2 H$_2$O
  - 0.27 g KH$_2$PO$_4$

**Thioflavin-S solution:**
- 0.05 g Thioflavin-S (Sigma-Aldrich T1892)
- 87.5 mL VE-water
- 12.5 mL Ethanol (96 %)

**UV-Multi-Well plates:**
- UV-Star® 96-Well Microplates, Greiner Bio-One

**UV/VIS spectrophotometer:**
- Synergy™ Mx Microplate Reader, BioTek Instruments
**Process:**

**Sample preparation:**

1. Measure $OD_{600}$ of your sample. (Caution: Only fresh samples are suitable for this assay!)
2. Dilute your sample to $OD_{600} 1.0$ in $1 \text{ mL PBS}$.  
3. Centrifuge (5000 g, 4 min) and wash the cell pellet twice in PBS. 
4. Resuspend the cell pellet in $500 \mu L$ Thioflavin-S solution. 
5. Incubate for 15 min at room temperature. 
6. Centrifuge (5000 g, 4 min) and wash the cell pellet the times in PBS. 
7. Resuspend the cell pellet in $1 \text{ mL PBS solution}$. 
8. Transfer $4 \times 200 \mu L$ into a UV-Star® 96-Well Microplate. 
9. Transfer also $4 \times 200 \mu L$ PBS and Thioflavin-S as control substances. 

**Preparation of the Synergy™ Mx Microplate Reader:**

1. Turn on the “Hamilton” marked PC and the Microplate Reader (mind the external power supply!). 
2. At the PC log into the account “Bioverfahrenstechnik” (password: Hamilton). 
3. Open the “Gen5” software – All following steps are based on software version 1.09! 
4. Click on “New experiment”. 
5. Choose “ThioflavinS+OD.prt” as experiment protocol. 
6. After finishing all internal tests, the Microplate Reader opens its tray.

**Measurement of $OD_{600}$ and Fluorescence:**

1. Set your plate on the tray of the Microplate Reader (Caution: Mind the correct orientation!). 
2. Press “Read” in the Gen5 software. 
3. Optional: Enter further identification parameters for later data analysis. 
4. Define a storage place and name for your experiment. 
5. Confirm the temperature inside the Microplate Reader with “OK”. 
6. $OD_{600}$ and Fluorescence (Excitation: 375 nm / Emission: 455 nm) of your samples are now detected. 
7. Both values can be checked in the plate matrix in the Gen5 software.
Transfer of results into Microsoft Excel:

(9) Select “OD600” as parameter in the plate matrix window.

(10) Press on the “Export to Excel” button to transfer your data into a new Excel book.

(11) Repeat this step with “Fluorescence” as selected parameter.

(12) Press again on the “Export to Excel” button to transfer your data into the Excel book.

(13) Divide the detected fluorescence by the measured OD600 to normalize your signals.

(14) Save your Excel work book.

(15) Shut down the PC, close the tray of the Microplate Reader, switch off the Reader and its external power supply.

Reagent-Overview:

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<th>Company</th>
<th>Ordering information</th>
<th>CAS-Number</th>
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<td>VWR</td>
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<td>10028-24-7</td>
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<tr>
<td>Thioflavin-S</td>
<td>Sigma Aldrich</td>
<td>T1892-x</td>
<td>1326-12-1</td>
<td>Causes skin irritation, causes serious eye irritation, may cause respiratory irritation</td>
</tr>
<tr>
<td>Ethanol 96% HiPerSolv</td>
<td>CHROMANORM</td>
<td>20825.x</td>
<td>64-17-5</td>
<td>Flammable</td>
</tr>
</tbody>
</table>

Literature:

Curriculum Vitae

Christian Reitz (Male)

Place of Birth: Berlin, Germany

Education:

10/2012 – 02/2017 Technische Universität Berlin (Berlin, Germany)
Doctoral student and scientific coworker

10/2004 – 03/2012 Technische Universität Berlin (Berlin, Germany)
Graduate Engineer (Dipl.-Ing.) in Bioprocess and Genetic engineering

08/1996 – 06/2003 Diesterweg Gymnasium (Berlin, Germany)
Abitur

Presentations (10/2012 – 02/2017):

8th Conference on Recombinant Protein Production in Palma, Mallorca, Spain (April 24th 2015)
“Simulating large scale conditions in a scale-down bioreactor: Impacts on cell physiology and product quality of recombinant Escherichia coli”.

“Simulating large scale conditions in a scale-down bioreactor: Impacts on the cell physiology of recombinant Escherichia coli”.

3rd BioProScale Symposium 2014 at the TU Berlin – in Berlin, Germany (April 3rd 2014)
“Simulating large scale conditions in a scale-down bioreactor: Impacts on the cell physiology of Escherichia coli”.

Sanofi Thesis Days 2013 in Saint-Raphaël, France (April 12th 2013)
“Zoning phenomena in large bioreactors”.

Publications (10/2012 – 02/2017):


**Poster presentations (10/2012 – 02/2017):**

**European Symposium on Biochemical Engineering Sciences (ESBES) 2016** – in Dublin, Ireland (September 11th – 14th)
“Industrial-scale Conditions can affect the quality of heterologous proteins in *Escherichia coli*: Lessons from scale-down bioreactors”.

**Tag der Biotechnologie** – in Berlin, Germany (July 14th 2016)
“Simulating large scale conditions in a scale-down bioreactor: Impacts on cell physiology and product quality of recombinant *Escherichia coli*”.

**4th BioProScale Symposium 2016** – in Berlin, Germany (April 6th – 8th 2016)
“Simulating large scale conditions in a scale-down bioreactor: Impacts on the cell physiology of *Escherichia coli*”.

**4th BioProScale Symposium 2016** – in Berlin, Germany (April 6th – 8th 2016)
“Modelling dissolved oxygen and glucose gradients in pulse-based fed-batch culture of *Escherichia coli*”.

**Tag der Biotechnologie** – in Berlin, Germany (July 16th 2015)
“Cellular responses in large-scale fed-batch bioprocess: Effects of substrate oscillation on the synthesis of interleukin2 in *Escherichia coli*”.

**DECHEMA Himmelfahrtstagung 2015** – in Hamburg, Germany (May 11th – 13th 2015)
“Simulating large scale conditions in a scale-down bioreactor: Impacts on cell physiology and product quality of recombinant *Escherichia coli*”.

**8th Conference on Recombinant Protein Production – RPP8** – in Palma, Mallorca, Spain (April 22nd – 24th 2015)
“Automated development of recombinant bioprocesses – combination of QbD and Model based DoE”.

**European Congress on Biotechnology - ECB16** – in Edinburgh, Scotland (July 13th – 16th 2014)
“Microorganisms respond in different ways to oscillations in large-scale bioreactors: Conclusions from Scale-down approaches”.

**7th Conference on Recombinant Protein Production – RPP7** – in Laupheim, Germany (March 06th – 08th 2013)
“Evaluation of Promoters for Enhanced Recombinant Protein Expression in *Bacillus halodurans*”.

**Supervised students (10/2012 – 02/2017):**

**Franziska Vera Ebert**
Master Thesis: “Response of recombinant *E. coli* exposed to oscillating cultivation conditions in a novel shake-flask based bioreactor”

**Qin Fan**
Master Thesis: “Impact of nutrient oscillations simulated in a two-compartment scale-down reactor system on the metabolic response of recombinant *Escherichia coli* and production of non-canonical amino acids”

**Ongey Elvis Legala**
Master Thesis: “Incorporation of Non-canonical Amino Acids into Recombinant Human Proteins Heterologously Expressed in *E. coli* by Bioprocess Perturbations”

**Franziska Vera Ebert**
Bachelor Thesis: “Impact of oscillating glucose-feeding on the production of non-canonical amino-acids in recombinant *Escherichia coli*”

**Houda Kalot**
Bachelor Thesis: “At-line monitoring of inclusion bodies formation in recombinant *E. coli* cultivations using the fluorescent dye Thioflavin-S”