The influences of fluctuations in process parameters and end-product inhibition on bacterial stress responses

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A doctoral dissertation completed for the degree of Doctor of Science (Technology) to be defended, with the permission of the Aalto University School of Chemical Technology, at a public examination held at the lecture hall V1 of the school on 12 May 2017 at 12.

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Abstract

The bacterial stress phenomenon is remarkable considering the industrial production organisms but also in prevention of the pathogen growth. Stress can be caused for example by abnormal temperature, pH, metabolic product, osmotic or hydrostatic pressure, lack of substrate, shearing forces, and oxidative radicals. The stress effects are diverse. The stresses occurring in a process can affect productivity, yield, and product quality. A sublethal stress can also improve the strain robustness towards subsequent stresses.

In this dissertation the subject of bacterial stress was approached through perspective of anaerobic *Clostridium acetobutylicum* and facultative anaerobe *Lactobacillus rhamnosus*. *C. acetobutylicum* is known of acetone-butanol-ethanol fermentation already from the beginning of the 20th century. The process shows potential as a source to produce butanol in response to renewable fuel demand. *L. rhamnosus* is widely utilized as a probiotic, in tablets or supplemented in functional foods.

The stress-related gene expression of *L. rhamnosus* was studied with scale-down methodology. In the scale-down the gradients of industrial scale processes were simulated in smaller scale using plug flow reactor in continuous cultivation and oscillating pH control in batch process. In the plug flow reactor pH and temperature were used as changing environmental variables. According to the gene expression results especially heat-shock response and phosphate uptake system were sensitive even to small scale pH changes. The pH change of 0.3 unit was enough to affect the expression of heat shock related genes. No relation between the expressions of the studied genes and freeze stability or acid tolerance was found.

The stress effects on *C. acetobutylicum* metabolism was investigated applying constraint-based genome-scale metabolic modelling. The study on metabolism was carried using data obtained from continuous cultivations. Excess butanol and glucose limitation were used as sources for stress in the cultivations. The solution space of the metabolic model for the studied cases was narrowed with additional constraints from experimental measurements and 13C- metabolic flux analysis results for internal metabolic fluxes. The solutions of flux space were investigated using different optimization objectives for the flux distribution of the model, such as maximization of the growth rate and maximization of the ATP maintenance. The flexibility of single fluxes with respect to different optimization objectives was identified for each case applying flux variance analysis.

Keywords  *Lactobacillus rhamnosus*, *Clostridium acetobutylicum*, bacterial stress, ABE, gene expression, metabolic modelling

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Avainsanat: Lactobacillus rhamnosus, Clostridium acetobutylicum, bakteerien stressi, ABE, geeniespressio, metaboliamallinnus
Preface

The work for this thesis was carried out during the years 2009-2016 in the Research group of Bioprocess Engineering, Department of Biotechnology and Chemical Technology, Aalto University School of Chemical Technology, and it started under the TEKES funded BioControl project. It would not have been possible without this funding and the following long-term assistant position. The final writing work of the thesis was supported by Tekniikan edistämissäätiö incentive grant of which I am also grateful.

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In addition to the co-authors, I am grateful for Tom Granström for taking me into his industrial collaboration projects during the years 2011-2012 and summer 2016 which delayed the thesis in a good way providing in-depth experience to fermentation techniques thus also indirectly benefiting the topics of this thesis, and providing funding. I am thankful for the technical and practical support provided by Seppo Jääskeläinen and Pekka Koivulaakso, HPLC analytics by Auli Murrola and Tommi Timo-harju, and contribution of Kristiina Kiviharju and Matti Kankainen to the first article. I also want to thank Maria Osmala for guiding me through the frustrating steps of COBRA toolbox installation, Johannes Haataja for providing technical support and the cover picture for the thesis, Jukka Lommi for carrying out preliminary cultivations for clostridial studies, and Forschungszentrum Jülich GmbH for providing 13CFLUX2 software. Special gratitude for peer support goes to Markus Räsänen and Siiri Viljanen. Markus and Siiri, you were there the whole journey sharing the passion for fermentations in and outside the lab. Your company was and is priceless.

As research and writing can be often lonely and frustrating I really appreciated the company of the precious people from Bioprocess technology, Molecular biotechnology, and Biomolecular materials groups sharing the frustrations, rare successes, passions, discussions, coffee breaks, lunch breaks, cakes, after-work beers, skiing trips... Also your company has been priceless and I am privileged to get to know many of you. Finally, I would like thank my friends, my parents, Harri and Anne, and my little sister, Mia, for their love and support. Mother and father, thank you also for your patience and contributions in listening my occasional babblings about the process things or research related problems.
“Yea, though I walk through the valley of the shadow of death, I will fear no evil: for thou art with me; thy rod and thy staff they comfort me.”

-Psalms 23:4

Espoo, April 3, 2017,

Janne Wallenius
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This thesis consists of an overview and of the following publications which are referred to in the text by their Roman numerals.


Author’s Contribution


The author planned the study together with M.Sc. Tuomas Uuksulainen under the supervision of D.Sc. (Tech.) Tero Eerikäinen. The author carried out the scale-down cultivations together with Tuomas Uuksulainen. The author was responsible for the modelling and interpretation of results. The author had primary responsibility for writing the manuscript.

Publication II: “The effects of pH oscillation on *Lactobacillus rhamnosus* batch cultivation”

The author planned the study together with D.Sc. Dorothee Barth under the supervision of D.Sc. (Tech.) Tero Eerikäinen. The author carried out the cultivations together with Dorothee Barth. The author carried out the freeze stability tests and gene expression analysis. The author interpreted the results and wrote the manuscript.

Publication III: “Constraint-based genome-scale metabolic modeling of *Clostridium acetobutylicum* behavior in an immobilized column”

The author built the model, developed the optimization procedure, carried out the model related analysis and validation under the supervision of D.Sc. (Tech.) Tero Eerikäinen. The author interpreted the results and wrote the manuscript.
Publication IV: “Carbon 13-Metabolic Flux Analysis derived constraint-based metabolic modelling of Clostridium acetobutylicum in stressed chemostat conditions”

The author planned the study under the supervision of D.Sc. (Tech.) Tero Eerikäinen. The author carried out the cultivations. The author performed the analyses excluding NMR, HPLC, HPAEC, and SEM analyses. The author carried out the modelling related tasks. The author was responsible for the EPS analysis. The author interpreted the results and wrote the manuscript.
Nomenclature

(p)ppGpp  Guanosine 3’-diphosphate 5’-triphosphate and guanosine 3’-diphosphate 5’-diphosphate

ABE  Acetone-butanol-ethanol

aCoA  Acetyl coenzyme A

ALE  Adaptive laboratory evolution

ATR  Adaptive tolerance response

CDW  Cell dry weight

CFA  Cyclopropane fatty acids

CIRCE  Controlling inverted repeat of chaperone expression

COBRA  Constraint-based reconstruction and analysis

CSP  Cold shock protein

FBA  Flux balance analysis

FVA  Flux variance analysis

GC  Gas chromatography

GC-MS  Gas chromatography combined with mass spectrometry

HPLC  High-performance liquid chromatography

HSP  Heat shock protein

LAB  Lactic acid bacteria

mRNA  Messenger RNA

NMR  Nuclear magnetic resonance
Nomenclature

ORF  Open reading frame

PBS  Phosphate buffered salt solution

PFR  Plug flow reactor

PMF  Proton motive force

PPP  Pentose phosphate pathway

Pst  Phosphate-specific transport system

qPCR Quantitative polymerase chain reaction

rcf  Relative centrifugal force

RCM  Reinforced clostridial medium

ROS  Reactive oxygen species

SD   Shine-Dalgarno sequence

sRNA Small RNA

STR  Stirred tank reactor

TCA  Tricarboxylic acid

TRAC Transcript analysis with the aid of affinity capture
Bacteria among other microorganisms, like algae and fungi, are utilized in many ways within the biotechnological industry. Compared to the other microorganisms they typically grow fast, are robust, and the cellular machinery and structure are simpler. Some of them like lactic acid bacteria are utilized due their natural metabolic properties in food industry, in dairy products, namely cheese, yoghurt, and buttermilk. Lactic acid and acetic acid bacteria are used in pickling process for products like sauerkraut, olive and cucumber pickles. Bacterial fermentation is used to prepare sausages, soy sauce and in processing of tea, coffee and cocoa beans. Lactic acid bacteria (LAB) themselves are added to functional food products and formulated to tablets as probiotic supplements.

Drugs, like for example insulin, growth hormone, vaccines and antibiotic can be produced with bacterial hosts. Most of the antibiotic compounds, such as tetracyclines, erythromycin, and streptomycin are produced by *Streptomyces* species, and antimicrobial peptides such as bacitracin by *Bacillus* species.\(^{52,229}\)

Bacterial hosts from geni of *Escherichia*, *Bacillus*, *Staphylococcus*, and *Pseudomonas* have also been used for enzyme production. Traditional bulk enzymes, such as proteases, lipases, amylases, and phytases are utilized in the dairy, bakery, detergent, textile, starch, and animal feed industries. Development of genetic engineering has enabled the production of recombinant enzymes, which vast majority of industrial enzymes are, and heterologous enzymes.\(^{4,223}\)

Some organic compounds can be produced more conveniently by bacterial process compared to organic synthesis, such as riboflavin and vitamin B\(_{12}\).\(^{231}\) Polymers like xanthan and polyesters are produced by bacteria with world production volumes of around 100,000 tonnes annually.\(^{187}\) The global market overview of industrial branches related to microbes
and microbial products is summarized in Figure 1.1. Bacteria have also been used for bioremediation, where environmental polluting compounds are degraded with microorganisms.179 *Bacillus thuringiensis* derived product under trade names like Dipel and Thuricide have been used in place of pesticides in biological pest control.41

![Figure 1.1](image)

**Figure 1.1.** The global markets of industrial branches related to microbes and microbial products. The annual market value corresponds to the given year.138,189–197

With diminishing fossil energy sources the demand for alternative, renewable, sources of especially fuel is increasing. One approach is production of organic solvents from replenishable biomass, among which bacterial fermentation shows potential. Biorefineries provide also possibilities for producing other bulk chemicals, such as organic acids, from the side-streams. For example, waste glycerol from biodiesel process can be utilized for propionic acid production.270,275

One major bottleneck in the processes is the costs of downstream pro-
cesses. Also the yield and productivity of the processes are crucial. These can be affected by the stressors inflicted on the host organism in the process. Besides industrial importance, the bacterial stress behaviour studies are also motivated by food microbiology safety aspects. The experimental part of this thesis focuses on *Lactobacillus rhamnosus* and *C. acetobutylicum*. Accordingly, the introductory part approaches the stress phenomena from the point of view of Gram-positive bacteria, and especially through LAB and butanol producing clostridial strains.

1.1 The stressors in bacterial bioprocesses

Different kinds of stressors, mechanical shear, induced from growth environment, or substrates and metabolic products, can affect a bioprocess. To withstand a stress an organism uses part of its resources and potential to cope with conditions outside the optimum, often reducing yields and productivities of the desired product. Under stress cells respond and adapt but also may develop a tolerance towards a condition. Certain kind of stress can induce a tolerance even towards another kind of stress condition.

1.1.1 Heat-shock

Elevated temperature causes a response which is called heat-shock. Microorganisms can be divided to psychrophiles, psychrotrophs, mesophiles, thermophiles, and hyperthermophiles depending on their preferred growth temperature range. The optimal growth temperature for psychrophiles is 15 °C or lower, for psychrotrophs between 20-30 °C, for mesophiles between 20-45 °C, for thermophiles between 55-65 °C, and for hyperthermophiles between 80-113 °C. Stress induced by reduced temperature is relevant especially when LAB are considered. Many fermentations with LAB are inoculated from frozen or lyophilized starters. Also bacteria used as probiotics are stored at low temperatures and viability is crucial for the probiotic effect.

1.1.2 pH related stress

Depending on the pH optimum for growth the bacteria can be divided to acidophiles, neutrophiles, and alkalophiles. Acidophiles have the growth optimum between pH 0-5.5, neutrophiles between pH 5.5-8.0, and alka-
lophiices between 8.0-11.5. Carboxylic acids, such as acetic acid, lactic acid, butyric acid, citric acid, malic acid, fumaric acid, succinic acid, some amino acids (e.g., aspartic and glutamic), propionic, and formic acids, are being produced by bacteria as part of their natural growth. However, acid accumulation is harmful to the cells. The stress effect from weak organic acids is more complex than strong acids such as HCl. Organic acids can cross the cellular membrane more easily in non-dissociated form and dissociate in the cytoplasm lowering cytoplasmic pH. The non-dissociated form of an organic acid can also be harmful for the cell. In case of LAB used as probiotics, acidic stress is also introduced in form of gastric and bile acids when entering the human stomach and intestine. On the other hand, in some fermentation processes, e.g. fermentation of green olives or noodle dough, LAB need to withstand alkaline environments of pH 8.5 or higher.

1.1.3 Product related stress

Also other metabolic products besides organic acids can induce stress. Organic solvents as metabolic end-products can be economically desirable, however for an organism the drawback is that they are in general toxic to the host at least in high concentration. Solvents throughout this thesis refer particularly to organic solvents. The most significant classes of organic solvents are alcohols, aldehydes, hydrocarbons, and their halogenated derivatives. Considering biorefineries and biofuel production, ethanol and butanol are important solvents and also their toxicity in such host organisms as E. coli, Saccharomyces cerevisiae, and C. acetobutylicum, is best understood. Also in expression of recombinant proteins, the production process itself causes burden on the host while the metabolic precursors are drained to the heterologous protein synthesis. Furthermore, non-optimization process itself causes burden on the host while the metabolic precursors are drained to the heterologous protein synthesis. Furthermore, non-optimal integration, i.e. lack of codon optimization and the differing amino acid demands between the recombinant proteins of the introduced biosynthetic pathways and native biomass related proteins can cause additional stress to the native system through limited translation.

1.1.4 Substrate related stress

In high concentrations the substrate sugars can afflict the osmotolerance of the bacterial cells. For example butanol producing C. beijerinckii is inhibited by higher concentrations than 150 g L\(^{-1}\) of sugar. Osmotic
pressure depends on total concentration of dissolved solutes in a solution. It is typically described in terms of osmolality. The osmolality of a solution is elevated with the concentration of sugars and, especially, concentration of salts. The ability to cope with different solution osmolalities is referred as the halotolerance (NaCl tolerance) of the organisms.\textsuperscript{261,263} In addition to osmotic pressure different impurities in the growth medium can act as inhibitors. In biofuel production the feedstock is preferably from a renewable source such as lignocellulosic hydrolysates. The hydrolysate typically contains several types of inhibitors, furans, phenolic compounds, and organic acids.\textsuperscript{159}

1.1.5 Starvation

While living with the excess amount of substrate sugars can be challenging for the bacteria, obviously also the lack of substrates causes stress. During the stationary phase of a batch process bacteria encounter naturally nutrient starvation. In general, many bacteria are well adapted to survive long-term starvation. Depending on the limiting compound, starvation leads to cell energy depletion, limitation of DNA/RNA synthesis and energy depletion, or limitation of protein synthesis in case of carbohydrate, phosphate or nitrogen limitation, respectively.\textsuperscript{89} The adaptation to starvation can also enhance tolerance towards many other stresses such as heat, acid, ethanol, oxidative, osmotic, freeze, disinfectant, and bile.\textsuperscript{57,89,256}

1.1.6 Mixing related stress

In large scale process inadequate mixing may cause stress due to inhomogeneous availability of substrate or oxygen limitation.\textsuperscript{64} On the other hand, the mixing itself can be harmful for the cells via shear stress. This may happen, especially, with aerobic processes where available oxygen is dependent on the agitation speed. The problem is more prominent with animal cell cultures\textsuperscript{107} than generally robust bacterial cells. However, when high agitation speeds are used in some cases the mechanical stress is too much even for the bacterial cells.\textsuperscript{163,266,268} For example, for a filamentous \textit{Streptomyces flocculus} strain agitation speed of 1000 rpm was studied to cause severe damage.\textsuperscript{268}
1.1.7 Oxidative stress and oxygen availability

The availability or lack of oxygen influences the mode of energy conservation in many bacteria. Depending on the need of or tolerance towards oxygen organisms can be divided into obligate aerobes, facultative anaerobes, aerotolerant anaerobes, and strict anaerobes. For the aerobic metabolism O\textsubscript{2} serves as terminal electron acceptor whereas in fermentation the terminal electron acceptor is a fermentation product, e.g. lactic acid or ethanol instead of O\textsubscript{2}. Organisms called microaerophiles, such as \textit{Campylobacter}, require low levels of O\textsubscript{2}, from below 2 to 10 %, to grow. Processes such as N\textsubscript{2} fixation and H\textsubscript{2} utilization are usually carried out with oxygen-dependent or sensitive enzymes and also biofilm formation is influenced by O\textsubscript{2}. The different relationships with O\textsubscript{2} depend on the inactivation of the O\textsubscript{2} sensitive enzymes and toxicity of reactive O\textsubscript{2} derivatives. The toxic products, reactive oxygen species (ROS), follow from the oxygen reduction. ROS can cause damage to cellular compounds, such as DNA, RNA, lipids, and proteins, through oxidation. Aerobes generally possess superoxide dismutase or superoxide dismutase and catalase enzymes to destroy the toxic compounds. The ROS are also utilized in the competition between microorganisms and thus they are related to antibiotic action and resistance.

1.1.8 Hydrostatic pressure

One area of stress which has aroused interest in the recent years is the effect of hydrostatic pressure. Pressure-based methods are typically used for the disinfection and killing of microorganisms. However, studies have shown that elevated yet not lethal pressure can actually enhance the oxygen transfer in a bioprocess and consequently the productivity. Sub-lethal pressure treatment has been also used to enhance probiotic \textit{Lb. rhamnosus} thermotolerance and to alter the end-product distribution of biofuel production related \textit{C. thermocellum}.

1.2 The stress responses of bacteria

The damage from stress may target various cellular structures, including the cell wall, cell membrane, proteins, RNA, and DNA. As various cellular targets can be damaged also different mechanisms are utilized by
the bacteria to endure the stresses. While some stresses damage in par-
ticular specific cellular structures or functions other stressors e.g. ROS react with many different targets. Considering different stressors, the damage targets and response mechanisms also overlap. According to Nezhad et al.\textsuperscript{105} the stress responses may result in following outcomes (adapted from\textsuperscript{105}):

1. Induction of proteins that repair damage, eliminate the stressor, or maintain the cell.

2. Transient increase in tolerance towards a stressor or stressors.

3. Transformation of the cell to a dormant state, e.g. spore formation.

4. Evasion (e.g. in case of pathogens) of host organism defenses.

5. Adaptation through mutations.

The outcomes 1.-3. occur in shorter time scale while the outcomes 4.-5. take place in time-scale of cell generations. The molecular level stress response mechanisms are summarized in Figure 1.2 and further discussed in following subsections 1.2.1-1.2.7. The responses are controlled through signaling systems and transcription factors. These mediators are further introduced in subsection 4.1.

1.2.1 Response to heat-shock

Exposure to high temperatures increases the synthesis of heat-shock proteins (HSPs). The HSPs consist of chaperones, such as GroEL and DnaK which assist the refolding of damaged proteins, and proteases e.g. Lon and ClpAP which destroy improperly folded and denaturated proteins.\textsuperscript{23} The shift to lethal temperature causes also damage of DNA, cytoplasmic membrane, ribosomes, and rRNA. However, the response is known to be only controlled by the level of unfolded proteins.\textsuperscript{137} In the high lethal temperatures cells continue producing HSPs at maximal rate as long as the protein synthesis is possible. In the elevated sublethal conditions the response occurs in different phases after the shift to higher temperature. First (I) in the induction phase the cellular concentration of HSPs increases rapidly. Then follows (II) the adaptation phase in which the
Figure 1.2. The different molecular level approaches to respond to stresses. The numbering of events correspond to the related subsections: 1.2.1 Response to heat-shock, 1.2.2 Response to cold-shock, 1.2.3 Acid stress response, 1.2.4 Starvation response, 1.2.5 Response to osmotic challenges, 1.2.6 Solvent stress response, 1.2.7 Response to oxidative stress.

response is turned down to a level consistent with the particular new elevated temperature. Finally, (III) in the steady-state phase a constant level of HSPs is maintained specific for a certain temperature of growth.137

1.2.2 Response to cold-shock

The shift to low temperature impacts the DNA, making it negatively supercoiled, reduces the membrane lipid fluidity, stabilizes the secondary structures in RNA, and hinders the assembly of ribosomes.137 The ribosome is considered to work as a sensor to cold shock.85 The cold shock proteins (CSPs) are induced after a temperature downshift. The CSPs can bind to single-stranded nucleic acids and enhance the RNA processing via destroying unwanted secondary structures.103 At low to moderate concentration, CSPs act as translational enhancers. The CSP concentration increases during the cold shock response and at a threshold point the translation of nearly all proteins is ceased due to a positive feedback mechanism until the cell is adapted to the new environmental conditions.103 In principle the adaptation pattern is similar to the heat shock adaptation with the addition that the non-CSP synthesis decreases in in-
duction phase (I) and increases again while the CSP level has reached the constant steady-state phase (III). Upon cold shock the cell membrane becomes rigid impairing the membrane-associated functions, i.e. transport, energy generation and cell division. The approaches to restore membrane fluidity comprise increase of unsaturated to saturated fatty acids ratio, relative increase of branched chain fatty acids, increase of cis-unsaturated to trans-unsaturated fatty acids ratio, change in composition of membrane carotenoids, modification of fatty acid chain lengths, and change in polar head groups of fatty acids. The bacteria can also accumulate compatible solutes e.g. trehalose, glycine betaine, and carnitine in response to cold shock. These compatible solutes function as chemical protectants towards denaturation and aggregation of proteins.

1.2.3 Acid stress response

Bacteria have developed a wide variety of mechanisms to respond to the stress caused by low pH. The internal pH homeostasis can be maintained with F$_1$F$_0$-ATPases. The transmembrane proton motive force (PMF) can be generated at the expense of ATP to pump out the excess protons from the cytoplasm. Vice versa the PMF from the passive influx of extracellular protons to the cell cytoplasm can be utilised for ATP generation. Decarboxylases contribute also to maintaining of the pH homeostasis by consuming intracellular proton in the decarboxylation reaction. For example in the external glutamate decarboxylation the reaction product, $\gamma$-aminobutyrate, is exported from the cell via an antiporter resulting to an increase of external pH as $\gamma$-aminobutyrate is more alkaline than glutamate. In addition to glutamate, arginine and lysine decarboxylation systems can be utilised. The deiminase and deaminase systems which function to catalyze the conversion of arginine to ornithine or deamination of glutamine to glutamate, can be utilised to produce NH$_3$ which forms NH$_4^+$ together with the intracellular protons resulting in an increase in intracellular pH. Urea can also be hydrolysed with urease to form NH$_3$. The reaction yields NH$_3$ and carbamate which further spontaneously decomposes to NH$_3$ and CO$_2$. Yet another approach to maintain pH homeostasis is to hinder the proton permeability of the cell membrane. Bacteria carry this out with the conversion of unsaturated fatty acid of the cell membrane to cyclopropane fatty acids (CFA). The exposure to acid may cause damage to proteins. Similarly as in the heat-shock response the damage inflicted on proteins is responded with induc-
tion of chaperones, such as GroEL and DnaK, and induction of proteases to destroy accumulated damaged proteins.\textsuperscript{144}

\subsection*{1.2.4 Starvation response}

When encountering nutrient limitation bacteria can form spores or modify cell morphology. For example \textit{Clostridium} and \textit{Bacillus} species sporulate in starvation whereas some LAB change cell morphology, i.e. cell surface and diminish in size, and \textit{E. coli} diminish in size.\textsuperscript{68,89} Starvation, including lack of amino acids, carbon source, iron, phosphate and fatty acids, is responded with ‘the stringent response’.\textsuperscript{25} In the stringent response the levels of two nucleotides, guanosine 3’-diphosphate 5’-triphosphate and guanosine 3’-diphosphate 5’-diphosphate referred together as (p)ppGpp, are elevated. The (p)ppGpp nucleotides regulate transcription by modulating RNA polymerase activity directly or indirectly. The consequences of the response are the arrest of ribosome biogenesis and cell growth, and the activation of survival-related stress genes.\textsuperscript{25} Some bacteria prepare to survive starvation by accumulating energetic polymeric compounds, such as polyphosphate, trehalose or glycogen. In case of carbohydrate starvation LAB have also been found to respond with amino acid catabolism.\textsuperscript{89}

\subsection*{1.2.5 Response to osmotic challenges}

Osmotic challenges strain the bacterial cell wall and cytoplasmic membrane. Bacteria can respond to the changes in external osmotic pressure by releasing or accumulating inorganic ions, such as K\textsuperscript{+}, or organic molecules, osmolytes. This can be carried out with increased uptake or synthesis of solutes.\textsuperscript{263} Among other, common known osmolytes are glutamate, trehalose, glycine betaine, proline, and ectoine.\textsuperscript{263} In high external osmotic pressure K\textsuperscript{+} is taken up to the cytoplasm via K\textsuperscript{+} -H\textsuperscript{+} -symporter and ATPase (\textit{E. coli}). The osmolytes are synthesized or taken up through transporters with wide substrate specificity, ProP and ProU, choline specific BetT, and betaine specific BetU. In external osmotic down-shift solutes are released from cytoplasm through mechanosensitive channels, such as MscS and MscL. Aquaporin AqpZ contributes to the osmoregulatory system by accelerating transmembrane water flux.\textsuperscript{263} The solutes causing the osmotic challenge also affect the response. Brown et al.\textsuperscript{31} studied the different responses when osmotic pressure was caused by ionic NaCl or non-ionic solute (e.g. sugars). NaCl caused a decrease in cytosolic
K\(^+\) while non-ionic solute increased the K\(^+\) uptake in *E. coli*. LAB have been studied to also increase the saturated/unsaturated fatty acid ratio of the cell membrane as a response to high external osmotic pressure.\(^ {105}\)

### 1.2.6 Solvent stress response

The solvents, for example alcohols such as ethanol or butanol, produced for biofuel purposes can be toxic to the cells, especially the cell membrane is the target of damage. The toxicity of the solvents increases with the chain length.\(^ {66,132}\) In case of butanol, it disturbs the membranes ability to maintain internal pH, hinders glucose uptake, and makes it permeable to ADP and to certain ions.\(^ {19}\) In general the response mechanisms against the solvent stress consists of cell membrane alterations, membrane repairing, and upregulated expression of solvent efflux systems.\(^ {66,238}\) The heat-shock proteins are also expressed as part of this response.\(^ {66,238}\) The approaches to respond with the cell membrane alterations differ between species. In clostridia the saturation of cell membrane fatty acids is increased while in other species such as, *Oceanomonas baumannii*, *Vibrio paraaemolyticus*, and *Oenococcus oeni*, the response has been found to occur vice versa.\(^ {66,238}\) Besides the changes in the fatty acid saturation/unsaturation, the observed cell membrane alterations are caused due to changes in biosynthesis of fatty acids, lipids, peptidoglycan, and bacterial outer membrane proteins.\(^ {139}\) Gram-positive bacteria tend to tolerate solvents better than Gram-negative bacteria. According to Liu and Qureshi\(^ {139}\) in solvent stress Gram-positive bacteria increase the cell membrane fluidity with cell membrane alterations while Gram-negative bacteria have the opposite approach.

### 1.2.7 Response to oxidative stress

As mentioned above aerobic bacteria respond to ROS with synthesis of superoxide dismutases or superoxide dismutases and catalase enzymes to destroy the toxic compounds.\(^ {261}\) In Gram-negative bacteria the ROS are recognized by the peroxide sensor OxyR, whereas in Gram-positive bacteria the equivalent sensor is the metalloregulator PerR. The regulator PerR controls a set of genes related to peroxide destruction. The corresponding enzymes include such as heme catalase KatA and alkyl-hydroperoxide reductase AhpC.\(^ {219}\) PerR is also associated with aerobic stress in anaerobes, for example in *C. acetobutyllicum*. To survive the
Microaerobic conditions *C. acetobutylicum* utilizes superoxide reductases, flavoproteins, NAD(P)H peroxidases, and other peroxidases. Many LAB species contain peroxidase and/or catalase to respond to ROS. In addition *L. helveticus* has been observed to respond to ROS with cell membrane alterations. The activity of oxygen consuming desaturase system resulted in increased fatty acid desaturation. It was also suggested that zinc uptake is related to defense system against oxidative damage. Scott et al. suggested that Zn(II) may protect thiol groups in proteins from oxidative conversion to disulfide bonds. In oxidative stress proteins’ zinc may also substitute for iron, which is cofactor of essential detoxification enzymes but on the other hand boosts the formation of hydroxyl radicals through Fenton chemistry.

### 1.3 The stress induced gene expression in bacteria

According to the central dogma a segment of genetic information from DNA is copied to RNA in transcription. RNA is further used as a template for protein synthesis in translation. Transcription and translation are the way by which cells express their genes. Typically there are a variety of differentially expressed genes related to stress responses. The expression patterns tend to have some overlapping parts between different types of stresses and some parts specific to a certain stress. The differential expression patterns give information about the cellular mechanisms that are relevant for responding to a certain stress. Different methods exist to study the gene expression at the RNA-level. Quantitative PCR (qPCR), Northern blotting or transcript analysis with the aid of affinity capture (TRAC) can be used to study limited set of genes quantitatively while RNA-Seq and microarrays can be used to study the whole transcriptome. qPCR or TRAC are well suited to study multiple samples with few genes of interest providing wide dynamic range and low quantification limits.

To adapt and survive in changing environmental conditions bacteria utilize signal transduction systems to transmit the stimuli for response regulation. The system typically consists of two components, membrane-associated histidine protein kinase which is regulated by environmental stimuli and cytoplasmic response regulator protein (Figure 1.3). The cytoplasmic histidine residue of the histidine kinase is phosphorylated with the expense of ATP. The phosphoryl group is further transferred to
an aspartate residue in the response regulator protein activating it. The activated response regulator is able to bind to its target DNA, and thus regulate gene expression.

Figure 1.3. The membrane-associated two-component signal transduction system: 1. External stimulus, 2. Phosphorylation of the histidine residue of the histidine kinase, 3. Phosphorylation of the aspartate residue of the response regulator protein, 4. Binding of the response regulator to its target DNA.

One approach to regulate gene expression is employing small RNAs (sRNAs). Many sRNA molecules alter the stability and/or translation of the target mRNA by binding to the complementary sequences. In positive regulation by sRNAs, the translation can be boosted when sRNAs bind the target mRNAs altering the secondary structure, and thus unmasking the ribosome binding sites. Alternatively, the binding can lead to negative control through destabilization of the target mRNA or blocking of the ribosome binding sites. Usually Hfq chaperone is required as a cofactor for sRNA to bind the target. For example a number of sRNAs have been shown to contribute to stress response towards toxic metabolites in *C. acetobutylicum*. Another way of regulate gene expression is through sigma factors that are part of the bacterial RNA polymerase and determine promoter specificity. Alternative sigma factors that are activated in presence of specific triggers, can replace a primary sigma factor and bind to distinct promoter sequences. Specific transcription factors may also be utilized to regulate certain stress responses. For example transcription factors SoxR and
Introduction

OxyR regulate the prokaryotic response to oxidative stress.\textsuperscript{21} Despite the fact that gene expression can provide information about the underlying cellular stress response mechanisms, the transcriptomic results do not typically correlate well with the actual proteomic outcome. The correlation between messenger RNA (mRNA) and protein is affected by translation efficiency which is dependent on physical properties of the transcript. The properties, such as the structure and temperature dependent conformation of the mRNA, influence the translation.\textsuperscript{92} The weaknesses in the part of mRNA sequence which helps the ribosome to align start codon to initiate protein i.e. Shine-Dalgarno (SD) sequence decrease translational efficiency.\textsuperscript{221} The density or number of ribosomes has also a major influence on translation.\textsuperscript{63} Other reasons for the absence of correlation are the differences in mRNA and protein half-lives and different regulation strategies for different open reading frames (ORFs). Greenbaum et al.\textsuperscript{87} suggest that ORFs with high variation are regulated on transcriptional level while the genes which show minimal variation are regulated on post-transcriptional level. The disagreement between proteomic and transcriptional analysis has been observed for example by Venkataramanan et al.\textsuperscript{249} in \textit{C. acetobutylicum} metabolite stress response studies and by An et al.\textsuperscript{11} in \textit{Bifidobacterium longum} bile stress response studies.

1.4 Metabolic shifts under stress conditions

Secreted metabolites, one of the direct consequences of stress-induced metabolic changes, can be studied with chromatographic methods. For example, gas chromatography (GC) can be used to analyze volatile compounds while high-performance liquid chromatography (HPLC) can be used to analyze soluble compounds in liquid. The internal metabolism can be studied with or without isotope tracers using gas (or liquid) chromatography combined with mass spectrometry (GC-MS) or nuclear magnetic resonance (NMR).\textsuperscript{79,237,269} If the metabolic network of the studied organism is known, intracellular metabolism can be further investigated by augmenting the measurements with modelling.\textsuperscript{237,269}

The external stress stimuli can affect the cellular metabolism by altering the size of the fluxes carried through reactions or by shifting the use of a certain pathways by enabling or disabling them. In subsection 1.2.3 the contribution of decarboxylation reactions and deiminase/deaminase sys-
tems to maintain pH homeostasis were already mentioned. Acid stress has been studied to affect also carbohydrate metabolism in *L. fermentum* IMDO 130101. The carbon sources, maltose and sucrose, could not be utilized in pH 3.5 compared to consumption in optimal growth pH 5.5.

Although, metabolomics approach is not as widely and coherently applied to study stress phenomena as the other omics techniques, and the underlying mechanisms for the metabolomic changes are not necessarily known. Commonly at least carbohydrate and amino acid metabolism appear to be affected in various stresses. Jozefczuk et al. studied the metabolomic responses of *E. coli* to various stresses, such as cold, heat, and oxidative stress. They observed that in general following the stresses the levels of metabolites related to glycolysis, pentose phosphate pathway (PPP), and tricarboxylic acid (TCA) cycle decreased while the levels of various amino acids increased. Increased levels of various amino acids have been also observed as a metabolic response to butanol stress in *C. acetobutylicum* by Wang et al. Besides the increased amino acid levels, fatty acid, i.e. hexadecanoic acid, octadecanoic acid and monostearin, levels were elevated and the results suggested that glycolysis was inhibited. Similar metabolic responses have been observed in yeast under ethanol stress. According to the results by Dash et al. under butanol and butyrate stress the arginine metabolism of *C. acetobutylicum* is down-regulated. Under butyrate stress also pyrimidine metabolism was down-regulated.

An example of a practical application is that the external stress can affect the flavor compounds produced by sourdough related LAB. Guerzoni et al. studied γ-decalactone, 2(5H)-furanones and aldehydes which were overproduced in LAB following oxidative stress, while acid stress induced the accumulation of isovaleric and acetic acid and higher alcohols. The 2(5H)-furanones and γ-decalactone are important aroma compounds.

### 1.5 Stress tolerance alterations

Tolerance towards a chemical is a complex trait, affected by several parameters such as pH, temperature, osmotic pressure, other small and large molecules and pressure. A tolerant phenotype can be a result of several simultaneous mechanisms in action, including molecular pumps, changes in membrane properties, changes in cell wall composition, altered energy metabolism, and changes in cell size and shape. The changes
that result a tolerant phenotype are mediated through alterations in gene expression (acclimation) or mutation (adaptation). The applied stress tolerance alteration strategies consist of adaptive cultivation, cross protection, addition of medium supplements, and genetic improvement of strains.

1.5.1 Adaptive cultivation

The adaptive cultivation approach has been applied for example to enhance acid, thermal, cold and solvent tolerance. In adaptive tolerance response (ATR) bacteria are pre-exposed to sub-lethal conditions. Subsequently they can tolerate harsher conditions. The ATR in LAB with acid stress adaptation has been especially widely studied. The stress tolerance may be growth-phase dependent, although acid stress related ATR has been induced in both exponential and stationary phase of growth. However, the cells in stationary-phase are more tolerant towards acidic pH. This may be due to cross-protection phenomena and general stress response triggered in the stationary phase.

1.5.2 Cross-protection

In cross-protection pre-exposure to sub-lethal stress enhance the tolerance towards a different subsequent stress. The phenomena has been observed with various bacteria. The probiotic LABs can be spray dried for storage purposes. The survival from the high temperature of the treatment and following storage has been enhanced for example with pre-exposure to salt (osmotic stress), H₂O₂ (oxidative), acid, and elevated temperature. Other examples of cross-protective responses are presented in table 1.1.

1.5.3 Medium additives

The available medium components influence also the capability of bacterial cells to cope with the stressors. Thus, the medium can be supplemented to boost the tolerance towards a certain stress. As it was mentioned above (Subsection 1.2) accumulating of some organic molecules, osmolytes, can improve tolerance towards osmotic stress. In recent studies the tolerance towards hyperosmotic stress has been investigated to improve in Lb. paracasei and in E. coli with addition of osmolytes to the growth medium. In case of Lb. paracasei the added osmolyte was pro-
Table 1.1. Examples of adaptive stress responses leading to enhanced cross-protective tolerance towards another stressor (partially adapted from reference\textsuperscript{199}).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sublethal stresses</th>
<th>Enhanced tolerance towards</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{Lb. johnsonni}</td>
<td>Heat</td>
<td>Freezing, Freezing, freeze-drying, Osmotic stress, ethanol, oxidative stress</td>
<td>253, 29, 143, 80, 33, 254, 239, 174</td>
</tr>
<tr>
<td>\textit{Lc. lactis}</td>
<td></td>
<td>Cold, Acid, Acid, Ethanol</td>
<td>29, 177, 176, 80</td>
</tr>
<tr>
<td>\textit{Listeria monocytogenes}</td>
<td>Heat</td>
<td>Freeze, freeze-drying, Ethanol, Osmotic stress, oxidative stress</td>
<td>141, 33, 186, 69, 133, 244, 16, 262, 200, 203, 59</td>
</tr>
<tr>
<td>\textit{B. cereus}</td>
<td></td>
<td>Heat, acid, ethanol, oxidative stress</td>
<td>54, 119, 32, 33, 177, 69</td>
</tr>
<tr>
<td>\textit{C. perfringens}</td>
<td></td>
<td>Heat, acid, ethanol, oxidative stress</td>
<td>253, 96, 82</td>
</tr>
<tr>
<td>\textit{E. coli} O157:H7</td>
<td></td>
<td>Heat, acid, ethanol, oxidative stress</td>
<td>31, 40</td>
</tr>
<tr>
<td>\textit{Shigella flexneri}</td>
<td></td>
<td>Heat, acid, oxidative stress, Osmotic stress, oxidative stress</td>
<td>141, 33, 186, 69, 133, 244, 16, 262, 200, 203, 59</td>
</tr>
<tr>
<td>\textit{Pseudomonas}</td>
<td></td>
<td>Heat, acid, oxidative stress, Osmotic stress, oxidative stress</td>
<td>141, 33, 186, 69, 133, 244, 16, 262, 200, 203, 59</td>
</tr>
<tr>
<td>\textit{L. monocytogenes}</td>
<td></td>
<td>Heat, acid, oxidative stress, Osmotic stress, oxidative stress</td>
<td>141, 33, 186, 69, 133, 244, 16, 262, 200, 203, 59</td>
</tr>
<tr>
<td>\textit{Salmonella typhimurium}</td>
<td>Osmotic stress</td>
<td>Heat, acid, ethanol, oxidative stress</td>
<td>54, 119, 32, 33, 177, 69</td>
</tr>
<tr>
<td>\textit{Salmonella spp.}</td>
<td></td>
<td>Heat, acid, ethanol, oxidative stress</td>
<td>54, 119, 32, 33, 177, 69</td>
</tr>
<tr>
<td>\textit{Vibrio parahaemolyticus}</td>
<td>Starvation</td>
<td>Freeze, Heat, acid, oxidative stress</td>
<td>253, 96, 82</td>
</tr>
<tr>
<td>\textit{Enterococcus faecalis}</td>
<td>Ethanol</td>
<td>Heat, acid, oxidative stress, Osmotic stress, oxidative stress</td>
<td>141, 33, 186, 69, 133, 244, 16, 262, 200, 203, 59</td>
</tr>
<tr>
<td>\textit{V. parahaemolyticus}</td>
<td>Ethanol</td>
<td>Heat, acid, oxidative stress, Osmotic stress, oxidative stress</td>
<td>141, 33, 186, 69, 133, 244, 16, 262, 200, 203, 59</td>
</tr>
<tr>
<td>\textit{B. subtilis}</td>
<td></td>
<td>Heat, acid, oxidative stress, Osmotic stress, oxidative stress</td>
<td>141, 33, 186, 69, 133, 244, 16, 262, 200, 203, 59</td>
</tr>
<tr>
<td>\textit{L. monocytogenes}</td>
<td></td>
<td>Heat, acid, oxidative stress, Osmotic stress, oxidative stress</td>
<td>141, 33, 186, 69, 133, 244, 16, 262, 200, 203, 59</td>
</tr>
<tr>
<td>\textit{Salmonella typhimurium}</td>
<td>Osmotic stress</td>
<td>Heat, acid, oxidative stress, Osmotic stress, oxidative stress</td>
<td>54, 119, 32, 33, 177, 69</td>
</tr>
<tr>
<td>\textit{Salmonella spp.}</td>
<td></td>
<td>Heat, acid, oxidative stress, Osmotic stress, oxidative stress</td>
<td>54, 119, 32, 33, 177, 69</td>
</tr>
<tr>
<td>\textit{Vibrio parahaemolyticus}</td>
<td>Starvation</td>
<td>Freeze, Heat, acid, oxidative stress</td>
<td>253, 96, 82</td>
</tr>
<tr>
<td>\textit{Enterococcus faecalis}</td>
<td>Ethanol</td>
<td>Heat, acid, oxidative stress, Osmotic stress, oxidative stress</td>
<td>141, 33, 186, 69, 133, 244, 16, 262, 200, 203, 59</td>
</tr>
<tr>
<td>\textit{V. parahaemolyticus}</td>
<td>Ethanol</td>
<td>Heat, acid, oxidative stress, Osmotic stress, oxidative stress</td>
<td>141, 33, 186, 69, 133, 244, 16, 262, 200, 203, 59</td>
</tr>
</tbody>
</table>
line and for *E. coli* glycine betaine. Similarly cold stress tolerance can be enhanced with cryoprotective additives. Different cryoprotectants, such as glycerol and dimethylsulfoxide among wide variety, are reviewed by Hubálek.\textsuperscript{108}

While NaCl causes osmotic stress, on the other hand it’s addition to the growth medium has been studied to enhance tolerance towards acetic acid in *E. coli*.\textsuperscript{104} The tolerance towards acetic acid has been also improved with the addition of amino acids, such as methionine, glutamate, lysine, glycine and arginine.\textsuperscript{245} The availability of oxygen and the choice of carbon source affects the acetic acid tolerance.\textsuperscript{127,245} For example glycerol has been studied to provide better acetic acid tolerance compared to glucose as a carbon source.\textsuperscript{127} The studies on the affect of oxygen are ambiguous. Enhanced survival in acidic conditions has been also achieved with supplementations of gum acacia and citrate.\textsuperscript{53,208}

Micronutrients, such as zinc and manganese, contribute to the oxidative stress tolerance.\textsuperscript{100,109} Zinc supplementation has been recently studied to provide enhanced tolerance towards formate, acetate, butyrate, and butanol in *C. acetobutylicum*.\textsuperscript{267} Zinc has been also observed to improve ethanol tolerance in yeast cells.\textsuperscript{273}

### 1.5.4 Genetic improvement of strains

Stress tolerance can be enhanced by improving strains genetically by targeted modifications or random approaches. The modification strategies principally consist of introduction of exogenous pathways to synthesize compounds with protective function, e.g. osmolytes, and expression of general stress proteins but also case-specific modifications have been applied.\textsuperscript{264} Different examples of enhanced stress tolerance by strain engineering are presented in table 1.2. The native or exogenous tolerance genes and heterologous pathways can be (over)expressed in a plasmid or through chromosomal integration.\textsuperscript{99,110} Random mutagenesis and selection based methods which rely on modifications in regulation or screening of strains enriched with genes from a genomic library provide another approach to achieve more robust strains.\textsuperscript{6,24,173}

Strains can be also engineered by natural or directed evolution. In adaptive laboratory evolution (ALE) sequential selection is carried out under environmental adaptation pressure. In contrast to ATR, ALE is carried out in longer period of time for several generations. ALE can be utilized to further adapt engineered strains with nonnative pathways.\textsuperscript{56,181} Strains
can be improved also with genome shuffling. In genome shuffling the genomes of selected seed strains can be shuffled utilizing fusion of protoblasts. The desired phenotypes can be screened and selected from the resulting populations. The shuffled strains are not considered as genetically modified organisms.130

Table 1.2. Examples of enhanced tolerances by strain engineering.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enhanced tolerance towards</th>
<th>Approach</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus</td>
<td>Acid</td>
<td>Genome shuffling</td>
<td>175</td>
</tr>
<tr>
<td>Lc. lactis</td>
<td>Acid</td>
<td>Expressed glutathione synthetase genes</td>
<td>271</td>
</tr>
<tr>
<td>C. acetobutylicum</td>
<td>Butanol, oxidative stress</td>
<td>Expressed glutathione synthetase genes</td>
<td>274</td>
</tr>
<tr>
<td>Lc. lactis</td>
<td>Acid, heat, cold</td>
<td>Introduction of trehalose biosynthetic pathway</td>
<td>35</td>
</tr>
<tr>
<td>Bifidobacterium breve</td>
<td>Acid, osmotic stress</td>
<td>Introduction of betaine-uptake system</td>
<td>220</td>
</tr>
<tr>
<td>Lc. lactis</td>
<td>Acid</td>
<td>Heterologous expression of dnaK</td>
<td>3</td>
</tr>
<tr>
<td>Lc. lactis</td>
<td>Acid, heat, ethanol, osmotic and oxidative stresses</td>
<td>Heterologous expression of shsp</td>
<td>241</td>
</tr>
<tr>
<td>Lc. lactis</td>
<td>Acid, osmotic and oxidative stresses</td>
<td>Heterologous expression of RecO</td>
<td>265</td>
</tr>
<tr>
<td>Lb. plantarum</td>
<td>Heat, cold, ethanol, butanol</td>
<td>Overexpression of three HSPs</td>
<td>71</td>
</tr>
<tr>
<td>C. acetobutylicum</td>
<td>Butanol</td>
<td>Overexpression of groESL</td>
<td>243</td>
</tr>
<tr>
<td>C. acetobutylicum</td>
<td>Butanol</td>
<td>Overexpression of groESL, grpE, htpG</td>
<td>149</td>
</tr>
<tr>
<td>C. acetobutylicum</td>
<td>Butanol</td>
<td>Genome shuffling</td>
<td>136</td>
</tr>
<tr>
<td>E. coli</td>
<td>Heat</td>
<td>Modifications in regulation</td>
<td>173</td>
</tr>
<tr>
<td>E. coli</td>
<td>Ethanol, ethanol and sodium dodecyl sulfate</td>
<td>Modifications in regulation</td>
<td>6</td>
</tr>
</tbody>
</table>

1.6 Lactic acid bacteria (LAB)

LAB are Gram-positive bacteria belonging to firmicutes with low (≤ 55 mol-%) genomic GC-content.102 Traditionally LAB have been considered as beneficial organisms, some strains are even health promoting, and they are associated with food fermentations such as dairy products, fermented meats, and beverages. They are nonsporulating, catalase-negative, aero-tolerant, yet generally nonrespiring cocci or rods which produce lactic acid as main fermentation product from carbohydrates.102,207 LAB are a diverse group considering the adaptation to different environments. Their
habitats cover extreme temperatures from around 50 °C to 0-2 °C. Certain LAB species can tolerate high salt concentrations (up to 25 % NaCl) and others low pH (around 3.9). Many Gram-positive bacteria respond to stresses with global stress-response regulator, i.e. $\sigma^B$, in contrast LAB respond with several conserved stress proteins, such as DnaK, GroEL, and Clp.\textsuperscript{102}

In general LAB process hexoses through two possible pathways (Figure 1.4). In homofermentative pathway only lactic acid is produced through glycolysis and in heterofermentative pathway, i.e. PPP, besides lactic acid, CO$_2$, and ethanol or acetic acid is produced. Glycolysis yields in theory two lactic acid molecules and two ATP molecules from glucose while heterolactic fermentation yields one molecule of each of lactic acid, CO$_2$, ethanol, and ATP from glucose.\textsuperscript{102,207} In certain conditions, e.g. under carbon limitation, homofermentative pathway is shifted to mixed acid metabolism in which formate, acetate, ethanol, and CO$_2$ can also be produced in addition to lactate (Figure 1.4).\textsuperscript{156}

1.6.1 *Lactobacillus rhamnosus*

*Lb. rhamnosus* are facultative heterofermentative rod-shaped lactic acid bacteria which are involved in food and feed fermentation.\textsuperscript{93} *Lb. rhamnosus* GG, a strain of *Lb. rhamnosus*, was isolated from human intestinal tract by Gorbach and Gordin.\textsuperscript{84} The strain has ability to attach the mucosal cells of the human intestinal tract mediated by protein surface layer called the S-layer and pili.\textsuperscript{39,83,84} It has also high tolerance towards acid and bile.\textsuperscript{84} *Lb. rhamnosus* GG strain is widely studied and commercially sold as probiotic product, formulated to capsules or as additives in dairy products. The probiotic benefits of *Lb. rhamnosus* GG are especially related to diarrhea in children.\textsuperscript{12,233,247} Ljungh and Wadström have reviewed\textsuperscript{140} the probiotic aspects of lactic bacteria, including also *Lb. rhamnosus* GG.

1.6.2 Acid tolerance in probiotic LAB

Many LAB are considered as probiotics and they are incorporated into food products.\textsuperscript{140} The probiotic LAB encounter acid challenge through the fermented products, common delivery food for probiotics, and through the acidity of stomach. The acid related ATR is considered to be multifactorial.\textsuperscript{202} Common elements of acid related ATR are increase in energy production through glycolysis augmentation, reduction of endogenous or-
**Figure 1.4.** Different glucose metabolism pathways in lactic acid bacteria: A) Homofermentative pathway, B) Heterofermentative pathway, and C) Mixed acid metabolism.\(^\text{156}\)

Organic acid production, increase in \(\text{NH}_4^+\) production, or active \(\text{H}^+\) export. General stress response proteins such as DnaK, GroES, and GroEL are commonly overexpressed subsequently to acid stress.\(^\text{202}\) The acid tolerance is increased in LAB during exponential growth and after entry in the stationary phase.\(^\text{89}\) The acid induced ATR conditions in studies have been roughly tens of minutes to one hour exposure to conditions from one unit lower than pH growth optimum to pH one unit higher than the subsequent stress which is close to possible stomach gastric acid pH.\(^\text{28,70,97,141}\) Human stomach gastric juice pH can vary between 1.5 and 3.5.
1.6.3 Freeze stability in probiotic LAB

For storage purposes in industrial practice probiotic LAB can be freeze-dried, refrigerated in fermented products or frozen, e.g. for starter culture use. The survival from these challenges contributes to the industrial performance. In the freezing process, high freezing rate is preferred to slow rate, which will result to formation of larger ice crystals that damage the cells. In addition to damage from ice crystals the cells encounter osmotic and chemical damage from unfrozen fraction of concentrated solutes.\textsuperscript{30,77} In freeze drying frozen water is removed by sublimation under vacuum. Besides freezing rate many factors influence freeze-drying survival, such as bacterial species, presence of lyoprotectants (see Subsection 1.5.3), initial cell concentration, the physiological state of the bacteria, freeze-drying parameters i.e. annealing temperature and time, and rehydration conditions.\textsuperscript{36,62,212} An optimum window of initial cell concentrations for freezing survivability exists, e.g. for \textit{Pseudomonas chlororaphis} between $1 \times 10^9$ and $1 \times 10^{10}$ CFU mL$^{-1}$.\textsuperscript{168} In general the stationary phase cells have higher tolerance towards freezing than those in exponential phase.\textsuperscript{178}

Cross-protection strategies, in which the cells are exposed to sub-lethal stresses prior freezing, can be applied to enhance survival. The survival from freeze-drying has been studied to increase when \textit{L. lactis} subsp. \textit{diacetylactis} SLT6 was exposed to sub-lethal heat stress of 45 $^\circ$C for 30 min prior freezing.\textsuperscript{276} Lower fermentation pH also enhances the freeze-drying survivability.\textsuperscript{167,204}

Other storage methods besides freezing and freeze-drying are spray-drying, vacuum drying, and fluidized bed drying. These drying techniques have certain advantages such as relatively low cost, no need of subzero transportation, and less cellular damage than in freezing and thawing.\textsuperscript{30}

1.7 \textit{Clostridium}

Bacteria belonging to \textit{Clostrium} genus, which is one of the largest bacterial genera, share rod-shaped morphology with Gram-positive-type cell wall, spore forming ability, anaerobic metabolism, and lack of dissimilatory sulfate reduction.\textsuperscript{68} The clostridia are infamous for causing severe disease with powerful toxins such as botulinum but they also possess biotechnological potential. Acetone-butanol-ethanol (ABE) ferme-
tation has been one of the largest biotechnological processes ever performed and it was the major production source for these solvents in the first half of the last century. Although the genus is extremely heterogeneous, clostridia have certain metabolic features that are characteristic for the genus. They are able to produce all 20 proteinogenic amino acids, short-chain fatty acids, and hydrogen from carbohydrates.68 Besides solvent producing strains, clostridia include human health affecting intestinal saccharolytic and proteolytic strains, cellulolytic strains, and strains that are able to carry out bioremediation of substances such as nitroaromatics, chlorinated compounds, and toxic metals.68,172

1.7.1  C. acetobutylicum

*C. acetobutylicum* is a nonpathogenic and solventogenic (solvent synthesizing) soil bacteria. It was first isolated between 1912 and 1914 by Chaim Weizmann. The organism could produce good yields of butanol and acetone, and it was used to supply the acetone needs of the First World War.113 The strain ATCC 824 is considered as the type strain. It was isolated in 1924 from garden soil and it is one of the best-studied solventogenic clostridia.160 *C. acetobutylicum* can utilize various carbon sources as substrate, including broad range of monosaccharides such as pentoses, disaccharides, starches, inulin, pectin, whey, and xylan but not crystalline cellulose.86,160,205

The growth of *C. acetobutylicum* can be divided to three different phases. In acidogenesis (Figure 1.5A) acetate and butyrate are produced and growth occurs exponentially. Subsequent to acidogenesis as a consequence to the decrease in pH, in solventogenesis (Figure 1.5B) growth reaches stationary phase, acids are reassimilated, and solvents, i.e acetone, butanol, and ethanol are produced. The genes for butanol and acetone formation are located in a large 210-kb plasmid, pSOL1, which can be lost. The loss of the plasmid leads to a degenerated strain that can not produce these solvents.42 In the third phase endospores are formed.145 A transcriptional regulator Spo0A is related to activation of cell differentiation including sporulation and solvent production.7,95 In clostridial signal transduction Spo0A is directly phosphorylated instead of the two-component signalling system described in section 4.1.61 This signal transduction system is triggered for example by low intracellular pH, the addition of butyrate, and high levels of carbon source.172
1.7.2 Acetone-butanol-ethanol process (ABE)

In the ABE fermentation acetone, butanol, and ethanol are normally produced by solventogenic clostridia in ratio of 3:6:1, respectively. The ABE fermentation balances from a batch process for *C. acetobutylicum* are presented in Table 1.3. The primary solventogenic clostridia are *C. acetobutylicum*, *C. beijerinckii*, *C. saccharobutylicum*, and *C. saccharoperbutylacetonicum*.

<table>
<thead>
<tr>
<th>Product</th>
<th>Acidogenesis (mol mol(^{-1}) (consumed glucose))</th>
<th>Solventogenesis (mol mol(^{-1}) (consumed glucose))</th>
<th>Total fermentation (mol mol(^{-1}) (consumed glucose))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td></td>
<td>0.3</td>
<td>0.22</td>
</tr>
<tr>
<td>Butanol</td>
<td></td>
<td>0.65</td>
<td>0.56</td>
</tr>
<tr>
<td>Ethanol</td>
<td></td>
<td>0.1</td>
<td>0.07</td>
</tr>
<tr>
<td>Acetate</td>
<td>0.5</td>
<td></td>
<td>0.14</td>
</tr>
<tr>
<td>Butyrate</td>
<td>0.75</td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td>H(_2)</td>
<td>2.5</td>
<td>1.4</td>
<td>1.35</td>
</tr>
<tr>
<td>CO(_2)</td>
<td>2.0</td>
<td>2.3</td>
<td>2.21</td>
</tr>
<tr>
<td>ATP/glucose</td>
<td>3.25</td>
<td>2.0</td>
<td></td>
</tr>
</tbody>
</table>

After the World Wars the economical feasibility of ABE fermentation started to decline as petrochemical production started to be more advantageous. Due to depletion of fossil fuels and environmental aspects, alternative bio-derived fuel options have risen interest again. Among possible biofuel alternatives, such as ethanol, methanol, and biodiesel, butanol has certain advantages. For example, it can be blended directly to gasoline and diesel, it’s less corrosive than ethanol or methanol, it does not have cold start problems as compared to ethanol or methanol blended gasolines, and low solubility of butanol in water reduces the groundwater contamination potential. Besides using butanol directly as transportation fuel or fuel additive, it can be used as C4 feedstock in chemical synthesis, e.g. as a precursor for paints, polymers, and plastics.

In the recent years there have been several attempts to renew the ABE industry in China. According to the review by Schiel-Bengelsdorf et al. in 2013 there were active butanol fermentation plants in Brazil and China. The key challenges in ABE process are feedstock costs, insufficient fermentation performance, sustainable process for solvent recovery, and process water recycle. The operational plants within ten years have been using corn, molasses, sugarcane, and agricultural wastes as feedstocks for fermentation. Besides the feedstock from the food...
crop sources, conversion of lignocellulosic materials is a potential option as they can be obtained as side-streams from agriculture and forest industry. Lignocellulose is a layer-structured material containing 10-55 % cellulose, embedded in a matrix of 11-65 % hemicellulose and 3.5-40 % lignin. The lignocellusic biomass needs to be pretreated i.e. hydrolyzed enzymatically or with acid to provide accessible sugars for the ABE fermentation.

The fermentation performance is dependent on the microbe. Performance can be enhanced through metabolic engineering, for example by increasing butanol tolerance (See table 1.2). However, enhanced butanol tolerance does not necessarily lead to better productivities of butanol. Other strategies for metabolic engineering are e.g. biosynthetic pathway related enzyme modification, engineering towards selective butanol production by removing competing endogenous pathways, sporulation control, and use of heterologous biosynthetic pathways in hosts which have more known genetic system and simpler physiology. The fermentation productivity can also be improved with simultaneous product recovery, as the product is toxic to the host organism, and consolidated bioprocess approaches in which enzyme production, saccharification, and fermentation are integrated into a single process.

The traditional distillation is energy intensive and expensive for butanol recovery. Thus, alternative methods have been developed. The developed options are adsorption, liquid-liquid extraction, pervaporation, perstraction, liquid membranes, and gas stripping. The advantage of the listed recovery methods is also that they can be carried out simultaneously with fermentation. The different recovery methods have been reviewed recently by Kujawska et al. Of the mentioned methods gas stripping shows the most potential to be applied in large scale due to simplicity, clean volatile products, and no mass transfer resistance. In gas stripping ABE solvents are removed from liquid phase to carrier gas/gases produced in fermentation, i.e. H₂ and CO₂, or sparged with nitrogen. Subsequently, the solvents are recovered by cooling the carrier gas in a condenser. The gas can be further recycled back to the fermentor for reuse.
1.8 Constraint-based metabolic network modeling

Modelling of bioprocesses can be carried out at different levels, as a black box model considering only the inflow substrates and outflow products neglecting the insight of biology or by applying biochemical reaction networks with certain amount of complexity. The models can be kinetic, i.e. have time as a variable, or assume steady-state conditions. The steady-state assumption is valid when modelling continuous static system, e.g. chemostat, while the kinetic approach may be more preferable in case of a dynamic system, e.g. batch or fed-batch. Kinetic models can be of stochastic or deterministic nature. The stochastic approach can be appropriate in kinetic modelling especially when the system size is small. Mayank et al. have reviewed the kinetic modelling approaches related to ABE fermentation. Kinetic modelling, i.e. kinetic flux profiling, approach has also been applied by Amador-Noguez et al. to study the TCA cycle and central metabolism of C. acetobutylicum.

When large scale network modelling is considered determining kinetic information for the entire network is not feasible. Instead, a constraint-based approach has been adopted to model genome-scale metabolic networks. In constraint-based modelling the modelled system, i.e. cell, is subjected to certain constraints which determine the solution space within which the system behaves. The information for the genome-scale metabolic network reconstructions are obtained from various sources. The primary sources are as follows (adapted from):

1. Biochemistry. The strongest evidence for a metabolic reaction is an isolated enzyme with proven functionality.

2. Genomics. Functionalities of an ORF can be predicted based on DNA or amino acid sequence homology or the location within genome utilizing the information about the neighbouring genes.

3. Physiology and indirect information. For example a known ability of the cell to produce certain compound.

4. In silico modelling data. A network needs to be able to simulate cell growth, thus required reactions need to be added to reproduce cell behaviour.
A metabolic network model can be represented as a stoichiometric matrix \( S \) of size \( m \times n \). The rows of the matrix represent \( m \) unique compounds and the columns represent \( n \) reactions. If a compound \( i \) is consumed in a certain reaction \( j \) it has a negative coefficient in the entry \( S_{ij} \). Likewise if the compound is produced it has a positive coefficient in the entry \( S_{ij} \). Zero is used as coefficient for compounds not participating a reaction.\(^1\)\(^{,}165\) An additional level of complexity can be obtained with integrating a regulatory network with a genome-scale metabolic model. These integration approaches have been recently reviewed by Vivek-Ananth and Samal.\(^251\)

A metabolic network model can be used to for example find functional states through optimization. Other capabilities of reconstructed metabolic network models are analysis of network properties, model-based interpretation of experimental data, and metabolic engineering.\(^1\)\(^{,}60\) One method to investigate the functional states is flux balance analysis (FBA) in which an optimization problem is formulated around the network. Mass balance equations are assumed to be at steady state, i.e.

\[
S\vec{v} = 0, \quad (1.1)
\]

where \( \vec{v} \) is the vector of fluxes through the network. In the optimization the equation 1.1 is subjected to upper and lower bounds of \( \vec{v} \) and an objective function. Objective function can be for example maximum growth rate.\(^165\) The use of different objective functions is discussed by Schuetz et al.\(^213\)

Several studies have been carried out where genome-scale metabolic models have been applied to investigate the behaviour of \( C. acetobutylicum \). The first genome-scale metabolic models of \( C. acetobutylicum \) were published 2008 by Lee et al.\(^129\) and Senger and Papoutsakis.\(^216\)\(^{,}217\) McAnulty et al.\(^151\) introduced the use of flux ratio constraints in these models. The responses of \( C. acetobutylicum \) to chemical stressors have been studied by Dash et al.\(^47\) using genome-scale metabolic model integrated with transcriptomic data. Kumar et al.\(^124\) analyzed the metabolic network of \( C. acetobutylicum \) under external stress using elementary mode analysis. The metabolic response to an external electron supply was studied by Gallardo et al.\(^78\) using a genome-scale model. In addition, other solventogenic clostridia, namely \( C. beijerinckii \), \( C. thermocellum \), \( C. ljungdahlii \), and clostridial co-culture of \( C. acetobutylicum \) and \( C. cellulolyticum \), have been studied with genome-scale metabolic models.\(^154\)\(^{,}157\)\(^{,}198\)\(^{,}206\) The cen-
tral metabolism of *C. acetobutylicum* has also been investigated in studies using isotopic tracer in $^{13}$C-MFA.$^{15,46,230}$

### 1.9 Scale-down methodology

In an ideal bioreactor the operation is performed in controlled and homogeneous environment. However, in large scale cultivations environmental gradients, i.e. in compound concentrations, pH, dissolved oxygen or temperature, can occur due to inefficient mixing.$^{64}$ In scaling-up when geometrical similarity of reactor is maintained only one fundamental variable can be kept constant. Typical scale-up criteria are constant power input per unit volume, constant volumetric oxygen transfer coefficient, or constant speed at the impeller tip. Following any of these criteria will result to lower agitation in a larger bioreactor compared to the smaller scale.$^{169}$

In scale-down approach larger scale is simulated with smaller scale experiments based on regime analysis. The smaller scale experiments are designed according to the fundamental limiting parameters in larger scale. Scale-down can be used in scaling a new process or in diagnosing problems in large-scale processes. In regime analysis the limiting subprocesses of a process are identified. For this purpose the time scales, or characteristic times, of subprocesses, e.g. substrate uptake and mixing, can be compared.$^{169}$

Special experimental configurations are needed for simulating the larger scale oscillatory gradients in laboratory environment. Gas gradients, i.e. dissolved oxygen or carbon dioxide, can be simulated for example oscillating the gas concentration in a single vessel or circulating culture broth through two connected vessels which have different gas concentrations.$^{169}$ Substrate and pH gradients can be simulated with a stirred tank reactor (STR) combined with a plug flow reactor (PFR).$^{10,131}$ The culture broth is pumped from STR through PFR and back to STR. The well mixed area of large scale is simulated with STR while PFR presents the stagnant zone.$^{169}$ In PFR for example a pH shift can be introduced to simulate pH gradient (Publication I).
Introduction

Figure 1.5. The two phases of *C. acetobutylicum* glucose metabolism A) Acido-
genic pathway and B) Solventogenic pathway. The essential active metabolic
routes are presented with unbroken arrows.
2. Aims of the Study

In this study two different bacteria, *L. rhamnosus* and *C. acetobutylicum*, were used as model organisms to study bacterial stress phenomena in various environmental conditions. The bacteria differ with respect to metabolism, *L. rhamnosus* being facultative anaerobe while *C. acetobutylicum* is strictly anaerobic. Also the process objectives differ for these bacteria. *L. rhamnosus* is grown to produce viable cell mass to be used as an additive in food industry or incorporated into capsules. *C. acetobutylicum* is known for ABE process where acetone, ethanol and butanol are being produced by the bacteria. Butanol is a potential biofuel candidate.

The aims of the study were:

- To study the gradients occurring in large processes with scale-down methodology using *L. rhamnosus* as a model organism, and in more detail:
  - To study the expression responses of a specific set of stress related genes under pH and temperature gradients.
  - To investigate if there is a relation between tolerance towards a subsequent stress and expressions of the genes.
  - To examine the sensitivity and delay of the response.

- To apply genome scale metabolic modelling methodology to study the flux space of *C. acetobutylicum* under butanol stress and glucose limitation.
Aims of the Study
3. Materials and Methods

3.1 Bacterial strains

The bacterial strains, *L. rhamnosus* (ATCC 53103) and *C. acetobutylicum* (DSM 792), were used as model organisms. For subsequent cultures 1 mL of *L. rhamnosus* glycerol stock was used to inoculate 50 mL MRS medium (M.R.S Broth, Lab M, UK). The pre-cultures were incubated for 15 h at 37 °C. The incubated pre-cultures were inoculated in hydrolyzed whey broth containing 5% hydrolyzed whey, 0.6% casein hydrolysate, and 0.0015% MnSO₄·H₂O.

*C. acetobutylicum* was inoculated from 1 mL glycerol stock and incubated for 24 h at 37 °C in 100 mL sterile reinforced clostridial medium (RCM) in a 125 mL air-tight flasks. The medium contained 10 g L⁻¹ meat extract, 5 g L⁻¹ peptone, 3 g L⁻¹ yeast extract, 30 g L⁻¹ D-glucose, 1 g L⁻¹ starch, 5 g L⁻¹ NaCl, 3 g L⁻¹ CH₃COONa, and 0.5 g L⁻¹ L-cysteine. *C. acetobutylicum* was further pre-cultured, 15 mL of the incubated RCM was inoculated to 350 mL of medium containing 60 g L⁻¹ D-glucose, 0.2 g L⁻¹ MgSO₄·7 H₂O, 0.01 g L⁻¹ NaCl, 0.01 g L⁻¹ MnSO₄, 0.01 g L⁻¹ FeSO₄·7 H₂O, 0.5 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ K₂HPO₄, 2.2 g L⁻¹ CH₃COONH₄, 0.01 g L⁻¹ biotin, 0.1 g L⁻¹ thiamin, and 0.1 g L⁻¹ 4-aminobenzoic acid. All the growth media for *C. acetobutylicum* was purged with nitrogen on preparation.

3.2 Bioreactor cultivations

The bioreactor cultivations were carried out as batch or continuous cultivations. The used reactor types were Biostat Q (1 L) and Biostat MD (2 L) (B. Braun Biotech International, Germany). The effects of pH oscillations...
on *L. rhamnosus* were studied using batch cultivations while the other cultivations were operated continuously. The scale-down studies of the temperature and pH fluctuation effects on *L. rhamnosus* were carried out using a plug flow reactor (PFR). The data for the *C. acetobutylicum* model construction in Publication III was obtained from continuous cultivations utilizing a cell immobilization column. The immobilization material was wood pulp rolled in a polyethylene mesh. The details of the column construct are further described by Survase et al.\textsuperscript{232} The butanol stress effects on *C. acetobutylicum* were studied using suspended cells in a continuous cultivation.

### 3.3 The stressors on *L. rhamnosus*

The stressors used in studies on *L. rhamnosus* stress behavior comprised pH oscillation by pH control in a batch cultivation and temperature and pH fluctuations using a PFR in a continuous cultivation. For the pH oscillation studies the pH of the medium was controlled to oscillate around the set point of 5.7 by addition of 8.16 % NH$_3$ and 28.3 % DL-lactic acid during the exponential phase of the cultivation while the bacteria were consuming glucose as their carbon source. The pH was oscillated with a frequency of 6/h and amplitude of 0.1 pH units during 2 to 3 h process time, with frequency of 3/h and amplitude of 0.3 pH units during 3.5 to 4.5 h process time and with a frequency of 2/h and amplitude of 0.5 pH units during 5.25 to 6.25 h process time.

In the scale-down study temperature fluctuations were induced to the continuous process using a PFR with a cooling jacket. The cooling jacket surrounded steel tubing which provided a plug flow reactor volume of 90 mL with an internal tubing diameter of 8 mm. Temperature of the water circulating in the jacket was controlled with a water bath (Haake K40, Germany). Temperature was measured with a digital thermometer (TES 1305 Thermometer, TES Electrical Electronic Corp., Taiwan) from the output line. The pH fluctuations were induced to PFR raising the pH of the growth medium conducted to the PFR with 3M NaOH and neutralizing the medium returning to the stirred tank reactor (STR) with 3M HCl. A circulation time of 60 s was used in the PFR studies.
3.4 The tolerance adaptation tests on *L. rhamnosus*

The effect on the robustness of the bacteria after an induced stressor was investigated in regards to acid tolerance (Publication I) and freeze stability (Publications I–II). The acid tolerance test was carried out using a method described earlier by Saarela et al.\textsuperscript{204} In this test 10 mL growth samples were gathered from a cultivation. The cells were spun down (10 min, 4,300 rcf) (Centrifuge 5804 R, Eppendorf, Germany) and the supernatant was discarded. The cell pellets were washed twice with phosphate buffered salt solution (PBS buffer, pH 7.2) before suspending in 2 mL PBS buffer. An aliquot of 0.2 mL of the cell suspension was further pipetted to 2 mL PBS buffer containing 0.003 g ml\(^{-1}\) malic acid (pH 3.2). The cells were incubated in malic acid solution at 37 °C for 60 or 90 min after which the survival rate was determined.

For the freeze stability test 2 mL growth samples were collected from a cultivation and 0.2 mL of sucrose solution (10 volume-%) was added to the samples. The samples were frozen rapidly placing the sample tubes into dry ice for 5 min after which they were stored in -80 °C. In Publication II also viability after freeze drying was studied. For freeze drying 1 mL samples were collected from a cultivation and they were kept at 20 °C for 30 min before storage at -80 °C. The samples were freeze-dried in 72 h with a laboratory-scale freeze-dryer (Christ Alpha 2-4, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) with constant pressure and coil temperature control. After drying the samples were stored in closed tubes in the desiccator at a pressure of 11 mmHg.

3.5 The stressors on *C. acetobutylicum*

The stress effects on *C. acetobutylicum* metabolism were studied using butanol and substrate limitation as stressors. The studies were carried out in continuous cultivations introducing also the stressors continuously through the inlet feed of the bioreactor. The continuous cultivation was operated with a dilution rate of 0.07 h\(^{-1}\). The butanol stress was induced with 10 g L\(^{-1}\) in the feed medium. The substrate limitation was induced with 30 g L\(^{-1}\) D-glucose concentration while for a reference cultivation 35 g L\(^{-1}\) D-glucose was used. The sampling for further analysis was carried out from a metabolically steady state cultivation.
3.6 Analytical methods

3.6.1 Cell dry weight

The cell dry weight (CDW) samples from a cultivation were immediately frozen and stored in a freezer prior analysis. The CDWs were determined from duplicate samples of 5 mL cell suspension. The samples were centrifuged at 7,650 rcf for 5 min at 4 °C and washed with Milli-Q water twice, and dried at 110 °C overnight.

3.6.2 Cell viability analysis

The cell viability of L. rhamnosus was determined preparing dilution series from differently treated samples. The dilutions of $10^{-4}$, $10^{-5}$, and $10^{-6}$ were plated on MRS agar plates. The cell counts were determined after incubation for 72 h at 37 °C. The cell counts were performed with two or three replicates.

3.6.3 HPLC

High-performance liquid chromatography (HPLC) was used to analyze substrate sugars and external metabolites, mainly organic acids. The samples were centrifuged and the supernatant was collected, diluted properly with Milli-Q water, and filtered through 0.2 μm filters before injection to HPLC column.

In Publication II HP Series 1100 (Hewlett Packard, USA) equipped with HP 1047A (Hewlett Packard, USA) RI detector and Rezex ROA-Organix Acid H+(8%) column (300 × 7.8 mm) (Phenomenex, USA) was used for analysis of glucose, galactose, ethanol, acetate, formate, acetoin, and lactate concentrations. The eluent was 5 mM H$_2$SO$_4$ and a flow rate of 0.6 L min$^{-1}$ was used.

In Publications III and IV HPLC (Waters Alliance 2695, USA) equipped with RI detector (Waters 2414, USA) and Aminex HPX-87P column (300 × 7.8 mm) (Bio-Rad, USA) was used for sugar analysis. The column temperatures of 70 °C and 85 °C with flow rates of 0.6 L min$^{-1}$ and 0.4 L min$^{-1}$ were used, respectively. The organic acid concentrations, acetic and butyric acid, were determined by HPLC (Waters Alliance 2690, USA) equipped with an RI detector (Waters 2414, USA) and Hi-Plex H column (300 × 7.7 mm) (Agilent Technologies, USA). The eluent was 5 mM H$_2$SO$_4$.
with a flow rate of 0.6 L min\(^{-1}\). The column was heated to 65 °C.

### 3.6.4 GC

The analysis of solvents produced in the ABE process (Publications III and IV) was carried out by gas chromatography (GC). In publication IV a gas chromatograph (Agilent Technologies 7890A) equipped with a flame ionization detector was used. The separation took place in a DB-WAXetr capillary column (30 m × 0.32 mm × 1 μm) from Agilent Technologies, Finland. The temperatures were 200 °C and 250 °C, for the injector and detector respectively. Injector volume of 1 μL was used. The GC analysis for the data presented in publication III was carried out similarly with the exception of different injector volume (10 μL) and gas chromatograph (Hewlett Packard series 6890).

### 3.6.5 Transcriptional analysis

For the transcriptional analysis of *L. rhamnosus* transcript analysis with the aid of affinity capture (TRAC)\(^{235,236}\) (Publication I) and quantitative PCR (qPCR) (Publication II) analyses were carried out. The samples were drawn using “rapid spin method” for both of the analyses.\(^{180}\) The suspensions were centrifuged immediately for 30 s with 16,060 rcf (Biofuge Pico, Heraeus Instruments, Germany) at a temperature of 4 °C. The supernatant was frozen in liquid nitrogen and the cell pellets were stored in -80 °C.

The cells were lysed mechanically for TRAC analysis with 100 μL glass beads [Glass Beads 106 μ and finer (acid washed), Sigma, USA] using Disruptor Genie (2 min) (Scientific Industries Inc., USA) in 100 μL lysis buffer (TRACKPACK\(^{TM}\) Lysis Buffer, PlexPress, Finland). For the qPCR analysis the cells were lysed enzymatically in 100 μL buffer containing 20 μL lysozyme (100 mg mL\(^{-1}\); Sigma, USA); 2.6 μL mutanolysin (25 U μL; Sigma, USA); 0.2 μL EDTA, pH 8, 0.5 M (MERCK, Germany); 1 μL Tris–HCl, pH 8, 1 M (Trizma base, Sigma, USA; HCl, J.T. Baker, Netherlands); and 76.2 μL H\(_2\)O (DEPC-treated). The cells were resuspended by vortexing for 10 s. The samples were incubated for approximately 12 min at 37 °C with shaking at 200 rpm (Minitron, Infors, Switzerland).

TRAC was performed from *L. rhamnosus* cell lysates as described by Rautio et al.\(^{185}\) with the exception that gene specific biotinylated and 6-FAM-labeled probes were used. The gene specific probes are presented
in publication I. Briefly, the TRAC protocol consisted of following steps. Gene specific biotinylated and 6-FAM-labeled probes were hybridized to RNA targets. The hybridized RNA targets were captured to streptavidin coated magnetic beads. The beads were washed. The probes were eluted to deionized formamide. The eluents were analyzed by capillary electrophoresis with the separation based on the detection probe sizes and quantitative detection based on fluorescence.

The qPCR analysis is described in detail, and also the gene specific primers used in qPCR are presented, in publication II. Briefly, the protocol from the lysed cells was as follows. RNA was extracted from the cell lysate and treated with DNase. The synthesis of cDNA was carried out from the RNA extractions. The expressions of the studied genes were analyzed from the sample cDNA with qPCR using gene specific primes.

Expression of a reference gene, ldhD, was used to normalize the expression results of the studied genes from both qPCR and TRAC. The gene ldhD, encoding L. rhamnosus primary metabolism related D-lactate dehydrogenase, has been separately studied to be a suitable reference gene for lactic acid bacteria stress studies.55,72

3.6.6 13C labelled proteinogenic amino acid analysis

In Publication IV C. acetobutylicum metabolism was studied using 10 % uniformly 13C labeled glucose in chemostat cultivation substrate feed. The labeled feed was continued for 1.7 residence times resulting in labelling of 82 % of the biomass. Samples containing at least 0.227 g cells were collected by centrifugation, 3,600 rcf for 5 min at 4 °C, for further analysis. The cell pellets were stored at -80 °C. The pellets were lyophilized at -56 °C and 0.010 mbar for 48 h with a freeze-drier (Christ LDC-2 Beta 1-8 K, Martin Christ Gefriertrocknungsanlagen GmbH, Germany). The freeze dried pellets were suspended into 10 mL of 6 M HCl and hydrolyzed in sealed glass tubes at 110 °C for 22 h. The hydrolysates were dried and dissolved in H2O for filtration through 0.2 μm filters and further vacuum-dried. For the NMR experiments the samples were dissolved in D2O. The pH of the samples was below 1 due to residual HCl.

The NMR spectroscopy related to proteinogenic amino acid spectra was carried as described earlier by Jouhten et al.114 at 40 °C on a 600 MHz Bruker Avance III NMR spectrometer equipped with a QCI cryoprobe. The relative abundances of intact carbon fragments in proteinogenic amino acid carbon signals were determined with the software FCAL (R.W. Glaser;
Materials and Methods

3.7 Modeling

3.7.1 Constraint-based reconstruction and analysis (COBRA)

In Publication III a genome scale constraint-based model for *C. acetobutylicum* DSM 792 was expanded from a previously published model by Senger and Papoutsakis.\textsuperscript{216,217} Transport and substrate utilization reactions obtained from the KEGG database\textsuperscript{116} were added. Also reactions for bifurcated TCA cycle and enabling glycine formation from threonine were adapted from a studies by Amador-Noguez et al.\textsuperscript{8,9}

The model for *C. acetobutylicum* was further extended in Publication IV. The amino acid composition of proteins from Lee et al.\textsuperscript{129} was adapted to constraint-based model as ratio constraints. Reaction reversibility constraints were adapted from thermodynamic properties.\textsuperscript{74} The model was augmented with reactions to enable serine formation pathway from 3-phospho-D-glycerate precursor, Entner-Doudoroff pathway, and formation of isoleucine from threonine origin.\textsuperscript{15} Also reactions to form exopolysaccharide were added. The model consisted of 451 metabolites and 604 reactions. For the studies on metabolism related stress responses caused by butanol stimulation and glucose limitation in Publication IV reactions were constrained according to the results from \textsuperscript{13}C-MFA simulations (Subsection 3.7.4) and measured external fluxes, i.e. consumed substrate and formed products. Also the biomass formation reaction was adjusted according to the measured relative protein, RNA, and glycogen amounts which were considered the most sensitive macromolecular components.\textsuperscript{48}

3.7.2 Flux balance analysis (FBA)

Genome-scale constraint-based models were used to study *C. acetobutylicum* metabolism using FBA with the COBRA toolbox\textsuperscript{22,210} in Matlab. In FBA a mathematical model, described in a stoichiometric matrix (S), is used to simulate metabolic fluxes in metabolic steady-state by linear programming. For the optimization problem formulation, in addition to linear equality constraint from steady-state assumption, lower and upper bounds for the fluxes are constrained and an objective function is set, e.g. maximization of growth rate or ATP formation equation. The flux boundaries
can be set according to the experimental or theoretical knowledge if such exists. Otherwise the fluxes can be set to be open by setting a big number like -1000 for the lower bound and 1000 for the upper bound as it was done for the models used in publications III and IV. The optimization problem can be expressed in the following form 3.1:

$$\max \ v_{objective}$$
$$s.t. \ S \cdot \vec{v} = 0,$$
$$v_{min}^* \leq \vec{v} \leq v_{max}^*.$$  

(3.1)

The open-source GLPK linear programming software was used to solve the optimization problems.

3.7.3 Flux variance analysis (FVA)

Usually in the case of large genome-scale models the solution space of fluxes with which the optimum for FBA can be obtained is vast. In FVA the maximum and minimum values of individual fluxes are obtained while still retaining the optimum value, or a certain percentage, of the objective flux. The FVA algorithm can be described as follows 3.2:

$$for \ i = 1..\text{length}(\vec{v}),$$
$$\max \ v_i$$
$$s.t. \ S \cdot \vec{v} = 0,$$
$$v_{min}^* \leq \vec{v} \leq v_{max}^*,$$
$$v_{objective} = \text{optimimum value}.$$  

(3.2)

$$\min \ v_i$$
$$s.t. \ S \cdot \vec{v} = 0,$$
$$v_{min}^* \leq \vec{v} \leq v_{max}^*,$$
$$v_{objective} = \text{optimimum value}.$$  

Also the FVA used in publications III and IV was carried out using COBRA toolbox in Matlab with the GLPK linear programming software to solve the optimization problems.
3.7.4 $^{13}$C metabolic flux analysis ($^{13}$C-MFA)

In $^{13}$C-MFA an isotopic tracer is used in substrate to study intracellular metabolic pathways via mapping of carbon flow through metabolic reactions. The central carbon metabolism model of C. acetobutylicum for $^{13}$C-MFA carried out in Publication IV was mainly adapted from the model described by Au et al. The metabolic model was defined by 37 free net fluxes and 81 free exchange fluxes. Additional constraints for the model were obtained from the biomass composition used earlier by Lee et al. The biomass precursor demands were derived from the macromolecular components ratios and building block prerequisites. As in COBRA models (Subsection 3.7.1) for Publication IV the macromolecular biomass compositions respect to the measured relative protein, RNA, and glycogen amounts were taken into account. The relative abundances of intact carbon fragments in the proteinogenic amino acids obtained via NMR spectroscopy (Subsection 3.6.6) were used with the external flux measurements (Subsections 3.6.3 and 3.6.4) in model fitting. The $^{13}$C-MFA was carried out using 13CFLUX2 software. The details of the simulations are presented more elaborately in Publication IV. Additional constraints from the $^{13}$C-MFA results were transferred to the COBRA models applied in Publication IV.
4. Results and Discussion

4.1 The effects of temperature and pH gradients on *L. rhamnosus* (I-II)

The scale-up of a microbial production process may affect the process performance as only few operational parameters can be maintained constant if geometrical similarity of the vessel is also conserved. The productivity can decrease due to nonhomogenous distribution of dissolved oxygen, substrate, and pH. Zones of high pH can occur due to the base addition for pH control purpose from the top of the reactor. The spatial gradients in a bioreactor regarding pH can for example affect intracellular pH, metabolism, glucose transport, ATP/ADP ratio, and activity of certain enzymes. Typically temperature gradients do not occur in cultures maintained at constant temperature. However, temperature gradients could possibly result to cross-protection effects as discussed in section 1.5.2.

4.1.1 Gene expression responses to pH and temperature shifts in *L. rhamnosus*

The possible gradients occurring in large-scale fermentations were simulated in smaller scale using two different approaches. The first approach was the use of PFR in a chemostat with different pH and temperature shifts (Publication I). In the second approach the pH of a batch process was controlled on a sinusoidal trajectory with various amplitudes and frequencies (Publication II). The differential expression of selected genes (Table 4.1) which were considered to be related to possible cellular stress response mechanisms were studied in these experiments.
Table 4.1. The genes of interest in the transcriptional analysis of *L. rhamnosus*.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Description of the gene product</th>
<th>Publication</th>
</tr>
</thead>
<tbody>
<tr>
<td>hrcA, LGG_01606</td>
<td>Heat-inducible heat-shock operon repressor</td>
<td>I and II</td>
</tr>
<tr>
<td>groEL, LGG_02239</td>
<td>Heat-shock related chaperonin</td>
<td>I and II</td>
</tr>
<tr>
<td>aldB, LGG_01898</td>
<td>Acetolactate decarboxylase</td>
<td>I</td>
</tr>
<tr>
<td>cfa, LGG_02109</td>
<td>Cyclopropane-fatty-acyl-phospholipid synthase</td>
<td>I and II</td>
</tr>
<tr>
<td>fat, LGG_02257</td>
<td>Acyl-ACP thioesterase</td>
<td>II</td>
</tr>
<tr>
<td>atpA, LGG_01181</td>
<td>F-type H-transporting ATPase subunit alpha</td>
<td>I and II</td>
</tr>
<tr>
<td>pstS, LGG_00906</td>
<td>Phosphate transport system substrate-binding protein</td>
<td>I and II</td>
</tr>
<tr>
<td>ldhD, LGG_00158</td>
<td>D-lactate dehydrogenase, reference gene</td>
<td>I and II</td>
</tr>
</tbody>
</table>

Table 4.2. Models for the gene expressions in response to pH and temperature shifts in PFR. *K* refers to temperature (Kelvin).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Model</th>
</tr>
</thead>
</table>
| atpA | \[ \ln(atpA) = -785.61615 - 496.44678 \cdot \ln(pH) + 757524.625 \cdot \frac{1}{K} - 157939328 \cdot \left( \frac{1}{K} \right)^2 \\
|      | + 152964.9531 \cdot \ln(pH) \cdot \frac{1}{K} \]                    |
| hrcA | \[ \ln(hrcA) = -3557.98682 - 19.70627 \cdot \ln(pH) + 2211282.5 \cdot \frac{1}{K} - 343034464 \cdot \left( \frac{1}{K} \right)^2 \\
|      | + 6603.88477 \cdot \ln(pH) \cdot \frac{1}{K} \]                      |
| groEL| \[ \ln(groEL) = 760.091 - 473.25604 \cdot \ln(pH) - 201350.4531 \cdot \frac{1}{K} + 32.65895 \cdot (\ln(pH))^2 \\
|      | + 111142.7656 \cdot \ln(pH) \cdot \frac{1}{K} \]                     |
| pstS | \[ \text{pstS} = -466426 + 248389 \cdot pH - 44121.1 \cdot pH^2 + 2622.499 \cdot pH^3 \] |

\(^a\)In publication I there was an error in the model formula

4.1.2 Responses to pH and temperature shifts using PFR

The pH shifts induced to the PFR were between 5.3-6.2 and the temperature shifts between 32.0-37.0 °C. Of the studied genes *groEL, hrcA, atpA*, and *pstS* responded with differential expressions to the pH and temperature stimulations while *cfa* and *aldB* responses were noisy and showed only small differences in their expressions. The chosen genes are related to heat-shock response (*groEL* and *hrcA*), pH homeostasis (*atpA* and *aldB*), phosphate uptake (*pstS*), and cell membrane alteration (*cfa*). The responses from *cfa* and *aldB* expressions indicate that the cyclopropane fatty acid composition of the cell membrane and acetoin production via 2-acetolactate were not affected by the studied stressor ranges. The *groEL, hrcA, and atpA* expression responses were presented with response surface models. The models for the differentially expressed genes are presented in table 4.2. The response surfaces for *groEL, hrcA, and atpA* expressions are shown in Figure 4.1 and the model for *pstS* expressions is elucidated in Figure 4.2. The statistical key figures for the different models are presented in publication I.

According to the response surface models the *atpA* expression was repressed when an upward shift of pH or a downward shift of temperature
Figure 4.1. Response surfaces of the expressions of genes atpA, hrcA, and groEL. The logarithmic values are transformed back to regular values in figures and the presented values for expressions are normalized values.
Results and Discussion

was inflicted to the PFR but when both shifts occurred at the same time expression seemed to be induced. The gene *atpA* encodes to a subunit of F-type H\(^+\)-transporting ATPase. The ATPase is related to the maintenance of the pH homeostasis of the cell, as it can induce proton pumping from cytoplasm to the extracellular space with the expense of ATP or vice versa. The expression of the genes encoding its subunits have been also studied to give responses to differing pH.\(^5\),\(^8\),\(^12\),\(^6\) The optimal activity of F\(_0\)F\(_\text{ATPase}^\) produced by closely related *Lactobacillus* subspecies, *L. casei* and *L. plantarum*, have been studied to range from pH 5.0-5.5.\(^17\) This is in consensus with the obtained results in publication I and II as apparently the expression of *atpA* was elevated to compensate the loss of activity outside the optimal pH range.

The *hrcA* expression was induced the most with the highest pH shift and downward temperature shift of 1-2 °C. The modelled expression reached the highest value when PFR temperature was between 35-36 °C and with the highest pH value. This was also in consensus with the measurements as the highest measured expression value for *hrcA* expression was with pH 6.2 and temperature 36.2 °C in PFR. The *groEL* expression was induced when there was an upward pH shift in PFR. With lower pH shift in PRF the expression was dependent on temperature. The expression was 

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**Figure 4.2.** Regression model of *pstS* expression. Measured and normalized *pstS* expression values are presented with cross-shaped dots.
induced with an upward temperature shift in PFR while the lowest groEL expression was with the lowest pH and temperature shifts in PFR.

The model structures do not necessarily correspond to a real phenomenon. For example in case of hrcA and groEL expressions, there was high correlation of 0.97 between raw expression data of groEL and its regulator hrcA. However, the expressions were presented with different model structures (Table 4.2) as the chosen structures provided the best fits among the different polynomial regression models.

The chaperonin GroEL, i.e. Hsp60 part of GroEL/GroES system, stabilizes proteins during heat-shock. It is expressed from the groE operon. The expression of the operon is controlled with negative control by repressor HrcA in Gram-positive bacteria. GroEL stabilizes the assembly of HrcA to dimer under normal conditions. The dimer binds to an upstream location, controlling inverted repeat of chaperone expression (CIRCE), of both groE and the operon of its own repressing their expressions. Under stress conditions HrcA is destabilized and released from CIRCE elements, allowing the heat-shock proteins to be expressed, while GroEL is spent to stabilize other proteins. The state of repression is resumed when GroEL is available again to stabilize the HrcA dimer formation.123 The described regulation mechanism explains the high correlation between expression measurements of hrcA and groEL in the PFR studies (Publication I), and synchronous expression patterns in the pH oscillation studies (Publication II). The GroEL expression has been recently studied in relation to increased heat resistance in L. rhamnosus.2

The operon of phosphate-specific transport system (Pst) has been studied to be under positive control of the Pho two-component signal transduction system in B. subtilis.182 The Pho regulon is associated with polyphosphate synthesis. The gene pstS encodes the substrate binding site of the phosphate specific ABC-transporter. Its expression was highly sensitive to external pH shift while the studied temperature range did not affect the expression. The pstS expression was modelled in respect to pH and it was induced almost linearly as the pH shift in PFR was increased. Esteban et al.65 also studied pstS to be expressed at a basal level which escapes the Pho regulation. The Pho regulon exists also in L. rhamnosus.116 Atalla and Schumann13 studied the pst operon to be alkali inducable in B. subtilis while other members of the Pho regulon remained unaffected by the alkaline environment. The phosphate concentration and pH dependency of the pst operon expression has been also ob-
served in *C. acetobutylicum*.\textsuperscript{73} Polyphosphate is a known stress response molecule.\textsuperscript{125} It has many functions, as it can for example substitute for ATP in kinase reactions, provide a reservoir of P\textsubscript{i}, chelate metals, buffer against alkali, and has a regulatory role in the physiological adjustments to growth and stress responses.\textsuperscript{121}

The Pst system has been studied to be associated also with the competence of biofilm formation.\textsuperscript{146,158,164} Neznansky et al.\textsuperscript{158} studied that PstS has a phosphate-independent role in biofilm formation in *P. aeruginosa*. The biofilm formation competence itself is dependent on external pH.\textsuperscript{106,128,161,224}

### 4.1.3 Responses to pH oscillations

The expression of six genes related to the stress response mechanisms were studied in *L. rhamnosus* batch cultivation with pH controlled on a sinusoidal trajectory increasing the amplitude and frequency as the cultivation time increased. The sinusoidal pH trajectory is shown in Figure 4.3 along with the differential expressions of the studied genes. The studied genes included *hrcA*, *groEL*, *atpA*, and *pstS* as they had shown sensitivity to the stress stimuli applied in PFR studies (Subsection 4.1.2). In addition, the expression of the genes *cfa* and *fat* related to the control of cellular fatty acid composition was studied.

A pH change during cultivation time 2 to 3 h with amplitude of 0.1 and frequency of 6/h was not enough to induce expression response in any of the studied genes (Figure 4.3). The sensitivity to the pH change of the genes *pstS*, *hrcA*, and *groEL* was in consensus with the PFR studies as they gave the most clear responses to the pH oscillations. The responses could be observed during two oscillation phases at cultivation times 3.5 to 4.5 h and 5.25 to 6.25 h. During 3.5 to 4.5 h pH was oscillated with an amplitude of 0.3 and frequency of 3/h while during 5.25 to 6.25 h pH was oscillated with an amplitude of 0.5 and frequency of 2/h.

Considering the responses observed in the expression of *pstS*, *hrcA*, and *groEL*, especially the heat-shock response and phosphate uptake were sensitive to the pH oscillations, even to small magnitude of pH unit amplitude. The heat-shock response is often associated with acidic conditions. However, in the pH downshifts the expression of *hrcA* was repressed while in the pH upshifts it was induced. The *groEL* expression also increased in the pH upshifts and was reduced in the following downshifts. This was in consensus with the results from PFR studies. There is a delayed response effect in the *groEL* expression which can be observed in Figure 4.3 during
Figure 4.3. Fold changes of gene expressions elucidated using Matlab spline function (Matlab® 7.0.4.365 (R14) Service Pack 2, The MathWorks Inc) along with the pH changes. The fitted expression is presented as red line, pH as blue dash line, measured fold changes as blue multiplication sign, sampling points as red plus sign, and reference point as black dot. Reprinted with permission of Springer.

pH oscillation of 3.5 to 4.5 h cultivation time, as the groEL mRNA accumulates during the oscillation phase. Cross-covariance analysis results also indicated a delay in the response (Publication II). The groEL mRNA half-life could be estimated to be longer than 7.5 min as the expression continued to increase after a pH oscillation upshift in cultivation time 5.25 to 5.50 h, although the pH downshift had already occurred (Figure 4.3). The regulator hrcA expression had also been already reduced at this time point after the pH upshift.

As observed in PFR studies (Subsection 4.1.2) the pstS expression was highly sensitive to the pH changes. The expression followed the sinusoidal pH trajectory without accumulation of mRNA or delayed effect as observed in the case of groEL expression. Considering the pstS expression as part of the Pho regulon, the results indicate that also the regulation is very sensitive to pH changes while unaffected by the range of the studied temperature changes in PFR studies (Subsection 4.1.2).

The expression of gene fat was elevated as function of increasing cultivation time and pH. The fold change of expression $f_{fat}$ was modelled with the following regression model:

$$f_{fat} = a + b \times pH + c \times t$$
\[ f_{fat} = -4.0148 + 0.226256 \cdot t + 0.816483 \cdot pH. \] (4.1)

The gene \textit{fat} is related to the cell membrane associated fatty acid synthesis. It encodes acyl-ACP thioesterase, which is part of the pathway to synthesize fatty acids such as C12:0, C14:0, C16:0, C18:0, and C18:1. The result of \textit{fat} expression indicate that the production of these fatty acids was elevated as the external pH and cultivation time increases.

As in PFR studies the \textit{cfa} expression was not affected by the external stressors. Surprisingly, also \textit{atpA} expression was unaffected by the pH oscillation considering the responses obtained in PFR studies. However, there was elevated expression of \textit{atpA} after the last pH oscillation phase which had the highest amplitude (Figure 4.3). Otherwise the magnitudes of the pH oscillation amplitudes were relatively small. Apparently, the \textit{atpA} expression was less sensitive to external pH changes than the expressions of \textit{pstS}, \textit{hrcA}, and \textit{groEL}.

### 4.1.4 Freeze stability and acid tolerance of \textit{L. rhamnosus}

In publication I and II the relation between the differentially expressed genes and survival from freezing was studied. Also the relation to survival from malic acid treatment, which corresponds to 1-6 week storage in apple juice,\textsuperscript{204} was investigated in publication I. The survival from freezing in publication II was determined using freeze-dried samples. It was shown that the viability from freeze-drying and regular freezing were comparable. The pH oscillation did not result in enhanced freeze tolerance in the freeze-dried samples. The tolerance towards freezing slightly increased as the cultivation time increased, for both reference and pH oscillated cultivations (Figure 4.4).

In the PFR studies it was observed that the freeze tolerance was reduced with the increased pH and decreased temperature in the PFR. On the contrary the malic acid tolerance was enhanced when pH was increased and temperature was decreased in the PFR (results not shown). However, no connection between the differences in freeze or acid tolerances and the differential expression of the studied genes was found.
4.2 The stress influences on the *C. acetobutylicum* metabolism (III-IV)

One of the major obstacles for commercial scale butanol production is the toxicity of the solvent to the host organism. The understanding of underlying stress phenomena and the stress consequences on the metabolism and cell physiology can provide ways to improve the strain or process.

The metabolism of *C. acetobutylicum* under butanol stress and slight glucose limitation was studied using constraint-based modelling methodology. The model was formulated and validated for secreted fluxes in publication III. In publication IV the model was extended and additional constraints from $^{13}$C-MFA were applied to narrow the solution space of the genome-scale model. The experimental data was obtained from chemostat cultivations.

4.2.1 Genome scale constraint-based model

In publication III the genome scale constraint-based model was used to simulate fluxes from continuous ABE fermentations with 25 different experimental setups. The fermentations were carried out with immobilized cells. The mean absolute error in the simulations for the modelled efflux solvents were 0.31 g L$^{-1}$, 0.49 g L$^{-1}$, and 0.17 g L$^{-1}$ for acetone, butanol,
and ethanol, respectively. For the residual efflux acids the error was 0.32 g L$^{-1}$ and 0.14 g L$^{-1}$ for acetate and butyrate, respectively.

The model’s capability to predict effluxes correctly was validated with an external data set containing six different experimental setups. The validation data set was obtained from continuous ABE fermentations with immobilized cells. The mean absolute errors for the validation data were similar to the mean simulation errors of 25 different experimental setups: 0.24 g L$^{-1}$, 0.60 g L$^{-1}$, 0.17 g L$^{-1}$, 0.63 g L$^{-1}$, and 0.32 g L$^{-1}$ for acetone, butanol, ethanol, residual acetate, and residual butyrate, respectively. It was observed that the model error increased for the validation data obtained from experimental setups which had high dilution rate.

The results from publication III provided a model framework for the studies in publication IV, as the model could simulate cell growth and reproduce effluxes quantitatively. However, in the publication IV the effluxes were constrained according to the experimental measurements since the focus of interest was on the investigation of internal fluxes. Additional constraints for the model used in publication IV were adapted from $^{13}$C-MFA results. The adapted 12 reaction constraints are shown in table 4.3 for different studied cases, i.e. reference, glucose limited, and butanol stimulated cultivation.

### 4.2.2 Chemostat experiments

The chemostat cultivations were carried out under butanol stress, with glucose limitation, and as a reference cultivation (Publication IV). In general the measured results between the reference and glucose limited experiment did not differ much in terms of external fluxes, measured macromolecular components, and cell mass (Table 4.4). The RNA, protein, and glycogen proportions of the macromolecular composition were relatively higher in the butanol stimulated experiment compared to the other two experiments. The cell dry mass obtained in the butanol stimulated was notably less, 0.85 g L$^{-1}$, compared to 2.19 g L$^{-1}$ in the reference and 2.27 g L$^{-1}$ in the glucose limited experiment. The butanol efflux in the butanol stimulated experiment appeared to be diminished while the butyrate efflux was increased. The ethanol and acetone effluxes were also decreased in the butanol stimulated experiment. The reference experiment had the highest glucose consumption rate with respect to the CDW. This was likely due to the high relative amount of viable cell mass. The glucose consumption rates were 88.83 mmol g$^{-1}$(CDW) for the reference,
Table 4.3. The flux constraints adapted from $^{13}$C-MFA results to the genome scale constraint-based models. The nomenclature uses KEGG identifiers.\textsuperscript{116}

<table>
<thead>
<tr>
<th>KEGG Identifier</th>
<th>Reaction</th>
<th>Lower bound, reference</th>
<th>Upper bound, reference</th>
<th>Lower bound, glucose limited</th>
<th>Upper bound, glucose limited</th>
<th>Lower bound, buOH stimulated</th>
<th>Upper bound, buOH stimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>R00342</td>
<td>(S)-Malate + NAD$^+$ &lt;=&gt; Oxaloacetate + NADH + H$^+$</td>
<td>-82.43</td>
<td>-28.54</td>
<td>-111.94</td>
<td>-5.94</td>
<td>-93.93</td>
<td>9.63</td>
</tr>
<tr>
<td>R01513</td>
<td>3-Phospho-D-glycerate + NAD$^+$ &lt;=&gt; 3-Phosphoxoxypyruvate + NADH + H$^+$</td>
<td>3.32</td>
<td>5.86</td>
<td>1.53</td>
<td>4.85</td>
<td>1.28</td>
<td>16.02</td>
</tr>
<tr>
<td>R00228</td>
<td>Acetaldehyde + CoA + NAD$^+$ &lt;=&gt; Acetyl-CoA + NADH + H$^+$</td>
<td>-27.57</td>
<td>28.37</td>
<td>-27.40</td>
<td>50.33</td>
<td>-105.37</td>
<td>45.51</td>
</tr>
<tr>
<td>R01061</td>
<td>D-Glyceraldehyde 3-phosphate + Orthophosphate + NAD$^+$ &lt;=&gt; 3-Phospho-D-glycerol phosphate + NADH + H$^+$</td>
<td>81.38</td>
<td>127.00</td>
<td>77.24</td>
<td>131.91</td>
<td>22.52</td>
<td>119.16</td>
</tr>
<tr>
<td>R04672</td>
<td>(S)-2-Acetolactate + Thiamin diphosphate &lt;=&gt; 2-(alpha-Hydroxyethyl)thiamine diphosphate + Pyruvate</td>
<td>-37.58</td>
<td>93.16</td>
<td>-95.27</td>
<td>99.37</td>
<td>-98.63</td>
<td>99.64</td>
</tr>
<tr>
<td>R00355</td>
<td>L-Aspartate + 2-Oxoglutarate &lt;=&gt; Oxaloacetate + L-Glutamate</td>
<td>-5.56</td>
<td>-0.60</td>
<td>-8.95</td>
<td>-0.80</td>
<td>-27.52</td>
<td>-0.75</td>
</tr>
<tr>
<td>R01655</td>
<td>5,10-Methenyltetrahydrofolate + H$_2$O &lt;=&gt; 10-Formyltetrahydrofolate + H$^+$</td>
<td>-3.93</td>
<td>-0.20</td>
<td>-2.24</td>
<td>0.05</td>
<td>-9.38</td>
<td>0.08</td>
</tr>
<tr>
<td>R01082</td>
<td>(S)-Malate &lt;=&gt; Fumarate + H$_2$O</td>
<td>-16.49</td>
<td>-1.62</td>
<td>-10.19</td>
<td>-0.78</td>
<td>-32.82</td>
<td>-0.16</td>
</tr>
<tr>
<td>R00344</td>
<td>ATP + Pyruvate + HCO$_3^-$ &lt;=&gt; ADP + Orthophosphate + Oxaloacetate</td>
<td>33.43</td>
<td>87.58</td>
<td>13.63</td>
<td>118.59</td>
<td>19.62</td>
<td>110.18</td>
</tr>
<tr>
<td>R10092</td>
<td>HCO$_3^-$ + H$^+$ &lt;=&gt; CO$_2$ + H$_2$O</td>
<td>5.74</td>
<td>73.27</td>
<td>26.15</td>
<td>75.69</td>
<td>-52.55</td>
<td>61.71</td>
</tr>
<tr>
<td>R00212</td>
<td>Acetyl-CoA + Formate &lt;=&gt; CoA + Pyruvate</td>
<td>87.25</td>
<td>130.16</td>
<td>66.27</td>
<td>153.74</td>
<td>22.20</td>
<td>149.69</td>
</tr>
</tbody>
</table>
73.32 mmol g⁻¹(CDW) for glucose limited, and 60.95 mmol g⁻¹(CDW) for butanol stimulated experiment.

Table 4.4. The measured values in different chemostat experiments.

<table>
<thead>
<tr>
<th></th>
<th>Butanol stimulated</th>
<th>Glucose limited</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDW (g L⁻¹)</td>
<td>0.85</td>
<td>2.27</td>
<td>2.19</td>
</tr>
<tr>
<td>Protein (w-%)</td>
<td>0.55</td>
<td>0.42</td>
<td>0.41</td>
</tr>
<tr>
<td>RNA (w-%)</td>
<td>0.08</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Glycogen (w-%)</td>
<td>0.17</td>
<td>0.08</td>
<td>0.07</td>
</tr>
<tr>
<td>Acetic acid (mmol g⁻¹(CDW))</td>
<td>12.93</td>
<td>12.99</td>
<td>10.20</td>
</tr>
<tr>
<td>Butyric acid (mmol g⁻¹(CDW))</td>
<td>9.90</td>
<td>3.23</td>
<td>2.81</td>
</tr>
<tr>
<td>Acetone (mmol g⁻¹(CDW))</td>
<td>7.86</td>
<td>12.47</td>
<td>15.99</td>
</tr>
<tr>
<td>Ethanol (mmol g⁻¹(CDW))</td>
<td>2.19</td>
<td>6.13</td>
<td>7.61</td>
</tr>
<tr>
<td>Butanol (mmol g⁻¹(CDW))</td>
<td>n.d.</td>
<td>31.60</td>
<td>37.23</td>
</tr>
<tr>
<td>Consumed glucose (mmol g⁻¹(CDW))</td>
<td>60.95</td>
<td>73.32</td>
<td>88.83</td>
</tr>
<tr>
<td>Produced exopolysaccharide (g L⁻¹)</td>
<td>0.06</td>
<td>0.33</td>
<td>0.37</td>
</tr>
<tr>
<td>Glucose feed (g L⁻¹)</td>
<td>30.81</td>
<td>29.99</td>
<td>38.07</td>
</tr>
<tr>
<td>Glucose out (g L⁻¹)</td>
<td>21.47</td>
<td>0.00</td>
<td>3.10</td>
</tr>
<tr>
<td>Butanol feed (g L⁻¹)</td>
<td>10.16</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Butanol out (g L⁻¹)</td>
<td>9.42</td>
<td>5.32</td>
<td>6.03</td>
</tr>
<tr>
<td>Dilution rate (1 h⁻¹)</td>
<td>0.07</td>
<td>0.07</td>
<td>0.07</td>
</tr>
<tr>
<td>Working volume (L)</td>
<td>0.35</td>
<td>0.35</td>
<td>0.35</td>
</tr>
</tbody>
</table>

aNot determined

4.2.3 Flux balance analysis results

The genome-scale model was constrained to represent each experimental setup in the chemostat experiments (Publication IV), i.e. the constraints were obtained from the experimental results for the macromolecular compositions, efflux values,¹³C-MFA results (Table 4.3), and uptake glucose values. The FBAs for the differently constrained models were carried out using two different objectives, the maximization of biomass synthesis and maximization of the ATP maintenance equation. The ATP maintenance equation was maximized with the biomass synthesis requirement of 0.95. The maximization of ATP and biomass yields have been commonly used as objectives for FBA.²¹³ The ATP maximization objective is also interesting from the stress response perspective. In the butanol stimulated experiment the butyrate efflux was elevated which resulted in increased ATP generation. Ventura et al. have observed previously that an increased amount of available ATP enables cells to be more resistant to butanol.²⁵⁰ The same effect has been observed also in yeast that an enhanced ATP availability contributes to butanol tolerance.⁸¹ The presence of butanol itself results in the decrease of the intracellular ATP concentration.²⁶ Cер-
tain mechanisms related to stress responses such as chaperonins GroEL and DnaK are also dependent on the ATP availability.\textsuperscript{159}

The FBA results with biomass synthesis maximization revealed that the experimental biomass accumulation rates were close to the simulated maximum growth rates in reference and glucose limited cases. The simulated maximum growth rates were 3.2 % less for the reference than the experimental value and 15.6 % higher for the glucose limited case. As expected, the maximal simulated value for the growth rate in case of butanol stimulation was much higher, 112.9 %, than the actual biomass accumulation while substrate resources are spent to cope with the stress stimulus.

When the ATP maintenance equation was used as the optimization objective for the FBA the highest ATP maintenance output within the cases, 79.10 mmol g\textsuperscript{-1}(CDW), was obtained in butanol stimulated case. The ATP maintenance outputs for the reference was 41.89 mmol g\textsuperscript{-1}(CDW) and for the glucose limited case 66.01 mmol g\textsuperscript{-1}(CDW). Regardless the fact that the biomass synthesis equation was dependent on the ATP formation, the result indicates that the highest biomass per ATP yield was in the reference while the lowest was in the butanol stimulated case.

The flux balance analysis provides only one solution while there can be a vast optimal solution space for the genome-scale constraint-based models.\textsuperscript{1} For the simulated data set in publication III on average 326.3 of the 592 reactions carried fluxes. Close to similar amount of flux carrying reactions was obtained in different experimental setups of publication IV with FBA using different optimization objectives, ranging from 326 to 330 active reactions.

\textbf{4.2.4 Flux variance analysis results}

The flux spaces of the models representing the different experimental setups with \textit{C. acetobutylicum} were investigated using FVA with three different requirements for the optimizations. The experimental setups represented butanol stress, glucose limitation, and a reference cultivation. The applied model was investigating anaerobic metabolism, as \textit{C. acetobutylicum} is known to be strict anaerobe. The objectives for the optimizations were growth rate of at least 0.95 (95 % of the experimental cell mass accumulation), maximization of ATP combined with the growth rate requirement, and maximization of the NADH/NADPH formation combined with the growth rate requirement. The NADH/NAD\textsuperscript{+} ratio has been studied to be very important to redistribute the metabolic flux.\textsuperscript{166}
The NADH/NADPH maximization was carried out, especially to further study the butanol stimulated case. The conversion of butanoyl-CoA to butanol requires oxidation of 2 moles of NADH per produced butanol. In the butanol stimulated case no butanol formation was observed, thus there should be an excess of NADH and NADPH. NADPH is oxidized to NADP⁺ in conversion of butyrylaldehyde to butanol. The NADH and NADPH were interchangeable within the model thus the optimization objective is referred as maximization of both of these compounds due to the limitation in the model. Although their maximizations were carried out separately, the resulting flux spaces barely differed between the two. The flux variations in the central carbon metabolism between the studied cases using different requirements for the optimizations are shown in Figures 4.5 and 4.6.

The glycolysis (Figure 4.5) allowed only slight variance within the involved fluxes in all the studied cases. The fluxes were especially stringent when the maximization of the ATP formation was considered. Only few of the fluxes related to the PPP were stringent. The growth rate requirement in the reference case demanded the Entner-Doudoroff pathway to be active while in the glucose limited and butanol stimulated case the variances in the related fluxes were too high to draw conclusions.

It is known that *C. acetobutylicum* has a bifurcated TCA cycle. The majority of the flux entering the TCA cycle (Figure 4.6) in the reference and glucose limited cases was converted from oxaloacetate to malate. In the butanol stimulated case the conversion of oxaloacetate to aspartate appeared more favorable. Considering the ATP maximization the conversion of aspartate further to uracil via pyrimidine metabolism would be beneficial. However, no uracil was detected experimentally within the detection limit of RI detector in HPLC analysis. A part of the malate was simulated to be converted to pyruvate in the reference and glucose limited case which was in consensus with the earlier studies by Au et al. The solvent and acid yields were relatively low compared to the earlier studies which also resulted in a relatively high flux entering the TCA cycle in all the studied cases. In the reference and glucose limited cases according to the constraints from the ¹³C-MFA the major part of oxaloacetate was converted to malate while in the butanol stimulated case the direction of the reaction could not be determined stringently. A minor part from oxaloacetate and acetyl coenzyme A (aCoA) was converted to 2-oxoglutarate via another branch of the TCA cycle (Figure 4.6) in all of the studied cases.
The \( C_1 \) units of the folate metabolisms were mainly from the pyruvate origin as was reported previously by Au et al.\textsuperscript{15} To obtain maximal ATP formation glycine would be synthesized from \( \text{CO}_2 \) and 5,10-methylenetetrahydrofolate, although according to the earlier studies glycine is formed from threonine.\textsuperscript{9,15} The majority of serine originated from glycine and partially from 3-phosphoglycerate (Figures 4.5 and 4.6). Considering the ATP maximization serine was mainly converted to pyruvate. Amador-Noguez et al.\textsuperscript{9} and Wang et al.\textsuperscript{257} have previously considered succinate formation as a one possible sink for TCA cycle. According to the simulations succinate efflux was not favorable in any of the studied cases, also it was not observed experimentally.

The fluxes for the efflux gases, \( \text{CO}_2 \) and \( \text{H}_2 \), could not be determined strictly using only growth rate requirement for the optimization. The simulation results for the efflux gases in different cases using different optimization objectives are presented in table 4.5. Strict values could be obtained for \( \text{CO}_2 \) efflux using the ATP maximization and for \( \text{H}_2 \) efflux using the NADH/NADPH maximization. The values for the efflux gases were relatively low but in consensus with the other efflux values.

**Table 4.5.** The simulated efflux gas values for different cases using different optimization approaches.

<table>
<thead>
<tr>
<th></th>
<th>Biomass maximization</th>
<th>ATP maximization</th>
<th>NADH/NADPH maximization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butanol stimulated, ( \text{CO}_2 ) efflux</td>
<td>[0..120.82]</td>
<td>[15.51..15.51]</td>
<td>[0..0]</td>
</tr>
<tr>
<td>Butanol stimulated, ( \text{H}_2 ) efflux</td>
<td>[0..364.07]</td>
<td>[0..0]</td>
<td>[310.48..310.48]</td>
</tr>
<tr>
<td>Reference, ( \text{CO}_2 ) efflux</td>
<td>[95.75..120.42]</td>
<td>[109.60..109.60]</td>
<td>[95.75..95.75]</td>
</tr>
<tr>
<td>Reference, ( \text{H}_2 ) efflux</td>
<td>[0..103.05]</td>
<td>[0..0]</td>
<td>[248.69..248.69]</td>
</tr>
<tr>
<td>Glucose limited, ( \text{CO}_2 ) efflux</td>
<td>[41.08..75.34]</td>
<td>[75.34..75.34]</td>
<td>[0..0]</td>
</tr>
<tr>
<td>Glucose limited, ( \text{H}_2 ) efflux</td>
<td>[0..68.81]</td>
<td>[0..0]</td>
<td>[118.81..118.81]</td>
</tr>
</tbody>
</table>

In the butanol stimulated case the NADH/NADPH maximization resulted in an increased flux from oxaloacetate to aspartate which was further metabolized in the cysteine and methionine metabolism via L-homoserine, in the pyridimine metabolism, or converted to threonine (Figure 4.6). NADH can be oxidized with ferredoxin oxidoreductase resulting to formation and efflux of \( \text{H}_2 \). The elevation in \( \text{H}_2 \) effluxes was obtained in the simulations with the NADH/NADPH maximization objective (Table 4.5).
Figure 4.5. A diagram of *C. acetobutylicum* glycolysis and pentose phosphate pathway related fluxes for the studied cases. The presented flux range values are obtained using FVA with different optimization objectives, i.e. maximization of growth rate (Biomass), ATP maintenance (ATP), and NAD\(^+/\)NADP\(^+\) formation rates (NAD/NADP). The dashed arrows refer to multiple reactions. Reprinted with permission of Elsevier Ltd.
Figure 4.6. A diagram of *C. acetobutylicum* TCA cycle and glycine and serine formation related fluxes for the studied cases. The presented flux range values are obtained using FVA with different optimization objectives, i.e. maximization of growth rate (Biomass), ATP maintenance (ATP), and NAD\(^+\)/NADP\(^+\) formation rates (NAD/NADP). The dashed arrows refer to multiple reactions and blue color is used to match arrows and neighboring tables. Reprinted with permission of Elsevier Ltd.
5. Conclusions and future prospects

The bacterial stress response and tolerance are complex phenomena with many overlapping subsystems such as protein refolding and degradation, cell membrane alteration, transportation, gene expression and metabolism. The phenomena relate to different important fields such as the productivity of industrial host organisms, usage of probiotics, and bacterial pathogenicity. The interface with the responses and tolerances to improve a process can be met with different approaches. One approach is to engineer strains with different strategies, introducing novel pathways, overexpression of target genes, and tuning of regulatory systems. Specific cues, i.e. pre-treatments and medium supplements, can also be used to manipulate the performance towards stress. Process conditions, variables such as temperature and pH, also affect the stress response outcome.

Even small variations in the process conditions are detected by the bacterial cells. The bacteria respond to pH and temperature gradients on transcriptional level. The responses of a set of genes in *L. rhamnosus* GG was investigated using two different scale-down study approaches, i.e. oscillation of medium pH with a sinusoidal trajectory in a batch process and in a chemostat with a use of PFR inducing temperature and pH shifts. Regression modelling was used to describe the expressions with respect to temperature and pH changes. A small alteration, at the scale of 0.3 pH units, is sufficient to result in changes in the expression of *pstS* gene which is part of the phosphate regulon and heat-shock related genes while with an alteration of 0.1 pH units no response within the studied set of genes could be detected. Considering a large scale process, the result indicates that pH gradients scale of 0.1 pH would not disturb the organism. In large scale processes the local pH in the alkali addition point and in the bulk liquid can vary roughly within magnitude of 0.2 and 0.8 pH units depending on the setup. 43 Although the response system is sensitive to
the external cues, responses do not necessarily trigger adaptive tolerance response. In the study with sinusoidal pH oscillation the robustness towards freezing was only slightly and negatively affected by the treatment. In the PFR study no direct relation between the expression patterns of the studied genes and survival from the acid stress or freezing could be found.

Constraint-based modelling can be utilized to investigate genome scale metabolic networks from a systems perspective. The models themselves provide a suitable framework to store experimental data and a platform to describe organisms with different levels of complexity. The current state of the modelling approach is still enriched with various ad-hoc solutions. On the other hand the flexibility of the methodology is also its strength. A genome scale constraint-based model for *C. acetobutylicum* was adapted and validated to reproduce experimental effluxes from chemostat cultivation. The model was further combined with contraints from $^{13}$C-MFA and experimental efflux results to investigate flux space under butanol stress and glucose limitation. The flux spaces were probed using FBA and FVA with different optimization objectives. The stringency of the fluxes considering stress robustness related objectives, e.g. maximal available ATP could be identified with the modelling approach. The experimentally constrained network provides a platform for strain engineering design and simulations of strain engineering outcomes *in silico*.

Considering a production process the alternatives to improve productivity or product quality in respect to stress are strain engineering, optimization of process conditions, or pretreating the bacteria, and addition of supplementals. In case of food industry strain engineering is a sensitive topic due to public opinion on genetically modified organisms. Obviously the process optimization is crucial in the production of high volume and low value products. The pretreatments can provide solutions to enhance stress tolerance if the additional process costs are within acceptable range. Stress tolerance can be enhanced also with medium supplements, for example exogenous amino acids or micronutrients. However, they introduce an additional cost and may cause challenges to downstream processing. Regarding ABE process, in situ recovery of toxic butanol shows potential.

The bacterial stress phenomenon, although widely studied, is still a topic that can provide new discoveries. In the future a more holistic view, integrating results from different specific areas of study, on the topic should be adapted. The interest of the studies are branching to vari-
ous applications with different emphases and focuses, such as combating pathogens, probiotic food technology, and bulk and fine chemical bioprocesses, while the underlying phenomena remain the same. The further investigation of the regulatory networks and systematic studies on the different levels of regulation could also provide more coherent insight on the subject.
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The bacterial stress phenomenon is remarkable considering the industrial production organisms but also in prevention of the pathogen growth. Stress can be caused for example by abnormal temperature, pH, metabolic product, osmotic or hydrostatic pressure, lack of substrate, shearing forces, and oxidative radicals. The stress effects are diverse. The stresses occurring in a process can affect productivity, yield, and product quality. A sublethal stress can also improve the strain robustness towards subsequent stresses. In this dissertation the subject of bacterial stress was approached through perspective of probiotic *Lactobacillus rhamnosus* and butanol producing *Clostridium acetobutylicum* applying gene expression studies and metabolic modelling.