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Jaakko Soini

EFFECTS OF ENVIRONMENTAL VARIATIONS IN *ESCHERICHIA COLI* FERMENTATIONS

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Abstract

Production of recombinant proteins and organic molecules by microbial fermentation is a widely used process in pharmaceutical, biofuel, food and chemical industries. Micro-organisms are cultivated in a bioreactor enabling a proper environment and conditions for production of the desired product molecule. Usually, however, the conditions in the bioreactor are inhomogeneous because of technical limitations such as insufficient mixing. This causes gradients in various parameters such as temperature, ph, dissolved oxygen or substrate concentration, which can have a negative effect on product quality and quantity. Understanding the effects of such environmental variations for the host organism and product molecule are crucial for the bioprocess control.

The aim of this dissertation was to study the metabolic and gene expression level response of *Escherichia coli* bacterium for variating conditions in the bioreactor. The responses for rapid temperature increase and oxygen limitation was studied by shift experiments. The effect of oscillating oxygen and glucose concentrations, typical for industrial scale processes, was studied with a scale-down model.

The main results were obtained in the oxygen downshift experiments. It was shown that a non-canonical amino acid norvaline, known to replace leucine in recombinant proteins, is accumulated in significant concentration under oxygen limitation. The accumulation of norvaline was also observed in the scale-down model indicating that norvaline could also be found in large scale processes. The quantitative gene expression results for the norvaline pathway genes showed no clear response. This indicates that the norvaline formation occurs due to the changes on metabolic rather than transcriptional level. The second key result of this dissertation was the finding, that the accumulation of formate, a typical anaerobic metabolite, was diminished by medium boosted with trace amounts nickel, selenium and molybdenum, enabling the activity of formate degrading enzyme complex. The results of this dissertation can be utilized in the industrial process optimisation and as a basis for further bioprocess studies.

Keywords: Escherichia coli, fermentation, formate, metabolism, norvaline, oxygen limitation, scale-down model

Soini, Jaakko, Ympäristöolosuhteiden muutosten vaikutukset *Escherichia coli* fermentointiprosessissa.
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Tiivistelmä

Vierasproteiinien ja orgaanisten molekyylien tuottaminen fermentoimalla on paljon käytetty menetelmä lääke-, bioenergia-, elintarvike- sekä kemianteollisuudessa. Mikrobit kasvatetaan bioreaktorissa, joka mahdollistaa sopivan kasvuympäristön ja tuotanto-olosuhteet halutulle tuotteelle. Usein bioreaktorin olosuhteet ovat kuitenkin epätasaiset teknisten rajoitteiden kuten riittämättömän sekoitustehon vuoksi. Tämä aiheuttaa eri muuttujien, kuten happi- ja ravinnepitoisuuden, pH:n tai lämpötilan vaihtelua ajan ja paikan suhteen, millä voi olla haitallinen vaikutus tuotteen laatuun tai saantoon. Ympäristötekijöiden muutosten isäntäsolulle tai tuotemolekyylille aiheuttamien vaikutusten ymmärtäminen ovat yksi ratkaisevista tekijöistä bioprosessien hallinnassa.

Tässä väitöskirjassa tutkittiin *Escherichia coli* -bakteerin aineenvaihdunnan sekä geeniekspression vasteita bioreaktorin ajan ja paikan suhteen vaihtelevissa olosuhteissa. Hapenpuutteen ja lämpötilan nousun vaikutusta tutkittiin kokeilla, joissa olosuhdetta kertaluontoisesti ja nopeasti muutettiin. Teollisille fermentointiprosesseille tyypillistä happi- ja ravinnepitoisuuksien jatkuvaa vaihtelua tutkittiin suurta bioreaktoria jäljittelevällä pienoismallilla.

Tärkeimmät tulokset liittyivät kokeisiin, joissa tutkittiin hapenpuutetta. Kokeissa kävi ilmi, että happirajoitetuissa olosuhteissa muodostuu huomattavia määriä epätavallista aminohappoa norvaliinia, jonka tiedetään korvaavan leusiinia vierasproteiineissa. Norvaliinin muodostumista havaittiin myös pienoismallilla tehdyissä kasvatuksissa, osoittaen että norvaliinia voi mahdollisesti löytyä myös suuren mitan prosesseista. Geeniekspressiomittaukset eivät osoittaneet muutoksia norvaliinin aineenvaihduntareitillä, osoittaen että havaittu norvaliinin muodostuminen tapahtuu aineenvaihdunnallisten muutosten seurauksena. Toinen tässä väitöstutkimuksessa saatu tärkeä tulos oli muurahaishapon kertymisen vähentyminen, kun kasvatusliuokseen lisättiin nikkeliä, seleeniä ja molybdeeniä aktivoimaan muurahaishappoa hajottavaa entsyymikompleksia. Tämän väitöstutkimuksen tuloksia voidaan hyödyntää teollisten prosessien optimoinnissa ja perustana uusille tutkimusaiheille.

Asiasanat: aineenvaihdunta, Escherichia coli, fermentointi, happipitoisuus, muurahaishappo, norvaliini, pienoismalli

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Oulu, September 8th, 2012 Jaakko Soini

Abbreviations

AA	amino acid
AAA	amino acid analysis
AHAS	acetohydroxy acid synthase
AMP	adenosine monophosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCAA	branched chain amino acid
CDW	cell dry weight
DNA	deoxyribonucleic acid
DOT	dissolved oxygen tension
$FAD/FADH_2$	flavin adenine dinucleotide
FDH	formate dehydrogenase
FHL	formate hydrogen lyase
FNR	fumarate and nitrate reduction
GTP	guanosine-5'-triphosphate
HPLC	high performance liquid chromatography
ILV	isoleucine-leucine-valine
mM	millimolar
mV	millivolt
$NAD^+/NADH$	nicotinamide adenine dinucleotide
NVA	Norvaline
OD	optical density
OPA	*ortho*- phtalaldehyde
OTR	oxygen transfer rate
PCR	polymerase chain reaction
PEP:PTS	phosphoenolpyruvate phosphotransferase system
PFR	plug flow reactor
(m)RNA	(messenger) ribonucleic acid
RQ	research question
SHA	sandwich hybridisation assay
STR	stirred tank reactor
TCA	tricarboxylic acid cycle
2CR	two-compartment reactor

List of original publications

This dissertation is based on the following publications:

I Soini J, Falschlehner C, Liedert C, Bernhardt J, Vuoristo J & Neubauer P (2008) Norvaline is accumulated after a down-shift of oxygen in *Escherichia coli* W3110. Microb Cell Fact 7:30.

II Soini J, Ukkonen K & Neubauer P (2008) High cell density media for *Escherichia coli* are generally designed for aerobic cultivations – consequences for large-scale bioprocesses and shake flask cultures. Microb Cell Fact 7:26.

III Soini J, Falschlehner C, Mayer C, Böhm D, Weinel S, Panula J, Vasala A & Neubauer P (2005) Transient increase of ATP as a response to temperature up-shift in *Escherichia coli*. Microb Cell Fact 4:1.

IV Soini J, Ukkonen K & Neubauer P (2011) Accumulation of amino acids deriving from pyruvate in *Escherichia coli* W3110 during fed-batch cultivation in a two-compartment scale-down bioreactor. Advances in Bioscience and Biotechnology 2: 336–339.

The author's contribution in original publications:

In articles I, II and IV the author was responsible for planning and performing the experiments. In article III the author was responsible for planning and performing the experiments for mRNA part of the work. All the manuscripts were drafted in co-operation with the author and co-authors.

Contents

1 Introduction

1.1 Background

Cultivation of living micro-organisms is widely used technology for production of biomolecules with versatile properties, e.g. biochemicals (Yu *et al.* 2011, Kind & Wittmann 2011), biofuels (Zheng *et al.* 2009, Ali *et al.* 2012) and recombinant proteins (Schmidt 2004, Ni & Chen 2009). In traditional processes micro-organisms were applied to preserve milk and vegetable products, baking and brewing of beer and wine without the knowledge of scientific mechanism behind the phenomena. Industrial microbiology evolved in the early 1900´s with the processes producing organic acids, solvents, vitamins and other chemicals and was followed in the middle of the century by the first antibiotic production process (Pelaez 2006). Antibiotic production also introduced completely new era of engineering science, namely biochemical or bioprocess engineering, by desire of more efficient design, acepticity and scale-up of the processes (Enfors & Häggström 2000). The progress in genetics and other biological sciences and the revolutionary recombinant DNA technology paved the way for modern biotechnology and brought in totally new insights for the biotechnical industry (Stulz *et al.* 2011).

However, the giant leaps in technologies available for the biotechnology industry have also brought up new challenges. The complex nature of host organisms and product molecules introduce a great challenge for optimisation and scale-up of bioprocesses (Enfors *et al.* 2001, Hewitt & Nienow 2007). Novel process analytical technologies on transcriptional (Jurgen *et al.* 2005, Rautio *et al.* 2007, Schweder 2011), proteomic (Aldor *et al.* 2005, Xia *et al.* 2008) or metabolomic (Schaub & Reuss 2008) levels provide an infinite source of information for better understanding of cultivation processes. The increasing amounts of biological multi-level data must be processed in such a form that it can be applied as engineering statements in order to improve the actual processes (Wang *et al.* 2009).

Escherichia coli is a facultative gram-negative bacterium naturally living in, for example mammalian intestine. It has been the model organism for biological research for decades because of its well-known biology, easiness to handle and short doubling time. It has also taken the position as one of the most used host organisms in bioprocess industry for the production of recombinant proteins

(Zerbs *et al.* 2009), biomolecules (Fan *et al.* 2012) and biofuels (Nawabi *et al.* 2011, Fan *et al.* 2012).

E. coli is cultivated in laboratories and in industrial fermentation processes throughout the world. Enormous amount of research and development is carried out to understand the effects of the different parameters on final product quality and quantity (Glassey *et al.* 2011). Virtually stable processes comprise vast amount of potential sources for variations such as the microbial strain, reactor conditions (Hewitt *et al.* 2000, Lara *et al.* 2006) and the medium composition (Qin *et al.* 2006), varying as a function of time and position. One of the most common challenges in *E.coli* high cell density cultivations is the availability of oxygen, when concentrated substrate is fed into the reactor accelerating the flux through glycolysis, and thus, increasing the oxygen uptake rate (Xu *et al.* 1999, Lin *et al.* 2001, Enfors *et al.* 2001). Temperature is also a key variable since it controls the rate of metabolism and causes a strong response to prevent the inactivation of enzymes (Arsene *et al.* 2000). Heat-shock research is practically significant e.g. in temperature inducible recombinant protein production systems (Hoffmann *et al.* 2002). Variations in environmental conditions can be studied for example by pulse experiments (Lara *et al.* 2009) or by allowing cells to move from one condition to another in a two –compartment system (Oosterhuis & Kossen 1983, George *et al.* 1993, Neubauer *et al.* 1995, Lara *et al.* 2006)

The response of the organism for varying conditions can be monitored at several levels. The first level is the understanding of the genomic information stored in the DNA sequence, which has been completely solved for *E.coli* K-12 (Blattner *et al.* 1997). The second level is the transcription, producing mRNA molecules, initiated by the environmental stimulus activating the expression of certain genes. Transcriptional monitoring gives rapid information for the evaluation of the process state (Jurgen *et al.* 2005, Gasser *et al.* 2007, Rautio *et al.* 2007, Pioch *et al.* 2008). Third, protein expression from mRNA molecules in translation produces the enzymes catalyzing the metabolic reactions (Gasser *et al.* 2007). The variations observed in genome, gene and protein expression levels can only partly explain the cellular level responses. Thus, metabolite level monitoring is required for a complete understanding of bioprocesses (Saito *et al.* 2010). The challenge in the metabolite analysis is that the genome is not applicable for target specification as is the case in proteomic and transcriptomic approaches. Also the lack of amplification option and the labile nature of many target molecules introduce problems in metabolite analysis (Hollywood *et al.* 2006). Transcriptional and metabolite levels were chosen as the target of this dissertation.

1.2 Objectives and scope of the research

This doctoral dissertation studies the response of *Escherichia coli W3110* to various practically significant changes in cultivation conditions. The aim of this study is to provide new information for cultivations of different type and scale to manage and develop cultivation processes. *E. coli* W3110 wildtype strain was used in the experiments of this study to monitor and compare the metabolic and transcriptional responses on varying environmental stresses. The stress conditions were temperature upshift, oxygen downshift and oscillations in glucose and oxygen concentrations during cultivation in a two-compartment scale-down bioreactor (2CR). The scale-down reactor mimics the conditions of large-scale fermentation processes characterised by insufficient mixing resulting in gradients of substrate concentrations. The specific focus areas of this dissertation are the formation of mixed-acid fermentation products, biosynthesis of canonical and non-canonical amino acids and process monitoring at mRNA level.

The above described research problem is divided into four research questions (RQ).

RQ1: How does the oxygen downshift affect the free amino acid pools in *E.coli* cultivations?

RQ2: How to use sandwich hybridisation assay as a tool for mRNA based monitoring of rapid changes in cultivation conditions?

RQ3: What is the response of *E.coli* to fluctuating conditions in a two-compartment scale-down reactor?

RQ4: How do the additional trace elements nickel, selenium and molybdenum affect the accumulation of mixed-acid fermentation products?

These research questions are addressed in four peer-reviewed scientific articles as indicated in Table 1.

Table 1. Answering research questions by scientific articles.

Research question	Article I	Article II	Article III	Article IV
RQ1	X			
RQ2	X		X	
RQ3		X		X
RQ4		X		

RQ1 is addressed in the Article I, which presents the changes in amino acid pools in *E.coli* W3110 cultivated under aerobic conditions with a sudden oxygen downshift. Oxygen downshift is also studied on transcriptional level with mRNA measurements. These results are also discussed under RQ2 together with mRNA measurements in connection to another environmental variation, the heat-shock studied in Article III. The RQ3 is addressed in Articles II and IV, where a two-compartment scale-down bioreactor is applied to mimic the oxygen and glucose concentration gradients occurring in large scale processes. Article II discusses RQ4, by providing a possible solution to avoid formate accumulation, a common problem in high cell density cultivations.

2 Materials & methods

2.1 *Escherichia coli* strain

The *Escherichia coli* K-12 strain W3110 [F^-, IN(*rrnD rrnE*)1, λ^-], kindly provided by the *E. coli* Stock Center (New Haven, USA) was used in Articles I-IV.

2.2 Methods

Batch cultivations in shake flasks were performed for the Article III. The growth was monitored by temperature and dissolved oxygen sensors with wireless data transfer by Senbit® technology (Vasala *et al.* 2006). Glucose limited fed-batch was used in Articles I-IV. A two-compartment scale-down reactor was used in Article II and IV to simulate the conditions occurring in large-scale fermentation. In each Article I-IV, the growth was monitored by spectrophotometric measurement of optical density at 500, 540 or 600nm and by gravimetric analysis of cell dry weight (CDW). The main features of cultivation types are listed in Table 2 and the sample preparation and analysis methods are listed in Table 3. More detailed procedures are available in the original publications.

Table 2. Main features in *E.coli* cultivation experiments in this research.

Cultivation type	Article(s)
Shake flask cultivation in defined mineral medium (M9) with oxygen and temperature monitoring with wireless data transfer by Senbit®	III
Glucose limited fed-batch fermentation in defined mineral salt medium (MSM)	I-IV
Two-compartment scale-down fermentations in defined medium (MSM)	II, IV

Table 3. Main sample preparation and analysis methods used in this research.

Analysis	Methods	Articles
mRNA sample preparation and quantification	Inactivation of the cells in pre-chilled (-20 °C) Ethanol/Phenol solution Total RNA separation by phenol/isopropanol extraction Computer-aided primer and probe design for PCR and sandwich hybridisation PCR for DNA fragments with T7 promoter sequence *In-vitro* transcription for standard mRNA production Detection probe 3´-end labelling by digoxigenin RNA quantification by fluorescent staining and detection Sandwich hybridisation assay for mRNA quantification	I, III
Amino acid analysis from crude cell extract	Inactivation of the cells by rapid introduction into liquid nitrogen Cell disruption by sonication OPA pre-column derivatisation of amino acids Separation of amino acids by reversed phase HPLC Quantification by fluorescence detection	I, IV
Intracellular amino acid analysis	Methanol inactivation and centrifugation to separate the cells Sample preparation and AA analysis as described above for crude cell extracts	IV
Glucose and metabolite analysis by HPLC	Cell removal by centrifugation/filtration Reversed phase HPLC for the separation of the molecules Refractometric detection for glucose and ethanol UV-Vis detection for metabolites	I, II, IV
Enzymatic glucose and acetate test	Cell removal by filtration Enzymatic analysis from the supernatant	III
Adenosine nucleotide analysis	Inactivation and cell disruption by acid/freeze-thaw treatment Analysis of adenosine nucleotides by reversed-phase HPLC	III

3 Literature review

3.1 Background

Escherichia coli has been the model organism for biological research for decades because of its well-known biology, easiness to handle and short doubling time and has also taken the position as one of the most used host organism in the bioprocess industry. It also has ability to express foreign proteins of up to 20% of the total protein content. As a drawback *E.coli* lacks the machinery for post-translational modifications and efficient secretion and therefore in recombinant protein production the product is often harvested as the protein aggregates, inclusion bodies (Schmidt 2004). *Escherichia coli* bacterium was founded by Theodor Escherich in 1800´s and named as it is know today in 1919 in a revision of bacteorogical nomenclature. There are versatile strains of *E.coli* species existing including pathogens causing diarrhoeal diseases, urinary tract infections and sepsis (Kaper *et al.* 2004). The most used strains for biotechnological applications are derivates from *E.coli* K-12 (e.g. W3110 strain) and B families (e.g. BL21)

3.2 Metabolic basis of *Escherichia coli* fermentation process

The cellular metabolism of *E. coli*, as any other host organism, comprises a complicated network of reactions and their regulation. Some of the reactions are more relevant for a fermentation process than the others and metabolic models describing a pathway always contain simplifications. The production of macromolecules, such as proteins, lipids, DNA and RNA, from carbon and nitrogen substrates comprises the key metabolic process for cell growth and production of polymeric products. Also an energy source if other than a carbon source, minerals, trace elements and vitamins are required for cell growth. In catabolism cellular energy, reducing power and precursor molecules are produced in enzymatically catalyzed reactions from the substrates present in the cultivation medium. Anabolic reactions produce the building blocks required for the biosynthesis of macromolecules and biomass. In order to optimize the fermentation process with efficiency and quality, it is of utmost importance to understand the relevant metabolic pathways and their regulation. (Enfors &

Häggström 2000) The metabolic network discussed in this dissertation is presented schematically in Figure 1.

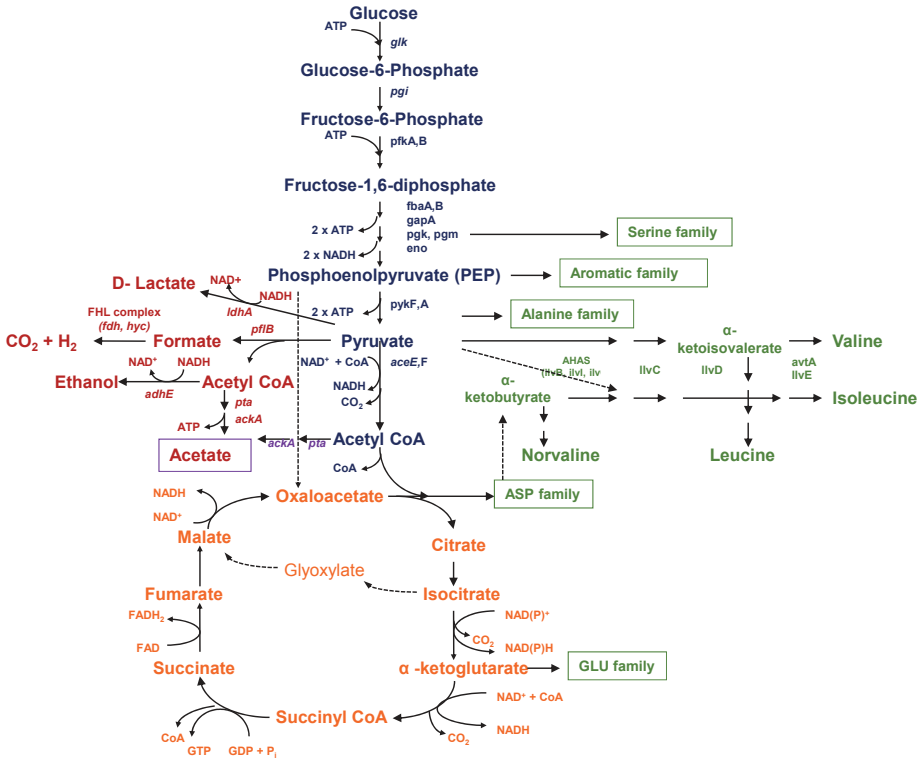

Fig. 1. Schematic presentation of metabolic pathways introduced and discussed in this dissertation work. The network is divided to glycolysis (blue), tricarboxylic acid (TCA) cycle (orange), mixed-acid fermentation (red) and amino acid biosynthesis (green).

3.2.1 Formation of energy and precursors in catabolism

The energy production of chemo-organotrophs (e.g. *E.coli*) occurs either by a respirative or fermentative pathway depending on the availability of and the ability to use external terminal electron acceptors. In respiration molecular oxygen is the terminal electron acceptor whereas in the fermentative metabolism a metabolic intermediate (e.g. pyruvate) or inorganic nitrate or sulphate is the electron acceptor and the energy is generated by a substrate-level phosphorylation.

The energy generated in catabolic reactions is stored in energy-rich intermediates, mostly adenosine triphosphate (ATP), which is dephosphorylated to adenosine di- and monophosphates (ADP and AMP) to release energy in energy demanding reactions. (Madigan *et al.* 2011)

The adenylate energy charge is a parameter representing the energetic status of a cell and it is calculated by equation 1 (Villadsen *et al.* 2011)

$$\text{Energy charge} = \frac{[ATP] + \frac{1}{2}[ADP]}{[ATP] + [ADP] + [AMP]}. \quad (1)$$

The glycolysis pathway from glucose to pyruvate is common for respiration and fermentative metabolism. Glucose is mainly taken up and phosphorylated via phosphoenolpyruvate phosphotransferase system (PEP:PTS) and cleaved and oxidised by a series of reactions to pyruvate (Frankel 1996). Glycolysis yields two moles of ATP by substrate-level phosphorylation and two moles of nicotinamide adenine nucleotide (NADH) when one mole of glucose is converted to two moles pyruvate. NADH is a redox carrier mediating the redox reactions. NADH is reduced in substrate-level oxidation from NAD^+ and oxidised back to NAD^+ by oxidative phosphorylation in respiration (Madigan *et al.* 2011). Under anaerobic conditions NADH is oxidised by substrate-level oxidation while pyruvate is reduced by mixed-acid fermentation (Enfors & Häggström 2000).

Pyruvate is a key metabolite and precursor of many metabolic pathways including citric acid cycle, overflow metabolism to acetate, mixed-acid fermentation and biosynthesis of many amino acids. In the citric acid cycle pyruvate is oxidized under aerobic conditions into carbon dioxide while the released electrons are transferred to NAD^+ and flavin adenine nucleotide (FAD) to form NADH and $FADH_2$. $FAD/FADH_2$ is an alternative redox carrier produced in citric acid cycle. This enables the production of ATP by oxidative phosphorylation. Citric acid cycle also produces many important pre-cursors (e.g. α-ketoglutarate and oxaloacetate) for anabolic reactions and guanosine triphosphate (GTP), an alternative energy carrier. (Madigan *et al.* 2011) The complete reaction of glucose completely oxidised to CO_2 in glycolysis and TCA cycle yields 12 redox cofactors 2 ATPs and 1 GTP (Villadsen *et al.* 2011). The overall reaction from glucose to carbon dioxide and redox and energy equivalents is illustrated in Equation 2

$$C_6H_{12}O_6 \longrightarrow 6CO_2 + 2NADH + (8\ NADH + 2\ FADH2) + 2\ ATP + GTP. \quad (2)$$

3.2.2 Overflow metabolism

A typical bottleneck in *E.coli* high cell density cultivations is the accumulation of acetate by overflow metabolism. (Luli & Strohl 1990, Shiloach *et al.* 1996, Xu *et al.* 1999) Acetate has negative effects on the process by inhibiting the growth in as low concentration as 0.5 g L^{-1} (Nakano *et al.* 1997) and reducing the product yields. Acetate is, in aerobic conditions, mainly produced by phosphotransacetylase (pta) and acetate kinase (ackA) pathway through acetyl-CoA (Dittrich *et al.* 2005, Valgepea *et al.* 2010). The overflow metabolism initiates when the citric acid cycle and respiration are unable to oxidize all the pyruvate coming from glycolysis (Hollywood & Doelle 1976). The growth rate of 0.14 h^{-1} was found sufficient to trigger acetate formation in fed-batch fermentation (Korz *et al.* 1995).

Overflow metabolism is typical for *E.coli* K strains, whereas it is low in *E.coli* B. It has been proposed that the higher re-assimilation rate of acetate by a higher activity of glyoxylate shunt and acetyl-CoA synthase in *E.coli* B strain explains the difference (van de Walle & Shiloach 1998) and this was further approved with a series of gene expression studies (Phue & Shiloach 2004, Phue *et al.* 2005, Phue & Shiloach 2005).

3.2.3 Mixed-acid fermentation

When external oxygen acceptors are not available (e.g. under anaerobic conditions) *E. coli* as an enteric bacteria shifts to so called mixed-acid fermentation. Mixed-acid fermentation produces oxidised fermentation products acetate, lactate, formate, ethanol, succinate and formate, which is further metabolized to H_2 and CO_2 by active formate hydrogen lyase complex (Enfors *et al.* 2001). NADH produced in glycolysis is oxidised to NAD^+, enabling the glycolysis pathway to continue. As illustrated in Fig. 1, all mixed-acid fermentation products are derived from pyruvate, except for succinate, which is produced from phosphoenolpyruvate via oxaloacetate (Xu *et al.* 1999).

D-Lactate is reduced from pyruvate by lactate dehydrogenase encoded by *ldhA*, jointly induced by acidic pH and anaerobic conditions. Acetyl-CoA derived from pyruvate can be used to produce ATP by conversion to acetate or NAD^+ by conversion to ethanol. Acetate is produced via phosphotransacetylase encoded by *pta* and acetyl kinase encoded by *ackA* (Lin & Iuchi 1991).

Pyruvate formate lyase encoded by *pflB* catalyzes the formation of formate and acetyl-CoA (Xu *et al.* 1999) through nonoxidative cleavage from pyruvate. Anaerobic conditions and presence of pyruvate are known to trigger *pflB* expression (Sirko *et al.* 1993). The disproportionation of formate to carbon dioxide and dihydrogen without exogenous electron acceptors is catalyzed by the formate hydrogen lyase (FHL) complex, consisting of formate dehydrogenase (FDH) and of six other proteins encoded by the *hyc* operon. FHL functions as a membrane-integrated electron transfer chain which finally releases dihydrogen by the HycE hydrogenase subunit (*hycE*). Three FDH isoenzymes exist in *E. coli*, all sharing the same mechanism for formate cleavage. All three isoenzymes of FDH are molybdo-seleno proteins and HycE is a NiFe hydrogenase. Therefore, a functional FHL complex is dependent on trace amounts of molybdenum, selenium, and nickel in the growth medium (Pinsent 1954b, Sawers 2005). *E.coli* is able to grow in anaerobic conditions without supplementation of carbon dioxide if the formate hydrogen lyase complex is functional and if the necessary metal ions of the complex, molybdenum, nickel and selenium are available (Hörnsten 1995).

The relative proportions of mixed-acid fermentation products is dependent on enzyme activities, depending eventually on environmental factors such as oxygen availability (Liu *et al.* 2011), substrate oxidation state (Alam & Clark 1989), pH (Clark 1989) and presence of redox agents (Levanon *et al.* 2005). The aim is to balance the number of reducing equivalents generated in glycolysis (Clark 1989).

The redox balance from glucose could be achieved by producing either lactate or acetate and ethanol in equal amounts. For less-reduced substrates acetate as a less-reduced product is preferred to keep the balance and for reduced substrates (e.g. sorbitol) ethanol (or succinate) would be preferred (Alam & Clark 1989). Lactate is the preferred product under acidic conditions (Mat-Jan *et al.* 1989).

3.2.4 Biosynthesis of amino acids

Amino acids are the building blocks for proteins and when they are not present in the cultivation medium, the organism must synthesise them from other substrates. The carbon skeletons for most amino acids derive from glycolysis and citric acid cycle intermediates and the nitrogen part is derived from an inorganic nitrogen source, such as ammonia.(Madigan *et al.* 2011) Amino acids can be classified based on the precursor molecule of their carbon skeleton.

Table 4. Amino acid families based on the carbon skeleton precursor.

Amino acid family	C-backbone precursor	Amino acids
Glutamate family	α-ketoglutarate	GLU, PRO, GLN, ARG
Aspartate family	Oxaloacetate	ASP, ASN, LYS, MET, THR, ILE
Alanine family	Pyruvate	ALA, VAL, LEU
Serine family	3- Phosphoglycerate	SER, GLY, CYS
Aromatic family	Phosphoenolpyruvate	PHE, TYR, TRP

The glutamate and aspartate families of amino acids are derived from citric acid cycle intermediates. Glutamate is produced from α-ketoglutarate. In the bacterial cultivations carried out in minimal medium with ammonium salt as a sole nitrogen source practically all nitrogen metabolism occur via glutamate and glutamine. Glutamate delivers the amino group for most of the amino acids and glutamine for histidine and tryptophan (Reitzer 1996).

Aspartate is produced from oxaloacetate with glutamate acting as the donor of the amino group. α-Ketoglutarate is produced as side product. Asparagine is produced from aspartate by transamination. Methionine, threonine and isoleucine pathways derive from aspartate through homoserine. Lysine pathway branches from the homoserine pathway (Patte 1996, Greene 1996). Exogenous methionine is a limiting factor in high-temperature cultivation (T > 45 °C) (Luders *et al.* 2009).

Alanine is produced by transamination of pyruvate. The biosynthesis of aspartate and alanine families is coupled by the joint pathway producing branched-chain amino acids valine, leucine and isoleucine deriving from pyruvate and α-ketobutyrate. The biosynthesis of isoleucine and valine occurs in parallel pathways, whereas the leucine pathway branches from α-ketoisovalerate, an intermediate of the valine pathway (Umbarger 1996). The first reaction of valine and isoleucine pathways is catalyzed by three isoenzymes of acetohydroxy acid synthase (AHAS) encoded by *ilvB*, *ilvG* and *ilvI*. The expression of the isoenzymes is regulated by the end-products derepressing the synthesis if any of the amino acids is lacking (Umbarger 1996). In *E.coli* K-12 strains AHAS II, encoded by *ilvG,* is inactive because of a frameshift mutation disturbing the regulatory system. This causes a phenomenon called valine toxicity allowing the presence of valine to block the pathway (Lawther *et al.* 1981, Andersen *et al.* 2001).

In addition to canonical amino acids, some non-canonical amino acids have been connected to the biosynthetic pathway of branched-chain amino acids. The

formation of norleucine (Barker & Bruton 1979, Tsai *et al.* 1988, Lu *et al.* 1988, Bogosian *et al.* 1989, Sycheva *et al.* 2007), norvaline (Apostol *et al.* 1997, Sycheva *et al.* 2007), β-methylnorleucine (Muramatsu *et al.* 2002a, Muramatsu *et al.* 2003) in *E.coli* have been reported in the literature. Norvaline has first been reported as a natural component in antifungal peptide produced by *Bacillus subtilis* (Nandi & Sen 1953). Later it was studied as a side product of isoleucine overproducing regulatory mutants of *Serratia marcescens* in a series of studies (Kisumi *et al.* 1972, Kisumi *et al.* 1976a, Kisumi *et al.* 1976b, Kisumi *et al.* 1977b, Kisumi *et al.* 1977c, Sugiura *et al.* 1981a, Sugiura *et al.* 1981b) The formation of modified branched chain amino acids received increasing attention as they appeared to be incorporated in certain recombinant proteins produced in *E. coli*, e.g. β-methylnorleucine into a recombinant hirudin (Muramatsu *et al.* 2002a, Muramatsu *et al.* 2002b, Muramatsu *et al.* 2003), norleucine into a recombinant human brain derived neurotrophic factor (Sunasara *et al.* 1999), interleukin 2 (Tsai *et al.* 1988, Lu *et al.* 1988, Fenton *et al.* 1994) and bovine somatotropin (Bogosian *et al.* 1989), and norvaline into a recombinant hemoglobin (Apostol *et al.* 1997).

3.2.5 Heat shock response

Heat shock response is a set of physiological changes of the cell to adapt into variations in the micro-environment such as temperature up-shift. It is induced by increased temperature but also by other environmental stresses. Heat shock response primarily affects protein synthesis and composition. There are approximately 20 genes which respond to temperature shift by transcriptional level regulation. Two sigma factors σ^{32} and σ^{24} are primarily responsible for guiding RNA polymerases to promoters of the heat shock genes (Gross C.A. 1996). Major heat shock proteins are chaperones and proteases, affecting protein folding and degradation. (Arsene *et al.* 2000)

The function of many heat shock proteins is connected to the hydrolysis of ATP, such as proteolysis by Lon, FtsH and Clp (Suzuki *et al.* 1997, Porankiewicz *et al.* 1999, Schumann 1999) and the action of the chaperone complexes GroEL/ES and DnaK/J/Grp (Braig 1998, Horwich *et al.* 1999). Although a number of *in vitro* studies have been performed revealing this ATP dependence (Sparrer *et al.* 1996) only limited information is available about the response of the *in vivo* ATP level after a temperature shift. Several authors (Findly *et al.* 1983, Lilly *et al.* 1984, Jones & Findly 1986) found in higher eukaryotes a fast decrease of the ATP level to less than 50% after a temperature up-shift parallel to the

induction of heat shock proteins. In *E. coli* the ATP concentration has been mainly followed in steady state conditions. For instance an ATP increase of several fold was reported in correlation with the specific growth rate (Gaal *et al.* 1997, Petersen & Moller 2000). Both ATP and the total adenylate concentration decreased after the cold shock experiment with a sudden downshift of the temperature (Rhodes *et al.* 1983).

IbpA and IbpB proteins are ATP independent proteins, encoded by a σ^{32} promoter regulated *ibp* operon. They are both small heat shock proteins and are known to recognize protein aggregates i.e. inclusion bodies in *E. coli* cells. These proteins act as chaperones and can suppress enzyme inactivation caused by heat shock by facilitating the preliminary step to protein refolding (Kuczynska-Wisnik *et al.* 2001). DnaK protein is a member of the hsp70 family and the *dnaK* gene is transcribed from the σ^{32} promoter. Together with two other heat shock proteins DnaJ and GrpE, it is involved in refolding proteins after damage caused by high temperatures. The DnaK-DnaJ-GrpE chaperone team is participating in the negative regulation of the σ^{32} -dependent heat shock response. This is achieved by binding dnaK to σ^{32} under non-heat shock conditions. By this function of DnaK as an anti-sigma factor, σ^{32} is not available to form σ^{32} -RNA polymerase complex. In the case of heat shock σ^{32} is immediately released. By this function DnaK has an important role in the autoregulation of the heat-shock response and can be used as an indicator for heat stress situations in cells. (Liberek & Georgopoulos 1993, Chattopadhyay & Roy 2002). The Lon protein, encoded by *lon* gene is an ATP-dependent cytoplasmic protease, which contributes to the removal of abnormal proteins from the cell. It is also regulated by the σ^{32} promoter. (Laskowska *et al.* 1996)

The physiological response of *E. coli* towards heat shock is more complex than just the synthesis of heat shock proteins, which includes transient growth decrease and changes in cell membranes all due to changes in the ratio of lipids to integral membrane proteins (Mejia *et al.* 1999). Early studies have shown that the maintenance energy requirement increases while the growth temperature increases (Harder & Veldkamp 1967, Topiwala & Sinclair 1971, Esener *et al.* 1983) and the yield coefficient $Y_{X/S}$ was decreased, possibly in connection to the higher protein turnover (Dawes & Ribbons 1964). Increased energy demand in temperature upshift experiments with recombinant *E. coli*, has also been explained by the extra burden caused by an increased synthesis rate of heat-shock and recombinant proteins (Hoffmann & Rinas 2001). A strong induction of acetate and formate production, together with up-regulation of glycolysis, citric acid cycle and ATP synthetase at proteomic level, was observed by the temperature upshift in a two-compartment

bioreactor setting (Luders *et al.* 2009). Acetate production was proposed to be caused by the up-regulation of *arcA* gene repressing the synthesis of citric acid cycle enzymes (Hasan & Shimizu 2008). Metabolic flux studies with a recombinant *E.coli* strain showed a rapid increase in respiration to respond to the increased energy demand, still resulting in a drop in energy charge (Hoffmann *et al.* 2002). A temperature upshift accelerates the glycolysis rate because of the increased energy demand. In *E.coli* cultivation a 20% increase in the glycolytic flux was observed together with a drop in the adenylate energy charge when the temperature was shifted from 30 to 42 °C (Wittmann *et al.* 2007).

The overproduction of heterologous proteins in *E.coli* often results in aggregation of product proteins and to a response similar to that of heat shock. (Laskowska *et al.* 2004) A heat shock response is also utilized as an induction technique for recombinant protein production (Hoffmann & Rinas 2000). In temperature up-shift fermentation without recombinant protein production or with the production of stable recombinant protein the heat-shock response is typically a transient change resulting in new steady-state conditions within 1 hour (Hoffmann & Rinas 2001).

3.3 Microbial fermentation processes

The basic principle in microbial fermentations is to produce high cell densities to ensure the high yield of the product (Fass *et al.* 1991, Seo *et al.* 2011, Liu *et al.* 2012) The high cell densities can be reached by considering the major bottlenecks, which are relatively well characterised for most common microbial host organisms such as *E. coli* (Shiloach & Fass 2005). A typical cell density level of batch cultivation in shake flask is approximately 1 g CDW L^{-1} (Enfors & Häggström 2000) whereas in an optimised fed-batch fermentation with a stirred tank reactor cell density up to 150 g CDW L^{-1} is reachable (Riesenberg & Guthke 1999). The estimated maximum level of cell density for *E. coli* is 200 g L^{-1} (Shiloach & Fass 2005) and densities close to that have been reported with special configurations such as dialysis reactors (Nakano *et al.* 1997).

3.3.1 Fermentation medium

The first prerequisite for high cell density is the supply of nutrients. The fermentation medium forms the chemical environment for the cultivation and is comprised of a mixture of substrates essential for the cell growth and formation of

the desired biosynthetic products. The macroelements carbon, oxygen, nitrogen, hydrogen and phosphate form 95% of the biomass weight. Microelements such as sulphur, potassium and magnesium are required at lower concentrations (< 1%). Trace elements are often activators of enzymatic reactions and they are required but only at very low concentrations. (Enfors & Häggström 2000) A complex medium contains undefined fractions, such as yeast extract or protein hydrolysates (Zhang & Greasham 1999) or currently highly interesting lignocellulosic feedstocks (Puligundla *et al.* 2011, Zhao *et al.* 2011, Fan *et al.* 2012). In a defined medium the exact chemical composition is known for improving the controllability and providing advantages in terms of up- and downstream processing, process scale-up and regulatory issues (Zhang & Greasham 1999).

Minimal medium is a defined medium with just a minimum amount of chemicals and it is commonly used for recombinant protein production in a lab-scale and also production scale fermentations (Enfors & Häggström 2000). Typical minimal medium for recombinant protein production fermentation contains mineral salts, trace elements and glucose as a carbon source and ammonia as a nitrogen source (Xu *et al.* 1999).

3.3.2 Cultivation modes

The main challenges in reaching the high cell densities in fermentations are the substrate toxicity in high concentrations (Fass *et al.* 1991) and production of inhibitory metabolites by the microbe (Xu *et al.* 1999, Enfors *et al.* 2001). To overcome these challenges fed-batch is the preferred operation mode for industrial fermentations (Sanden *et al.* 2003, Schmidt 2004, Liu *et al.* 2012). On the other hand batch cultivation in a shake flask is the dominating cultivation mode used in small scale experiments (Panula-Perälä *et al.* 2008).

Batch cultivation is the simplest cultivation mode with all carbon and energy sources and other nutrients, minerals and trace elements added from the beginning of the cultivation. After the initial lag-phase, caused by the adaptation of inoculated cells into the new environment, the cells grow with the maximum specific growth rate in the exponential phase if all essential nutrients are available. Oxygen, pH controlling agent and in some cases antifoam agent can be added into the bioreactor. When one of the substrates is consumed or one of the products reaches inhibitory level, the growth declines rapidly and the culture moves to the stationary phase. (Enfors & Häggström 2000)

When high cell densities are the aim, more optimized cultivation modes, such as fed-batch or dialysis fermentation are required (Shiloach & Fass 2005). The most common type of fed-batch process is the glucose-limited fed batch. The initial batch phase is followed by a controlled feeding of a concentrated glucose solution to control the growth rate and to avoid overflow metabolism. The glucose is the limiting substrate and other nutrients are added in the batch phase. The advantages of glucose-limited fed-batch mode are the possibilities to control the oxygen consumption and to avoid formation of inhibitory by-products such as acetate by limiting the glucose overflow (Xu *et al.* 1999, Lin *et al.* 2001, Shiloach & Fass 2005). That being said, most of the industrial bioprocesses are fed-batch processes (Bylund *et al.* 2000).

3.3.3 Process monitoring and control

The growth rate can be controlled by substrate feeding strategies based on many physical parameters such as dissolved oxygen tension (DOT) (Bocking *et al.* 1999), cooling power requirement (Schaepe *et al.* 2011), pH (Johnston *et al.* 2002) or CO_2 evolution rate (Taherzadeh *et al.* 2000). Also a pre-determined feeding profile based on the data from previous cultivations can be applied (Faulkner *et al.* 2006). Direct feedback control based on the measurement of a specific growth limiting product or substrate from the cultivation broth requires a reliable and sensitive sensor inside the bioreactor (Liu *et al.* 2001). By a constant feed rate, linear growth rate and declining specific growth rate can be achieved resulting in a quasi-linear increase in the biomass until increased maintenance rejects the growth (Bylund *et al.* 2000, Han L. 2002). In a certain phase of the cultivation, components required for growth such as vitamins, salts, amino acids or ammonia become limited and the growth declines. The addition of these compounds into the batch and fed-batch phases is restricted by the inhibitory effect of the concentration of e.g. salts (Enfors & Häggström 2000).

3.4 Stress in high-cell density fermentations

E. coli is a bacterium naturally living in heterogeneous conditions. In the course of evolution, it has created a mechanism to alter the metabolism rapidly when environmental pressure requires. Typical parameters prone for variation in *E.coli* cultivation processes are temperature, pH, dissolved oxygen and substrate availability. There is an increased desire for a better understanding of the consequences of heterogeneous environment in bioprocesses.

3.4.1 Oxygen solubility in aerobic fermentation

Most microbial processes are run under aerobic conditions with continuous oxygen supply into the fermentation medium (Enfors & Häggström 2000). The oxygen is first transferred from the gas phase to a liquid phase and subsequently to the site of oxidative phosphorylation in the cell. The limiting step is the oxygen solubility into the liquid, because oxygen is only dissolved in limited amounts in aqueous solutions (Enfors *et al.* 2001). A simplified two-film model (Eq. 3) considering only the gas-liquid interface mass transfer is often used for oxygen transfer rate (OTR) calculations (Garcia-Ochoa & Gomez 2009).

$$OTR = K_L a(C^* - C) \quad (3)$$

Since the mass transfer coefficient (K_L) and specific air bubble area per volume (a) are difficult to analyse separately the combined parameter $K_L a$ is used to characterise the oxygen transfer properties of a bioreactor. The OTR depends also on concentration difference between the gas-liquid interface (C*) and bulk liquid outside the interface (C) (Enfors & Häggström 2000). The solubility of oxygen (C*), is derived from Henrys Law and it is dependent on the temperature and the composition of the solvent. Typical saturation concentration oxygen in aqueous solution in 37 °C is 7 mg L^{-1} (Villadsen *et al.* 2011). In fermentation the dissolved medium components (e.g. salts) decrease the solubility from theoretical value (Garcia-Ochoa & Gomez 2009). The bulk liquid oxygen concentration is often measured online as partial pressure of oxygen pO_2, which in aerobic cultivations is closely connected to the consumption rate of glucose, or another carbon source. If the glucose uptake per cell is high, the oxygen supply becomes insufficient at high cell densities. This problem is enhanced in large-scale fed-batch processes, where substrate gradients occur because of the feeding of highly concentrated substrate solutions (Neubauer & Junne 2010). The imbalance between rapid glucose uptake and limited oxygen solubility can also be diminished by a genetically engineered *E. coli* with the PTS pathway for glucose uptake replaced by galactose permease (GalP) and glucokinase (glk) activities enabling the batch cultivation up to high cell densities (Flores *et al.* 2007).

3.4.2 Temperature

E. coli has the maximum specific growth rate at 37 °C but in many industrial processes lower temperatures are used to prevent protein aggregation (Schumann

1999, Baneyx & Mujacic 2004) and to maintain the balance between oxygen solubility and growth rate in high cell density cultivations (Shiloach & Fass 2005). Some recombinant protein expression systems contain a temperature sensitive promoter such as P_L or P_R promoter from bacteriophage lambda induced by the temperature up-shift (e.g. from 30 to 42 °C) causing significant stress and metabolic unbalance (i.e. heat-shock response) for the host organism (Valdez-Cruz *et al.* 2010). The heat-shock response is dependent on the magnitude of the shift (Villaverde *et al.* 1993, Wittmann *et al.* 2007) and the exposure time (Heitzer *et al.* 1992, Hoffmann & Rinas 2000, Wittmann *et al.* 2007) but also on the heating rate (Caspeta *et al.* 2009).

3.5 Scale-up and scale-down of bioprocesses

High cell density cultivations are widely used to increase the productivity of the fermentation processes by providing an effective method for the high-level production of recombinant proteins. Many approaches have been investigated to solve problems connected to *E. coli* cells, growing in high cell concentrations, including substrate inhibition, limitations in oxygen transfer, accumulation of acetate and other toxic byproducts and heat dissipation (Yoon *et al.* 2003).

3.5.1 Large-scale effect

In aerobic fermentation process the conditions are kept aerobic by a controlled feeding rate of the growth limiting substrate, aeration and agitation power. However, industrial scale bioprocesses are especially characterized by insufficient mixing and consequently prolonged mixing times enabling the formation of gradients in oxygen concentration but also other parameters (Enfors *et al.* 2001). For instance more than 10,000 fold variations have been reported for glucose concentration in a bioreactor (Enfors *et al.* 2001, Lapin *et al.* 2006). Glucose excess causes uncoupling of catabolic and anabolic reactions. The glucose uptake is higher than the biomass formation rate and subsequently ATP and extracellular metabolites are produced (Weber *et al.* 2005). Another relevant issue is the oxygen limitation, due to the low oxygen solubility causing the accumulation of mixed-acid fermentation products. (Neubauer *et al.* 1995, Xu *et al.* 1999, De Mey *et al.* 2010).

In addition to that also temperature (Caspeta *et al.* 2009), pH (Onyeaka *et al.* 2003) and dissolved CO_2 (McIntyre & McNeil 1997, Baez *et al.* 2009) have been

studied as parameters prone to form gradients relevant for bioprocess performance. The metabolic oscillations have been explained also by the frameshift mutation in the *ilvG* gene in the branched-chain amino acid pathway of *E.coli* K-12. The oscillations in dissolved oxygen, branched-chain amino acid concentrations and energy charges in the cell were detected and repaired by *ilvG* repaired strain and isoleucine feeding (Andersen *et al.* 2001).

Only a few confirmed global differences between laboratory and large-scale cultivations have been reported. The major difference in cultivating *E. coli* on a large scale compared to a small-scale well-mixed bioreactor is a reduced biomass yield (Xu *et al.* 1999, Hewitt *et al.* 2007). Studies in chemostat (Schaub *et al.* 2008, Lara *et al.* 2009, De Mey *et al.* 2010) and fed-batch cultivations (Lin *et al.* 2001) have indicated that the fast response to high glucose concentration is a high glycolytic flux resulting in immediate increase of pyruvate.

3.5.2 Scale-down models

The scale-associated differences in fermentation processes are studied by scale-down-models, providing a lab/bench scale tool to study the significant phenomena in large scale processes.

There are several approaches to study the large-scale fermentation conditions with scale-down models. By rapid sampling units and pulse feeding experiments, the oscillations can be studied in a single bioreactor (Neubauer & Junne 2010). The effect of heating rate for thermo-inducible processes was studied by Caspeta *el al*. (2009) with a scale-down model enabling the various heating rates during the temperature up-shift mimicking the large-scale processes with limited heat transfer capacity. As a conclusion the slowest heating rate (0.4 °C/min) mimicking the 200 m^3 process gave the highest productivity indicating that a slow heating rate allows for more efficient adaptation of the cells into new conditions. This was further confirmed by transcriptomic and metabolic level measurements indicating that the molecular level response was weaker and produced less by-products. (Caspeta *et al.* 2009)

Most common type is the two-compartment model, with a regular stirred tank reactor (STR) connected to another STR or to a plug flow reactor (PFR) (George *et al.* 1993, Onyeaka *et al.* 2003, Lara *et al.* 2006). Onyeaka et al (2003) studied the heterogeneities of pH, glucose and dissolved oxygen concentrations simultaneously. The conclusion was that the plug-flow reactor connected to a laboratory-scale bioreactor mimics the large-scale cultivation (20 m^3), in terms of

cell viability and biomass yield, when residence time for oxygen limitation and high glucose concentration was in the 50 s. The addition of the pH control agent into the inlet of the PFR, resulting in local zones with alkalic conditions, caused the similar result concerning the viability while the biomass yield was lower. The constant pH, as a result of feeding the control agent in STR, resulted in a higher biomass yield and lower viability (Onyeaka *et al.* 2003).

4 Research contribution

The effect of various environmental fluctuations in the cultivation processes is studied in this dissertation. The specific focus areas of this dissertation are the formation of mixed-acid fermentation products, biosynthesis of canonical and non-canonical amino acids and process monitoring at mRNA level. The research problem is divided in four research questions (RQ), which are addressed in the content of one or two peer-reviewed articles.

4.1 RQ1 – How does the oxygen downshift affect the free amino acid pools in *E.coli* cultivations?

Research question 1 is discussed in the Article I. Oxygen limitation in microbial cultivations is typically caused by the conflict between the increasing oxygen demand of exponentially growing cells and the limited oxygen solubility in water. Oxygen transfer rate can be increased by engineering solutions in vessel design as well as by pre-defined, or feedback controlled, aeration and agitation rates. Also oxygen enriched aeration or overpressure can be applied. In addition, oxygen consumption can be influenced by the glucose feeding rate. In mechanically agitated bench scale reactors, the oxygen supply into the cultivation medium is typically sufficient to keep the conditions far from oxygen limitation up to relatively high cell densities. However, often at least spatial oxygen limitations occur in high-cell density cultivations. A typical response for oxygen limitation in *E. coli* cultivations, when glucose is available as a carbon source, is the accumulation of anaerobic metabolites such as acetate, lactate, formate, molecular hydrogen, ethanol and succinate, by mixed acid fermentation. Article I studies the metabolic response to the oxygen downshift in *E.coli* cultivations which was monitored by metabolic and transcriptional levels.

In the reference cultivation of the Article I, representing typical bench-scale fermentation, oxygen levels remained between 30–60% out of the potential saturation level throughout the cultivations. This resulted in cell densities higher than 50 g L^{-1} of cell dry weight (Fig. 2A). The oxygen downshift experiment was performed by sudden decrease of the stirring rate resulting in rapid drop of dissolved oxygen to 0%. Glucose concentration remained high (> 4 g L^{-1}) due to the reduced growth rate even if the feeding rate was equal to reference cultivation (Fig. 2B). As a response to oxygen limited conditions, acetate, lactate, formate, succinate and ethanol were produced in significantly higher concentrations

(Fig. 2D) in comparison to the reference cultivation (Fig. 2C) indicating the existence of the oxygen limitation.

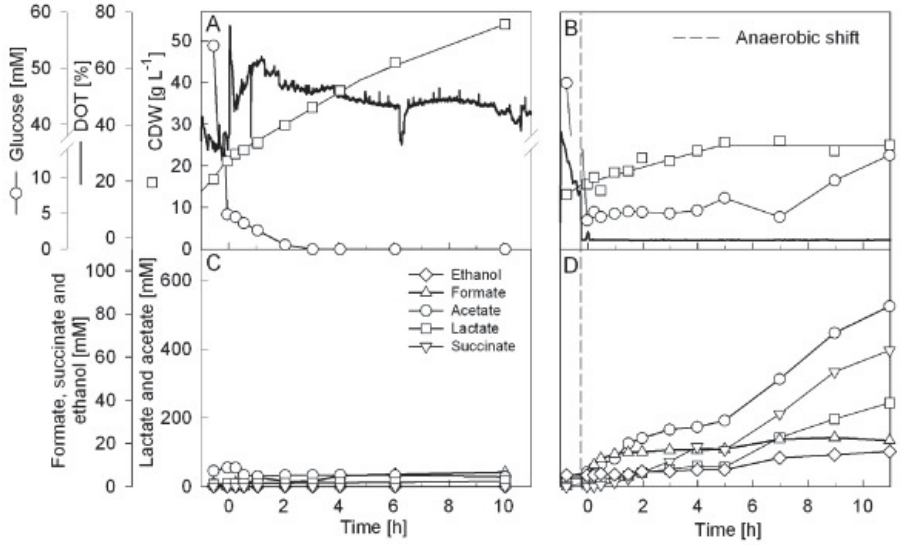

Fig. 2. High cell density cultures of *E. coli* W3110 without (A, C) and with (B, D) a stirrer downshift resulting in oxygen limited culture. The graphs show the data for cell dry weight (g L^{-1}), dissolved oxygen tension (DOT), glucose concentration (A, B) and the anaerobic metabolites acetate, lactate, formate, ethanol, succinate (C, D, symbols shown in C). Anaerobic conditions in graphs (B, D) were caused by a downshift of the stirring rate whereas the aeration rate was kept constant. The glucose feed was started at 0 hours. The stirrer downshift was performed 10 min before the feed start. (I, reprinted with a permission from BioMed Central).

The formation of organic acids under oxygen limitation is initiated from pyruvate. In the oxygen downshift experiment of Article I, clear accumulation of pyruvate was observed (See Fig. 4), as expected. Therefore, also some amino acids derived from pyruvate were quantified from crude extract samples that were collected from the oxygen downshift experiment. Interestingly, an increased profile was observed for certain amino acid concentrations after switching to oxygen limited growth conditions in pathways closely connected to pyruvate. In addition, an interesting finding was made in the oxygen downshift experiment of Article I. An

additional amino acid peak was detected shortly after elution of valine and methionine from amino acid analysis column (See Fig. 3) and identified as non-canonical amino acid norvaline. Norvaline was quantified from crude extracts of the reference and the oxygen downshift cultivations. Norvaline accumulates rapidly as a response to the oxygen limitation and concentration of 1mM was detected 6 h after the oxygen down-shift (See Fig. 4B)

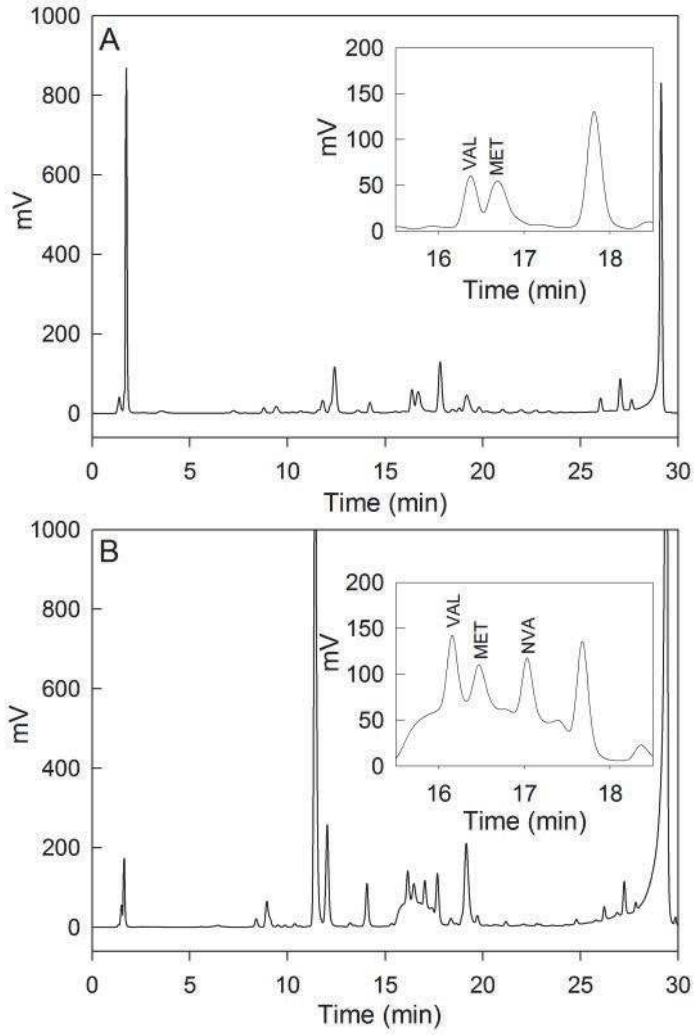

Fig. 3. HPLC chromatogram of crude extracts (containing cells and medium) from *E. coli* W3110 cultivations in glucose containing mineral salt medium. Samples were harvested 10 h after the oxygen downshift (B) or from corresponding phase of aerobic reference cultivation (A). (I, reprinted with a permission of BioMed Central).

Of canonical amino acids alanine, valine and leucine belonging to the alanine family and deriving from pyruvate were accumulating. Also, a significant increase was detected for aspartate, and asparagine, belonging to aspartate amino acid

family. In contrast, isoleucine, threonine and methionine belonging in the same amino acid family did not increase (See Fig. 4) Serine pathway branches from glycolysis before pyruvate and no change was observed in the concentrations. Glutamine, belonging to glutamate family originating from α-ketoglutarate, was rapidly formed after the oxygen downshift and the concentration was higher than in the reference fermentation.

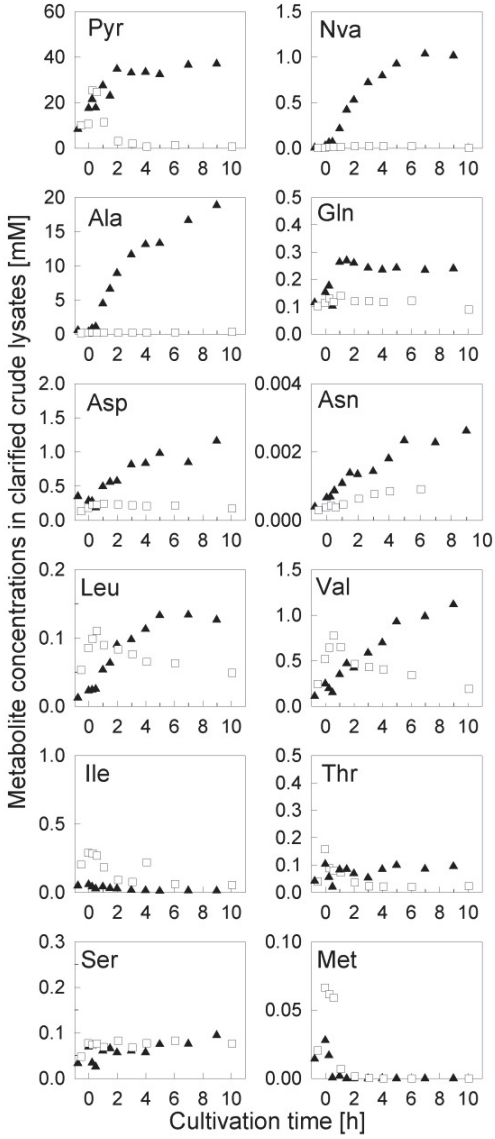

Fig. 4. Concentrations of free amino acids and pyruvate during fed-batch cultivations of *E. coli* W3110 without (reference, open squares) and with a stirrer downshift resulting in an oxygen limited culture (closed triangles). The analysis was performed from clarified crude cell extract samples collected during the fermentation. Zero time point represents the glucose feed start. (I, reprinted with a permission of BioMed Central).

4.2 RQ2: Sandwich hybridisation assay as a tool for mRNA based monitoring of rapid changes in cultivation conditions?

Research question 2 is addressed in Articles I and III by presenting results from mRNA based monitoring technology. The environmental stimuli triggering the transcriptional variations were oxygen limitation and temperature up-shift.

4.2.1 Response to oxygen limitation at mRNA level

Article I shows the response to an oxygen downshift at mRNA level. This was studied to enable a wider understanding of the response to an oxygen downshift and to test a potential monitoring tool for process control. A sandwich hybridisation assay (SHA) was applied in the experiments.

A group of mRNAs showed a clear induction after the oxygen downshift as illustrated in Fig. 5. The most significant change was the induction of *pflB* encoding for pyruvate formate lyase, which responded immediately after the oxygen depletion and thereafter very sensitively to small changes in the DOT levels. Also *ldhA* and *frdA* expression, closely related to the formation of lactate and succinate, behaved similarly but the change was smoother and the expression level lower. Also, some other mRNAs of the central carbon metabolism pathway were quantified. The *aceA* level was decreased shortly after the oxygen downshift. No clear response was seen for *ppc* and *pykF*. Also some genes involved in the branched chain amino acid pathway were studied but there were no major changes except for *ilvG* expression, which decreased in the end of batch phase and remained low after the oxygen downshift for approximately 0.5 h but was strongly induced after that. Furthermore, *ilvA* responded to oxygen downshift with a fast peak followed by smoother increase started approximately 1 h afterwards.

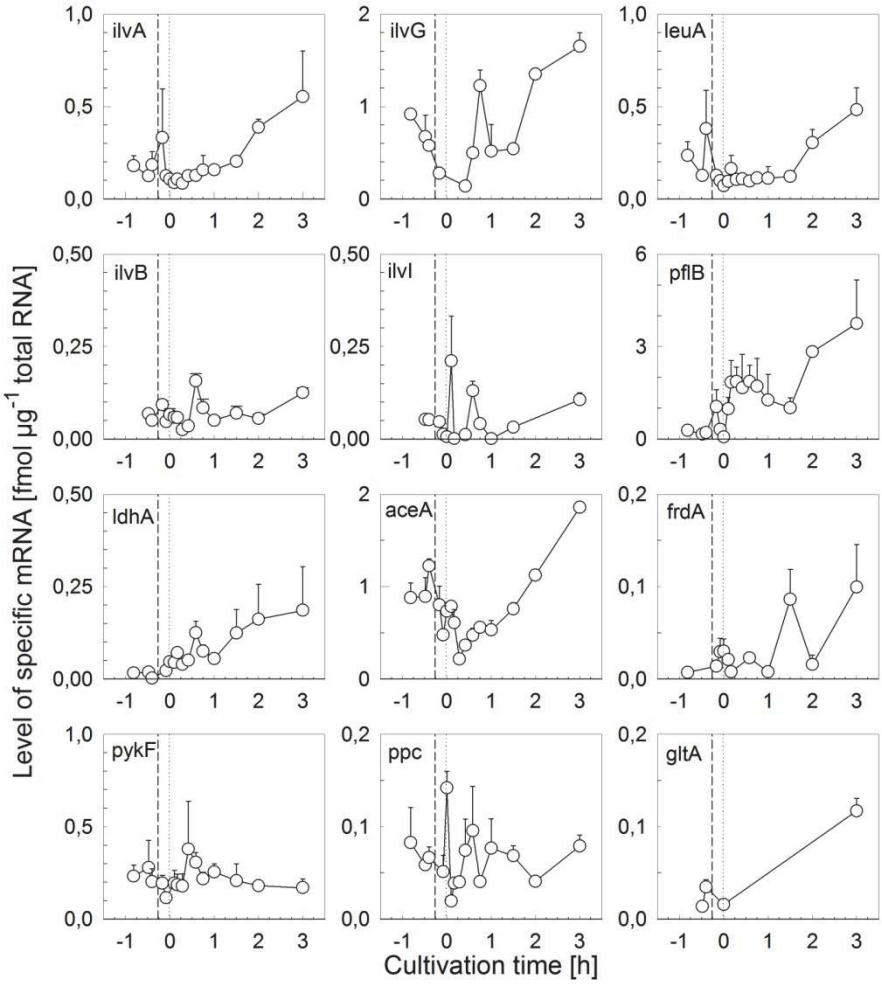

Fig. 5. Dynamic responses of a number of mRNAs after the stirrer downshift during cultivation of *E. coli* W3110. The mRNA levels were analysed by bead-based sandwich hybridisation and quantified by use of *in vitro* transcribed standard. The dashed and dotted vertical lines represent the time of oxygen downshift and glucose feed start, respectively. (modified from Soini *et al.* 2008).

4.2.2 Response to heat-shock

In Article III SHA measurement was applied as a method to evaluate for a heat shock response in a similar manner as an oxygen downshift in Article I. The aim of Article III was to measure the response of adenosine nucleotides and a set of heat-shock relevant genes to a temperature up-shift in *E. coli*. In response to a heat shock, *E. coli* strongly induces the production of a number of proteins with protecting and degrading functions, which are ATP dependent. The immediate effect of a temperature upshift was the increase of the specific rates for growth (μ), glucose uptake (q_S) and acetate production (q_A) (See Table 2).

Table 5. Cultivation parameters from batch cultivations of E.coli W3110 before and after the temperature upshift from 30 to 42 °C.

Parameter	Before upshift	After upshift
μ [h^{-1}]	0.38	0.71
q_S [mmol g^{-1} h^{-1}]	5.1	17.4
q_A [mmol g^{-1} h^{-1}]	2.5	8.0

The increased rate of the glycolysis also increased the adenosine nucleotide formation and rapid response was observed for ATP and ADP in the range of 5 minutes. The ATP was rapidly shifted from 1.5 μmol L^{-1} (diluted to OD_{500}=1) to more than 2.5 μmol L^{-1} and then decreased below 0.5 μmol L^{-1}. For ADP the concentration was shortly increased up to 1.3 μmol L^{-1} after the upshift but decreased to 0.2 μmol L^{-1}. AMP was only measured in low amounts approximately 30 min after the heat shock. The Adenylate Energy Charge decreased slowly from near 1.0 level to 0.6. Total nucleotide pool (AXP) further confirmed the rapid r-esponse to temperature upshift (See Fig. 6)

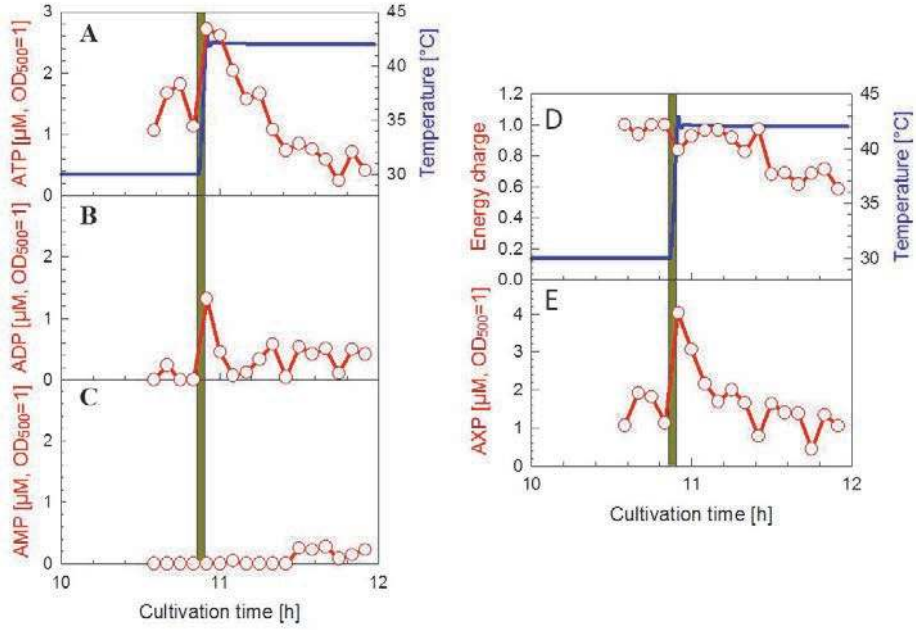

Fig. 6. Response of the adenosine nucleotide pool to a temperature up-shift from 30 to 42 °C in *E. coli* W3110. (A) ATP and cultivation temperature, (B) ADP, (C) AMP, D) Energy charge, E) AXP (combined adenosine nucleotides). (III, reprinted with a permission of BioMed Central).

The expression profiles of typical heat shock genes were analysed in article III to evaluate the heat-shock response. These experiments were performed in shake flasks with on-line monitoring of dissolved oxygen and temperature. A fast temperature up-shift was reached by diluting the culture with a pre-heated medium which explains the decrease of OD_{540} in Fig. 7. The expression of *dnaK* and *ibp* were both induced very rapidly and reached their maximum mRNA levels within only 1 min after the heat shock, but *lon* reached the maximum RNA level only after 5 min. The level of *dnaK* was continuously expressed significantly higher than before the shift, but *ibp* and *lon* were very low or not any more detectable after 10 min.

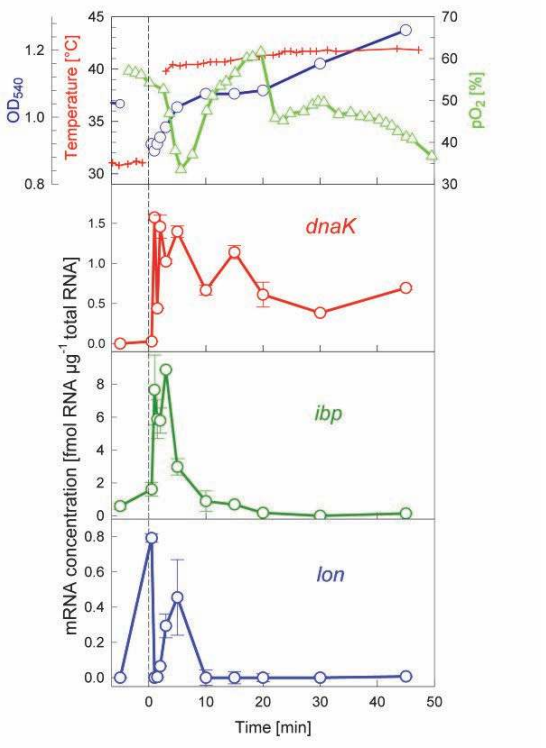

Fig. 7. Response of mRNAs in a stirred-flask cultivation of E. coli W3110 with a temperature shock from 30 °C to 42 °C. (III, reprinted with a permission of BioMed Central).

4.3 RQ3: The response of *E.coli* to fluctuating conditions in two-compartment scale-down reactor

Research question 3 is addressed in Article IV. In this article large scale bioreactor conditions are mimicked by using a two compartment scale-down reactor (2CR). *E. coli* W3110 was cultivated in 2CR fermentations with a regular well-mixed STR fermentation as the reference. Both in the reference and 2CR cultivation the glucose was limiting in the STR compartment in the fed-batch phase as illustrated in Fig 8. In the 2CR fermentation the glucose solution was fed in the PFR compartment right before the first sampling port and the glucose concentration in PFR compartment varied between 1.0 and 1.5 g L^{-1}. Final biomass concentration and biomass yield per glucose ($Y_{X/S}$) were calculated for the interval between the

feed start and the end of the cultivations 8 hours after the feed start and they both were 5% lower in the scale-down reactor cultivations compared to fermentations performed only in the stirred tank reactor. The cell density (cell dry weight) in the reference cultivation was 53.1 g L^{-1} and in the scale-down simulator 50.7 g L^{-1}. The biomass yields were 0.45 g g^{-1} and 0.43 g g^{-1}, respectively.

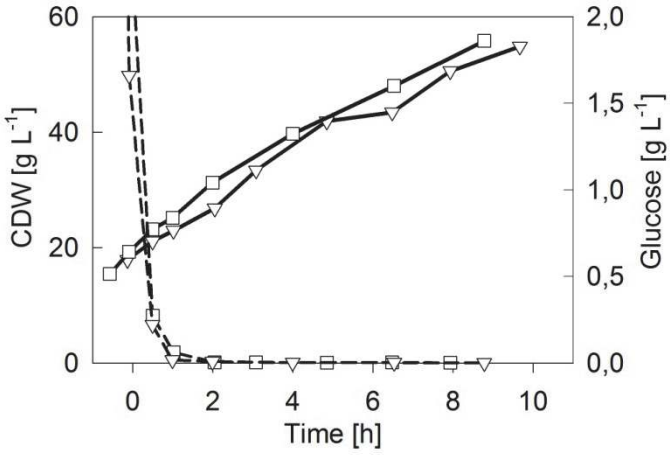

Fig. 8. Cell density (solid line) and glucose concentrations (dashed line) in *Escherichia coli* W3110 cultivated in reference STR (square) and scale-down STR-PFR (triangle) bioreactors (unpublished data).

4.3.1 Mixed acid fermentation products in 2CR fermentations

In the experiments conducted for Article IV typical mixed acid fermentation products were quantified from the STR compartment (unpublished data). Formate was accumulated up to 20 mM (see Fig. 9). This supports the presence of oxygen limitation in the PFR compartment. The highest acetate concentrations were measured before the feed start and thereafter the level was similar both in the reference and in the 2CR cultivations. No major changes were seen for lactate or ethanol.

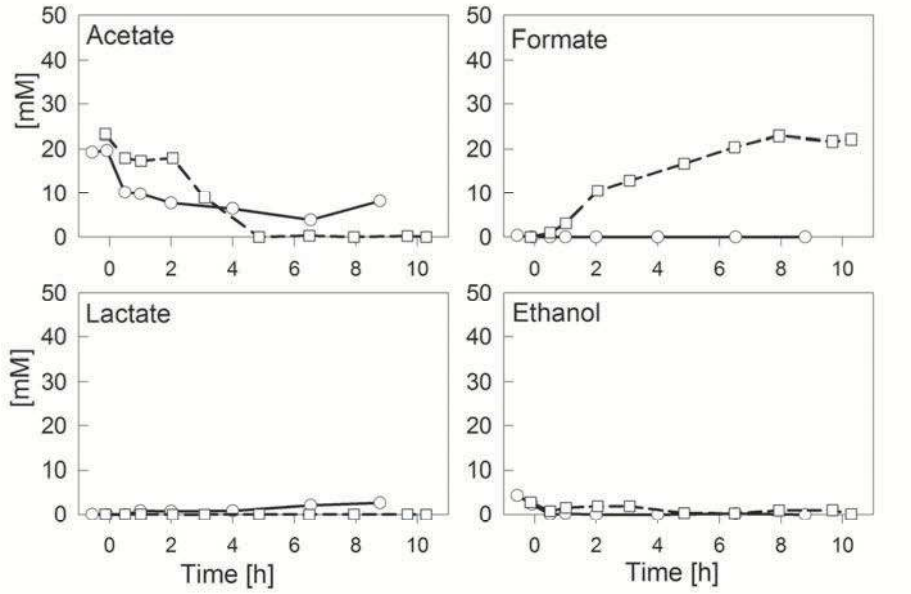

Fig. 9. Accumulation profiles of common anaerobic metabolites into the medium in the reference (sphere and solid line) and scale-down (square and dashed line) fed-batch cultivations with *E. coli* W3110 (unpublished data).

Article IV demonstrates the effect of scale-down simulator conditions for the accumulation of alanine and the branched-chain amino acids isoleucine, valine and leucine. Also, the non-canonical branched chain amino acid analogue norvaline was quantified from the crude cell extracts. Fig. 10 presents the amino acid concentrations quantified from the reference cultivation compared with those of the scale-down bioreactor. Valine, alanine and norvaline accumulate in the scale-down simulator in comparison to the reference cultivation. The accumulation profile of valine was similar under both cultivation conditions, but the valine concentration was significantly higher in the two-compartment system. The concentration of alanine was constantly increasing in the scale-down simulator whereas a clear decline was observed for the reference cultivation. Norvaline accumulation started immediately after the PFR circulation.

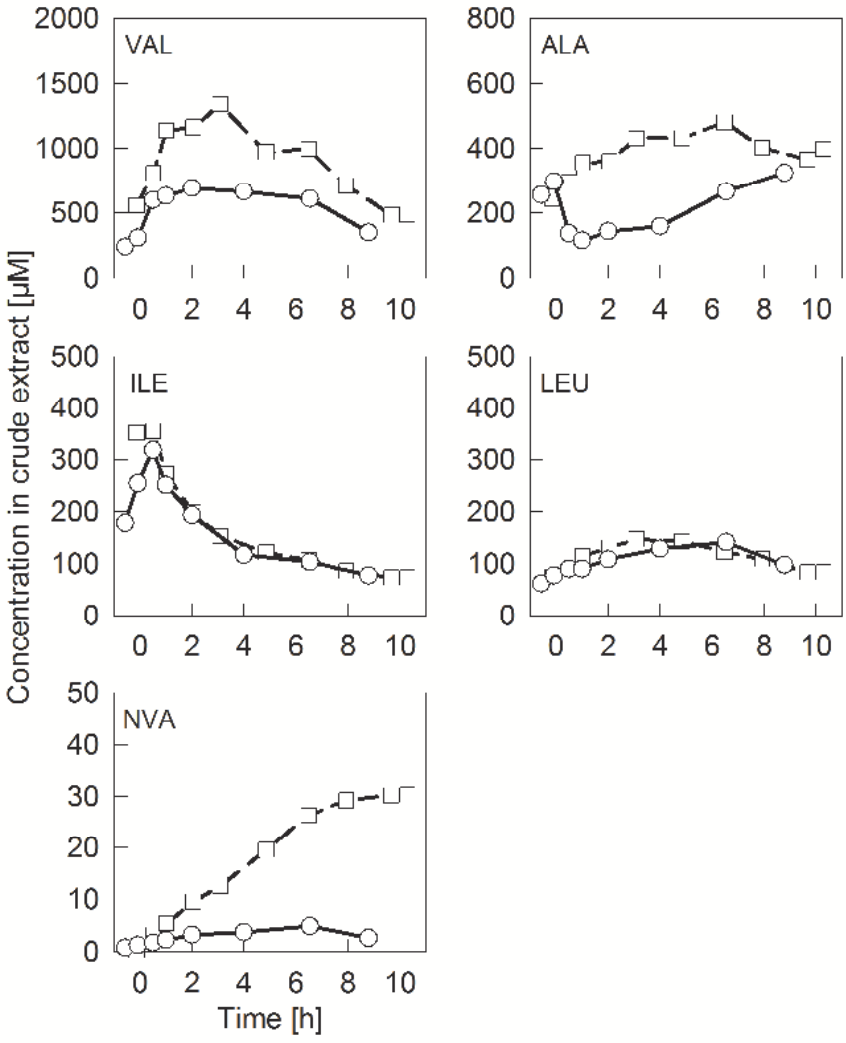

Fig. 10. The accumulation profiles of a set of amino acids in crude extracts (cells and medium) of *E. coli* W3110 from the cultivation in the two-compartment reactor (square and dashed line) and respective reference cultivation in the STR (sphere and solid line). (IV, reprinted with a permission of Scientific Research Publishing).

Leucine and isoleucine concentrations were not affected by the scale-down cultivation conditions. Isoleucine was accumulating during the batch phase and

decreasing during glucose limited fed-batch phase. Leucine concentration was relatively stable in comparison to other amino acids.

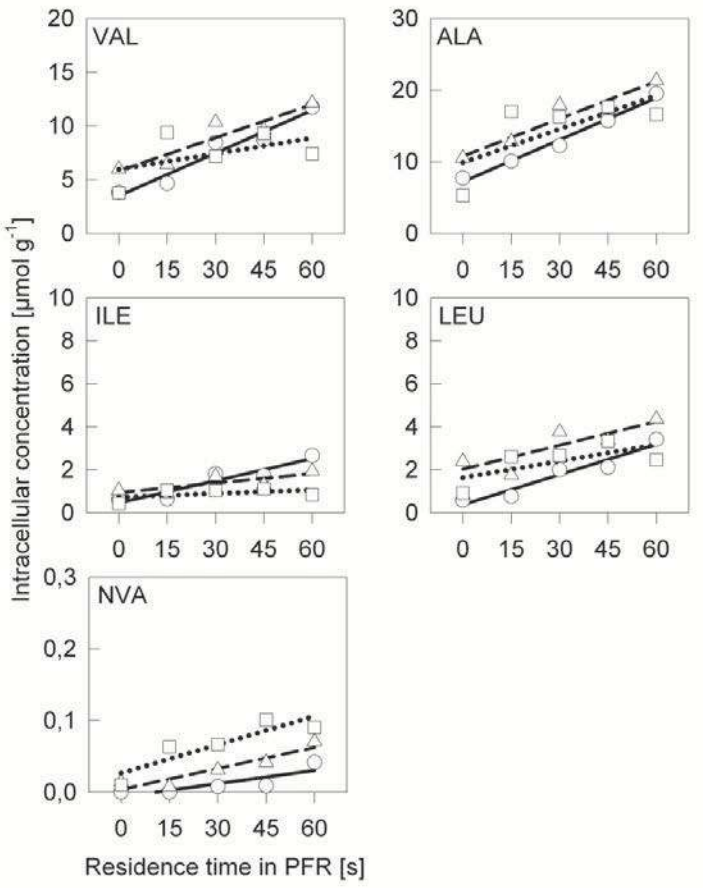

Fig. 11. A set of intracellular free amino acid concentrations in scale-down cultivation samples from the cultivation of *E. coli* W3110 in the two-compartment reactor. The samples were taken 0.5 h (sphere and solid line), 2 h (triangle and dashed line) and 5 h (square and dotted line) after the PFR recirculation was initiated at cell density of 19 g L^{-1} (CDW). The peristaltic pump was set to flow rate 1 L min^{-1}, and this rate was used to approximate the residence times in the PFR compartment shown on X-axis. (IV, reprinted with a permission of Scientific Research Publishing).

To study the dynamics of amino acid metabolism in more detail, the intracellular levels were determined and formation rates were calculated. Fig. 11 shows the

intracellular amino acid concentrations supporting the proposal that alanine and valine are produced as a response to conditions during PFR recirculation. Actually all studied amino acids accumulated in the PFR compartment, i.e. their synthesis was stimulated by the glucose pulse. In addition to alanine and valine also leucine and isoleucine were produced during the passage through the PFR compartment even if the accumulation on crude cell extract level was not seen. Also norvaline was produced in relatively low quantities.

4.4 Answering RQ 4 – Can formate accumulation be prevented by the addition of trace metals Ni, Se, Mo into cultivation medium

Research question 4 is addressed in Article II. Three trace metals, selenium, nickel and molybdenum were added into the cultivation medium and the effect on the formate accumulation was evaluated. For this study two different simulators were used, causing oxygen limitation in E. coli cultures. Two-compartment reactor (2CR) was used to imitate a large-scale bioreactor with limited mixing and fed-batch cultivation in a regular stirred tank reactor with oxygen downshift caused by a sudden decrease of the stirrer rate to provoke permanent oxygen limitation. The *E. coli* W3110 strain was applied also in Article II.

4.4.1 2CR experiment

The control cultivation in a defined mineral salt medium was a glucose limited fed-batch with constant feeding of a concentrated glucose solution and the DOT was kept at 30%. Article II shows the effect of a medium optimisation by the addition of selected trace elements nickel, selenium and molybdenum to avoid the formate accumulation. Nickel, selenium and molybdenum are known to be involved in the activity of formate hydrogen lyase responsible for metabolising formic acid further to dihydrogen and carbon dioxide. The growth parameters were similar between the cultivations (Fig. 12A). In the scale-down system presented in chapter 4.3 more than 20 mmol L^{-1} of formate accumulated in the medium without the addition of the extra trace elements (Fig 12B). The addition of the extra trace elements resulted in a significant decrease in the formate secretion to a hardly detectable level (< 0.1mM). The effect on the other mixed-acid fermentation products was relatively low (see Fig. 12C-F). The addition seems to have an effect for lactate and succinate but the accumulation levels were very low in 2CR cultivation.

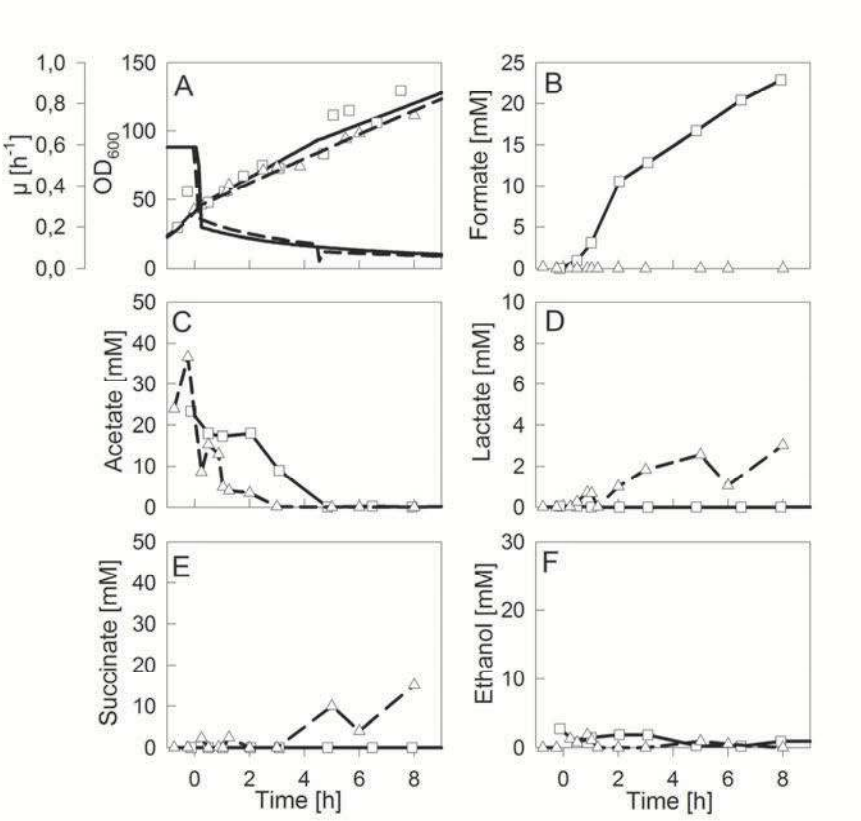

Fig. 12. Growth curves and anaerobic metabolite concentrations in fed-batch cultivations of *E. coli* W3110 in the STR-PFR system with (triangle and dashed line) or without (square and solid line) addition of the trace elements selenium, nickel and molybdenum. (A) Specific growth rate and optical density; (B) accumulation of formate, (C) acetate, (D) lactate, (E) succinate, and (F) ethanol. Time of feed start represents the time point 0 h in the graphs. (II, reprinted with permission of BioMed Central).

4.4.2 Oxygen downshift experiment

Figure 13 illustrates the effect of the formate hydrogen lyase (FHL) related trace elements for anaerobic metabolite concentrations in fed-batch cultivations of *E. coli* W3110 with a permanent downshift of the DOT. In the cultivation with a

permanent oxygen downshift, formate accumulated relatively fast but later ceased and stayed at about 17 mM. The addition of FHL related trace elements selenium, nickel and molybdenum into the cultivation medium significantly decreased the accumulation of formate also on the oxygen downshift experiment. In the cultivation, with the addition of FHL related trace elements formate only slightly accumulated within the first 1.5 hours, but decreased afterwards below the detection limit (Fig. 13B). The addition of the FHL-related trace elements had a major effect on the accumulation patterns of other mixed-acid fermentation products. In contrast to the 2CR system, lactate concentration was especially increased heavily by the addition. More than 400 mM of lactate concentrations were measured after the oxygen downshift in the medium supplemented with selenium, molybdenum and nickel. This means that 83% [w/w] of the glucose consumed in the PFR compartment was used to produce formate, acetate and lactate. It was also observed that the final cell yield of biomass per substrate was lowered by more than one third), from 0.38 g g^{-1} in the control cultivation to 0.23 g g^{-1} in the cultivation with the addition of the extra trace elements, corresponding to a lower cell dry weight of about 7 g L^{-1} (Fig. 13A). Also, changes in the accumulation pattern of the other acids were observed. As shown in Fig. 13c, acetate accumulated with a very low rate directly after the oxygen downshift, but approached a constant higher rate about 1 hour after the shift, so that the concentration was only slightly lower compared to the control cultivation at 4 hours after the onset of oxygen limitation. Succinate accumulation was similar with and without the additional trace elements, but its level stayed constant from two hours after the onset of limitation onwards, so that the final level was lower compared to the control cultivation (Fig. 13E). The ethanol concentration (Fig. 13F) increased during the whole cultivation similar to lactate, but the concentration was only less than one tenth of the lactate concentration. In contrast, ethanol reached a maximum of 10 mM in control cultivations without the addition of the extra trace elements.

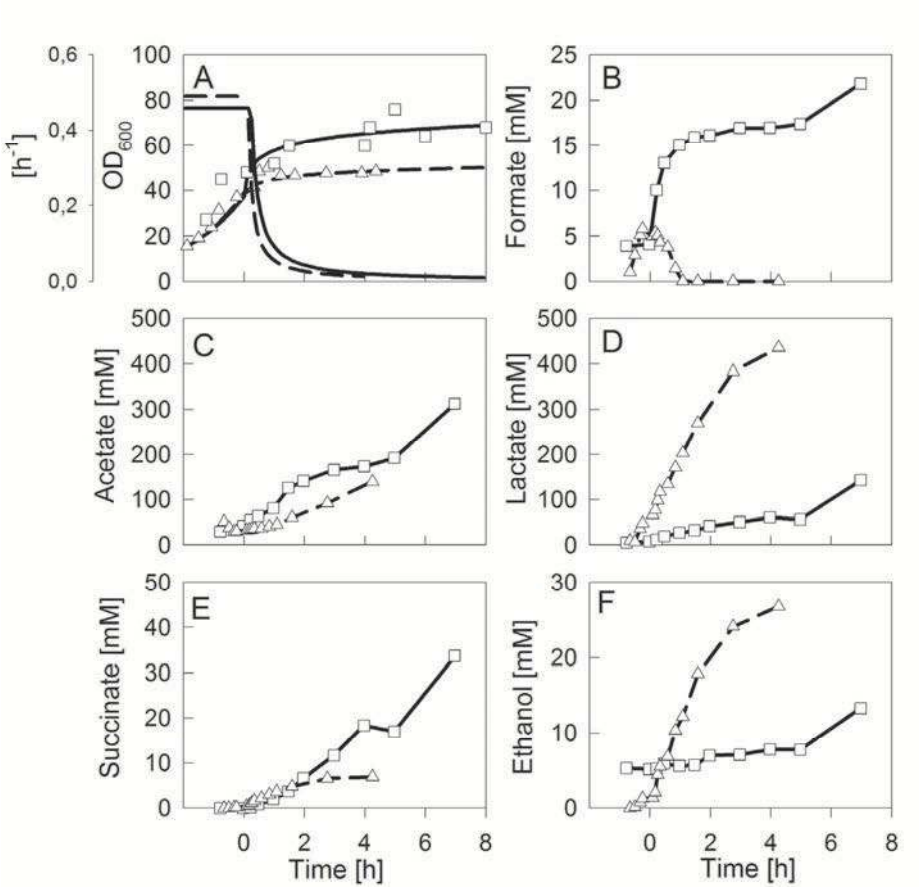

Fig. 13. Growth curves and anaerobic metabolite concentrations in fed-batch cultivations of E. coli W3110 with a permanent downshift of the DOT at the time of feed start (zero hours). Cultivations were performed with (△ and dashed line) or without (□ and solid line) addition of the trace elements selenium, nickel and molybdenum. (A) Specific growth rate and optical density; (B) accumulation of formate, (C) acetate, (D) lactate, (E) succinate, and (F) ethanol. Time of feed start represents the time point 0 h in the graphs. (II, reprinted with permission of BioMed Central).

5 Discussion

5.1 Norvaline accumulation in anaerobic conditions due to the oxygen limitation

Earlier research suggests that non-canonical amino acid norvaline biosynthesis is triggered by the cellular conditions leading to an accumulation of pyruvate (Apostol *et al.* 1997). The pyruvate accumulation in *E.coli* caused by anaerobic conditions is also well-reported (Lin *et al.* 2001, Schaub *et al.* 2008, Lara *et al.* 2009, De Mey *et al.* 2010) but a direct link between anaerobic conditions and norvaline accumulation has been missing until now. This research contributes to this gap by showing that norvaline is produced up to concentration of 1 mM, when *E.coli* cells growing in a mineral salt medium are exposed to sudden oxygen limitation. The accumulation level of norvaline is significant since it is at the same level with valine and clearly higher than other branched chain amino acids. It is also clearly higher than the norvaline concentration measured from recombinant hemoglobin fermentation earlier (Apostol *et al.* 1997).

Norvaline accumulation, as presented in this study, is probably caused by overflow metabolism, resulting from pyruvate accumulation in connection to an oxygen downshift. This is supported by the increased concentrations of other pyruvate derived amino acids such as alanine, leucine and valine. Also, the mRNA measurements of genes involved in the branched-chain amino acid pathway did not show significant response to an oxygen downshift indicating that the norvaline pathway activation occurs on a metabolite level rather than by transcriptional induction.

Norvaline accumulation in *E. coli* W3110 wildtype strain is interesting since it has earlier only been observed in mutant *S. marcescens* strains producing isoleucine (Kisumi *et al.* 1976b, Sugiura *et al.* 1981b), recombinant *E.coli* strains producing leucine-rich recombinant protein (Apostol *et al.* 1997) or modified *E.coli* strain with knock-out mutations in ILV pathway (Sycheva *et al.* 2007). The conditions triggering the norvaline accumulation in this research comprise of oxygen limitation caused by reduced mixing in high-cell density cultivation together with glucose excess. These conditions and therefore also the norvaline accumulation results are relevant in typical fermentation types. Shake-flask cultivation often run into oxygen limitation (Panula-Perala *et al.* 2008), which might trigger norvaline formation. Local oxygen limitation due to the glucose

excess and insufficient mixing is also a common bottleneck in large-scale fermentation (Enfors *et al.* 2001). This research indicates that the pyruvate accumulation caused by oxygen limitation is the key factor in norvaline formation.

The experiments carried out in this research also indicate that the aspartate family amino acids aspartate and asparagine were increased by the oxygen limitation. However, threonine, methionine and isoleucine belonging to the same group, did not increase. If the threonine biosynthesis is low, these results combined with the generally accepted pathway of 2-ketobutyrate, originating from threonine pathway (Umbarger 1996) does not explain the norvaline formation. Alternative isoleucine pathways have been reported for other organisms such as *S. marcescens* (Kisumi *et al.* 1977a) and *G.. sulfurreducens* (Risso *et al.* 2008) producing 2-ketobutyrate from pyruvate via citramalate. Such an alternative pathway would also explain a norvaline formation in pyruvate accumulating conditions. Pyruvate accumulation would cause the 2 –ketobutyrate accumulation, which in connection to inactive isoleucine pathway would result in a norvaline formation by biosynthetic enzymes from leucine pathway.

5.2 Norvaline accumulation 2CR scale-down system

Based on the results obtained in oxygen downshift fermentation, it was interesting to test the effect of 2CR scale-down simulator conditions for the accumulation of norvaline and some other branched-chain and pyruvate-based amino acids. As expected, norvaline accumulation started immediately after the PFR circulation due to the limitation of oxygen in the PFR compartment. Leucine and isoleucine concentrations were not affected by the scale-down cultivation conditions. The high accumulation of valine is interesting because of the valine toxicity in K-12 strains of *E. coli* (Andersen *et al.* 2001). This study demonstrates how valine accumulates rapidly up to a concentration clearly over 1 mM. The accumulation of valine could lead to growth inhibition and it also partly explains the rapid decrease of isoleucine since the valine toxicity prevents the isoleucine biosynthesis (Lawther *et al.* 1981) Also alanine was strongly accumulating in the 2CR system, indicating the leakage from pyruvate.

It can be seen from the results that the rapid increase of intracellular leucine and isoleucine concentrations in the PFR compartment are in contradiction to crude cell results is STR samples, which showed no increase. This could be explained by further re-assimilation as is the case for common anerobic

metabolites such as acetate and lactate, which are produced under oxygen limitation but rapidly consumed in the aerobic environment.

5.3 mRNA level response to environmental variations

Oxygen limitation response

The accumulation of norvaline after the oxygen downshift raised the question whether the response for the oxygen limitation could be observed on mRNA level since it has not yet been discussed in articles related to norvaline before (e.g. Apostol *et al.* 1997, Sycheva *et al.* 2007). However, the sandwich hybridisation results did not show significant changes of gene expression profiles of BCAA pathway genes (*ilvA*, *ilvG, leuA, ilvI* and *ilvB*). The norvaline accumulation seemed to be rather a direct consequence of a metabolic overflow resulting from a high metabolic flux into the glycolysis and the following accumulation of pyruvate.

When an aerobic culture is shifted to anaerobic conditions or to oxygen limitation, pyruvate dehydrogenase is rapidly inactivated due to cessation of the respiratory chain. The accumulating pyruvate can be disposed to either lactate by lactate dehydrogenase *ldhA* or to acetyl-CoA and formate by pyruvate formate lyase *pflB* (See Fig. 1). In this study the strongest response for an oxygen downshift was detected for *pflB* as expected based on the earlier studies (Schweder *et al.* 1999, Lara *et al.* 2006). The response was very sensitive to minor changes in the dissolved oxygen concentration. The expression of *ldhA* increased smoothly during the oxygen limitation period but clear induction was not observed. This was in contradiction with scale-down cultivations by Lara *et al.* (2006) proposing that *ldhA* would be the first one expressed under oxygen limitation because it is not regulated by FNR (Lara *et al.* 2006).

Heat shock response

In this part of study the gene expression analysis was applied as a tool to estimate the strength of the heat-shock response in wildtype *E.coli*. In temperature upshift experiments, heat-shock genes *dnaK*, *lon* and *ibp* were applied as transcriptional markers for changes in environmental conditions. They all responded rapidly with temperature up-shift as expected. The rapid response followed by a decline to the

new steady state level is in concert with earlier results obtained for heat-shock response (Hoffmann & Rinas 2000, Hasan & Shimizu 2008, Caspeta *et al.* 2009). Often these genes are studied in connection to protein aggregation in recombinant protein production processes (Schweder *et al.* 1999, Jurgen *et al.* 2000), which causes a similar response in *E.coli*.

The temperature up-shift experiment in this study was shown to accelerate the glucose uptake and glycolysis because of the increased energy demand as was also shown by another study with a recombinant strain (Wittmann *et al.* 2007). The ATP level, increasing from 6.8 to 11.4 μmol g^{-1} CDW (corresponds to from 1.5 to 2.5 μmol L^{-1} in OD_{500} 1 reported in "Results") obtained in this study after the temperature up-shift is on similar level as the results of Wittmann et al (2007) who measured ATP concentrations of approximately 13 μmol g^{-1} before and 8 μmol g^{-1} after the temperature upshift. In contradiction to the findings of this research, (Wittmann *et al.* 2007) experienced a decrease in ATP concentration and energy charge, indicating that the recombinant strain could not fulfill the increased energy demand. In wildtype strain applied in this research, ATP concentration increased rapidly after temperature upshift. This was probably due to the lack of burden caused by recombinant protein production.

5.4 Controlling of formate accumulation by medium optimisation

Formate accumulation is known to be a relevant issue for large-scale fed-batch cultivations with local gradients in dissolved oxygen tension (Enfors *et al.* 2001) but also in laboratory scale cultivations often running under oxygen limitation (Vasala et al. 2006, Panula-Perala et al. 2008). Therefore this study answers whether the medium optimisation by the addition of trace elements selenium, molybdenum and nickel, required for active FHL complex (Pinsent 1954a), would bring benefit in form of reduced formate accumulation in 2CR and oxygen downshift cultivations.

Formate accumulation up to 1000 mg L^{-1} (corresponding 22 mM) was obtained in a study applying similar 2CR system (Bylund *et al.* 2000) as applied in this research. As this kind of 2CR scale-down system has been earlier shown to provide a relevant model for large-scale bioreactors (Enfors *et al.* 2001), it was hypothesised that potential beneficial effects could be utilised in large-scale bioprocesses, where the cells are exposed to oscillations in the glucose and oxygen levels. In 2CR cultivations formate is typically accumulating but the other mixed-acid fermentation products are not. This indicates that there is oxygen limitation in the PFR

compartment but other anaerobic metabolites such as acetate and lactate are rapidly consumed under aerobic conditions (Enfors *et al.* 2001).

In this study, the accumulation of formate in partially oxygen limited cultivations was reduced below the detection limit by the addition of FHL-related trace elements. Formate decreased close to the detection limit in the 2CR scale-down system with optimised medium. On the other hand, more than 20 mM of formate accumulated in the cultivations in the two-compartment system without addition. These findings are in line with an earlier study with a similar approach (Bylund *et al.* 2000). This study did not indicate any significant negative effects on any bioprocess parameters when the *E.coli* culture was exposed to glucose and oxygen oscillations in 2CR system. The lactate and acetate concentrations were increased but the changes were marginal.

The growth reduction in 2CR cultivation was 5% which is less than what was reported earlier by Hewitt et al (2007), who reported a 15% to 38% decrease depending on the strength of the induction with recombinant *E. coli* strain (Hewitt *et al.* 2007) or by Xu *et al.* (1999) who reported a 12% decline. This loss of cell mass could be explained by the very high lactate accumulation, which is increased by approximately 36 g L^{-1}, but could be a result of carbon loss through the FHL-related carbon dioxide production. The lactate production could be explained by the reducing condition of the dihydrogen production by the FHL complex. Similar explanation was proposed for *E. coli* producing excessive ethanol from sorbitol (Alam & Clark 1989).

This study shows that the formate degradation catalysed by FHL complex results in the formation of carbon dioxide and hydrogen. It seems that this triggers lactate formation by some mechanism under oxygen limitation. In a 2CR system the lactate formation was not significant and it did not affect the biomass yield significantly. Therefore the addition of FHL-related trace elements selenium, molybdenum and nickel can be considered in large-scale fermentations as a tool to avoid formate accumulation to toxic concentrations and carbon-losses. However, for cultures suffering more severe oxygen limitation such addition might cause problems via strong lactate accumulation resulting in biomass yield reduction.

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