THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Towards membrane engineering as a tool in cell factory design

A case study on acetic acid tolerance in *Saccharomyces cerevisiae*

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Cover: Schematic illustration of membrane engineering

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Abstract

The sustainable production of fuels, chemicals, and materials using renewable resources is a necessity if we are to reduce our ecological footprint and the rate of climate change. Lignocellulosic biomass, the major constituent of plant cell walls, is a renewable raw material with great potential due to its high abundance. The conversion of lignocellulosic material into desired products using microorganisms is a promising option, although many microbial production processes fail to reach the titers required for process economy due to cellular inhibition. The inhibitory action of some compounds is related to the physiochemical properties of the cell membrane. Inhibitors may enter the cell by passive diffusion through the lipid bilayer of the cell membrane, or may inhibit cells by partition in the lipid bilayer, altering the membrane properties.

The aim of the research described in this thesis was to evaluate the possibility of engineering the lipid composition of the cell membrane to create microbial cell factories with maintained production capacity when exposed to compounds whose mechanism of inhibition relates to the physiochemical properties of the membrane. Attempts were made to increase the tolerance of *Saccharomyces cerevisiae* to the lignocellulose-derived inhibitor acetic acid, by engineering the cell membrane in order to reduce the rate of acetic acid diffusion. Studies of the acetic-acid-tolerant yeast *Zygosaccharomyces bailii* revealed that its high tolerance relies on its ability to remodel the cell membrane lipid composition so as to greatly increase the fraction of sphingolipids. Further evidence that sphingolipids reduce the rate of acetic acid diffusion was obtained by molecular dynamics simulations of model membranes with increasing fraction of sphingolipids. The lipid metabolism of *S. cerevisiae* was then engineered in an attempt to increase the fraction of sphingolipids in the cell membrane. However, sphingolipid synthesis was unchanged or decreased in these strains. The effect of sphingolipids on acetic acid tolerance in *S. cerevisiae* could therefore not be elucidated, but insight was gained into sphingolipid regulation. To understand the variation in membrane permeation, in particular the extent to which compounds partitioning in the cell membrane change the rate of acetic acid diffusion, the effects of ethanol and n-butanol were investigated. It was found that target titers in ethanol and n-butanol production significantly increased the rate of acetic acid diffusion; n-butanol having a stronger effect than ethanol. Molecular dynamics simulations were then used to suggest mechanisms for the experimental observations.

Key words: Lignocellulose, robustness, inhibitors, *Zygosaccharomyces bailii*, lipidomics, membrane permeability, molecular dynamics simulations, carbon-14 uptake, ethanol, butanol

List of publications

This thesis is based on the following papers, which are referred to as **Papers I-IV** in the text.

Paper I: Lindberg L‡*, Santos AX*, Riezman H, Olsson L, Bettiga M (2013) **Lipidomic Profiling of *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii* Reveals Critical Changes in Lipid Composition in Response to Acetic Acid Stress**. *PLOS ONE 8(9): e73936. DOI: 10.1371/journal.pone.0073936.*

Paper II: Lindahl L, Genheden S, Eriksson LA, Olsson L, Bettiga M (2016) **Sphingolipids contribute to acetic acid resistance in *Zygosaccharomyces bailii*.** *Biotechnol Bioeng. 113(4):744-53. DOI: 10.1002/bit.25845.*

Paper III: Lindahl L, Santos AX, Olsson H, Olsson L, Bettiga M (2017) **Membrane engineering of *S. cerevisiae* targeting sphingolipid metabolism.** *Scientific reports, 7:41868. DOI: 10.1038/srep41868*

Paper IV: Lindahl L, Genheden S, Faria-Oliveira F, Allard S, Eriksson LA, Olsson L, Bettiga M (2017) **Alcohols enhance the rate of acetic acid diffusion in *S. cerevisiae*: Biophysical mechanisms and implications for acetic acid tolerance**. *Manuscript in preparation.*

A correction to **Paper I** has been published. This correction, **Paper I correction**, is given directly after **Paper I** in the thesis.

‡ The former name of the author was Lindberg.

* Contributed equally.

Author's contributions

Paper I: I designed the study and performed the experimental work with the exception of the lipid analysis. I analyzed the data and wrote most of the manuscript.

Paper II: I designed the study and performed the experimental work with the exception of the computer simulations. I analyzed the data and wrote most of the manuscript.

Paper III: I designed the study and performed the experimental work, either by myself or by supervising Helén Olsson, with the exception of the lipid analysis. I analyzed some of the data myself, and supervised the analysis of the remaining data, and wrote most of the manuscript.

Paper IV: I designed the study and performed the experimental work with the exception of the computer simulations. I analyzed the data and wrote most of the manuscript.

Preface

This PhD thesis fulfills one of the requirements for a PhD degree at the Department of Biology and Biological Engineering, Chalmers University of Technology, Sweden. The project started in 2012 as part of a project funded by the Swedish Energy Agency with the aim of developing acid-tolerant yeast strains with efficient xylose consumption. Later, the project was funded by the Chalmers Area of Advance Energy and the Swedish Research Council. The main part of the project has been carried out at the Division of Industrial Biotechnology, at the Department of Biology and Biological Engineering, at Chalmers University of Technology, under the supervision of Dr. Maurizio Bettiga and Professor Lisbeth Olsson.

Lipid analysis was performed in collaboration with Professor Howard Riezman and Dr. Aline Santos at the Department of Biochemistry, University of Geneva, Switzerland. Molecular dynamics simulations were performed in collaboration with Professor Leif Eriksson, and Dr. Samuel Genheden, at the Department of Chemistry and Molecular Biology, University of Gothenburg. The interpretation of the simulations was supervised by Dr. Samuel Genheden. Measurements of acetic acid uptake rate were performed in collaboration with Dr. Stefan Allard, at the Department of Chemistry and Chemical Engineering, Chalmers University of Technology.

Lina Lindahl
April 2017

Contents

Chapter 1

Introduction

Wealth has increased steadily since the industrial revolution, along with the principles of "take, make, and dispose", defining the era termed linear economy (Andrews, 2015). The concept of circular economy is now being promoted, which is based on recycling and reducing the net effects on the environment (Murray et al., 2015). An important part of the circular economy is the sustainable production of fuels, chemicals, and materials from renewable resources, within a concept called bioeconomy (Erickson, 2015).

Lignocellulosic biomass, found in all trees and plants, is the most abundant renewable carbon source on Earth (Lopes, 2015). Products produced from lignocellulosic biomass have lower net carbon dioxide emissions than fossil-based products, due to the fixation of carbon dioxide during growth. Lignocellulosic biomass consists of the carbohydrate polymers cellulose and hemicellulose, together with lignin, a polymer made up of aromatic compounds. The components of biomass can be converted in a sustainable way into desired products by microorganisms, which catalyze reactions efficiently under mild conditions, without the need for hazardous chemicals, or the use of finite natural resources (Keasling, 2010). However, during pretreatment to break down the biomass to fermentable sugars, inhibitory compounds are formed that have negative effects on the physiology of the microorganism, reducing cell growth and productivity (Almeida et al., 2007; Jönsson & Martín, 2016). Maintaining high productivity of the microorganism in the presence of these lignocellulosic-derived inhibitors and other product- and process-related stresses is a major challenge in designing economically feasible microbial processes (Dunlop, 2011; Zaldivar et al., 2001).

My research described in this thesis focuses on the capacity of microbial cell factories to maintain high productivity in the presence of compounds whose inhibitory mechanism relies directly or indirectly on the physiochemical properties of the cell membrane. Hydrophobic compounds partition in the lipid bilayer of membranes and inhibit cells by affecting the structure and properties of the membrane. Hydrophilic

compounds with sufficiently high hydrophobicity to penetrate the lipid bilayer inhibit cells once inside, and therefore rely on membrane permeability for their inhibition.

The general aim of my work described in this thesis was to evaluate the potential of engineering the lipid composition of the cell membrane to create microbial cell factories with higher tolerance to compounds whose inhibitory mechanism relates to the physiochemical properties of the cell membrane. Attention was focused on acetic acid, an abundant lignocellulosic-derived inhibitor, severely affecting the yields and productivity of microbial processes using lignocellulose as the substrate (Almeida et al., 2007). The effects of acetic acid are also of interest as organic acids are one of the platform chemicals suggested from renewable resources (EuropeanCommission, 2015; Sauer et al., 2008), and organics acids are widely used in food preservation (Piper, 2011). The yeast S*accharomyces cerevisiae* was the microorganism chosen due to its established role as an industrial microbial cell factory (Kavšček et al., 2015).

The specific aim of my research was to investigate the possibility of increasing the acetic acid tolerance of *S. cerevisiae* by engineering the lipid composition of the cell membrane to enable a reduction in the diffusion rate of acetic acid. To address the specific aim of this work, a number of research questions were defined and studied. Traditionally, the influx of acetic acid into the cell has been described as passive diffusion across the cell membrane (Casal et al., 2008; Warth, 1989). The first research question was thus defined in order to gain knowledge on ways in which the diffusion rate of acetic acid could be reduced.

1. Which membrane lipid composition reduces the rate of acetic acid diffusion through the lipid bilayer of cell membrane? To address this question, the acetic-acid-tolerant yeast *Zygosaccharomyces bailii* was used as a model to describe the physiological features enabling cell growth in the presence of high concentrations of acetic acid. A comparative study of *S. cerevisiae* and *Z. bailii* was designed to investigate the physiology and membrane lipid profile of the two yeasts in detail, in the absence and presence of acetic acid. A group of membrane lipids called sphingolipids were found to be highly abundant in the cell membrane of *Z. bailii*, and their abundance increased further upon exposure to acetic acid, causing a considerable overall rearrangement of membrane lipids (**Paper I**). This observation prompted the second research question.

2. What is the relation between the fraction of sphingolipids in the cell membrane, and the acetic acid diffusion rate and cell growth of *Z. bailii* in the presence of acetic acid? To address this question, a cross-disciplinary approach was adopted, taking advantage of the predictive power of *in silico* molecular dynamics

simulations of lipid bilayers and *in vivo* inhibition of sphingolipid synthesis (**Paper II**). This led to the definition of the third research question.

3. Under which conditions will a reduction in the acetic acid diffusion rate significantly improve acetic acid tolerance? To address this question, tolerance was defined as the avoidance of intracellular acetic acid accumulation. A model including the physiological mechanisms in *S. cerevisiae* and *Z. bailii* influencing acetic acid accumulation, such as entry and removal, was then created (**Paper II**). With this model in mind, the fourth research question was defined.

4. How can *S. cerevisiae* be metabolically engineered to increase the fraction of sphingolipids in the cell membrane? To address this question, a combined strategy targeting both structural genes and pathway regulation was adopted (**Paper III**).

In the three first studies (**Papers I-III**) acetic acid stress was treated as an isolated problem. However, in an industrial process many factors may inhibit the microorganism, and synergistic effects often occur. The fifth research question was thus formulated to obtain knowledge on the extent compounds which partition in the cell membrane affect the acetic acid diffusion rate.

5. To what extent do ethanol and n-butanol affect the rate of acetic acid diffusion in *S. cerevisiae*, and what are the biophysical mechanisms of their effect? To address this question, the diffusion rate of acetic acid across the cell membrane of *S. cerevisiae* was measured in the presence and absence of ethanol and n-butanol **(Paper IV)**. Molecular dynamics simulations were then used to provide biophysical mechanisms for the observed effects.

A general overview of the research presented in this thesis is given in Figure 1.

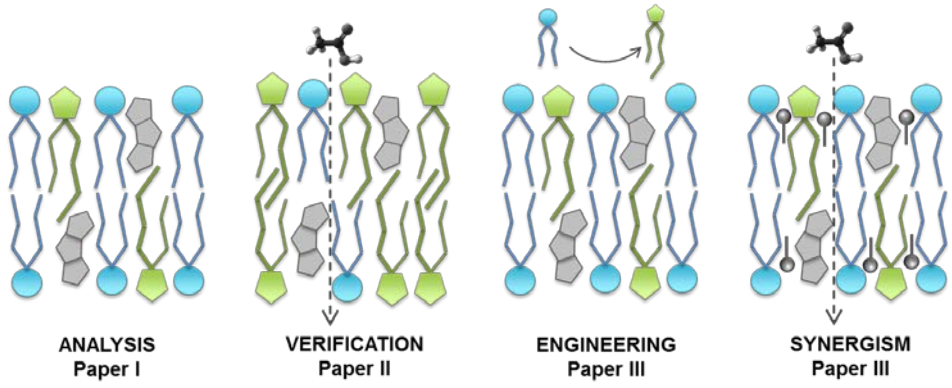


Figure 1: Overview of the research presented in this thesis. The lipid composition of the cell membrane of the acetic-acid-tolerant yeast *Z. bailii* was compared with that of *S. cerevisiae* (**Paper I**). It was then confirmed that the high fraction of sphingolipids found in the cell membrane of *Z. bailii* was responsible for the reduced acetic acid diffusion rate, and was necessary for the high acetic acid tolerance of *Z. bailii* (**Paper II**). An attempt was made to increase the fraction of sphingolipids in the cell membrane of *S. cerevisiae* (**Paper III**). Finally, the synergistic effects of ethanol and n-butanol on the acetic acid diffusion rate and acetic acid tolerance of *S. cerevisiae* were investigated (**Paper IV**).

The long-term goal of research in this field is to develop robust microbial cell factories. **Chapter 2** therefore discusses the challenging conditions to which microorganisms are exposed during industrial processes. Furthermore, as the overall aim of the present research was to investigate membrane engineering as a tool for the development of robust cell factories, extra emphasis is placed on inhibitors whose inhibitory mechanism relates to the physiochemical properties of the cell membrane. In addition, as the specific aim of this work was to use membrane engineering to increase the tolerance of *S. cerevisiae* to acetic acid, one section is dedicated to the description of the physiological mechanisms of acetic acid inhibition. In **Chapter 3**, the present work on membrane engineering is related to other ways of improving microbial robustness, by summarizing the current literature on the strategies used to improve acetic acid tolerance in *S. cerevisiae*. To provide the reader with the knowledge required to understand the concept of membrane engineering, **Chapter 4** presents a broad description of the biology of membranes. In this chapter, membrane organization, membrane lipid structure and lipid biosynthesis are described together with second functions of membrane lipids, and the reason why membrane homeostasis is so important. The methods used in membrane research are also briefly discussed. **Chapter 5** presents the essence of my findings, together with facts and my interpretation of the results available in the literature. Firstly, the identification of lipids suitable for membrane engineering is discussed, and two engineering strategies are described: changing the abundance of the cells' own lipid species, or introducing novel lipid species from other organisms into the microorganism of interest. Secondly, the conditions under which reduced membrane permeability may affect the intracellular concentration of a specific compound, and the interaction between inhibitors related to the physiochemical properties of the cell membrane are discussed. Finally, the state of the art concerning the field of membrane engineering is presented, and challenges and opportunities in membrane engineering are discussed.

Chapter 2

The requirement for robust microbial cell factories

Robust microorganisms are a prerequisite for the sustainable production of fuels and chemicals, from renewable resources, at high yields and productivities using microbial cell factories. In an industrial process, the production organism must be able to cope with inhibitors present in the raw material and cell inhibition by the product or product intermediates. In addition, industrial process configurations often subject the microorganism to various kinds of stress. The degree of inhibition varies between different organisms (Nicolaou et al., 2010), and this should be taken into consideration when selecting the microorganism to use in a specific process.

2.1 Industrial microbial cell factories

During the past decades, extensive knowledge has been obtained on a number of microorganisms, which are now continued to be used due to the great deal of information available on the microorganisms, the optimized genetic tools, their compatibility with current process setups, and their generally recognized as safe (GRAS) status. The bacterium *Escherichia coli*, the yeast *Saccharomyces cerevisiae*, and filamentous fungi belonging to the *Aspergillus* genus are a few examples of well-known microbial cell factories that are now used to produce a range of industrial products (Gustavsson & Lee, 2016). The research described in this thesis focuses on *S. cerevisiae*, a yeast that was domesticated thousands of years ago, and traditionally used for wine making, brewing and baking. This yeast is particularly suitable for the production of biofuels and biochemicals from lignocellulosic materials as, in addition to being a widely used industrial microorganism, it also has a high ethanol production capacity, and a relatively high tolerance to inhibitors derived from lignocellulose (Almeida et al., 2007; Olsson & Hahn-Hägerdal, 1993).

Emerging alternatives to traditionally used microorganisms are natural producers of the desired product, or organisms naturally tolerant to the specific process conditions. For example, the yeast *Scheffersomyces stipitis* is popular due to its natural ability to consume

xylose. The food spoilage yeast *Zygosaccharomyces bailii* has also been investigated as a cell factory for weak acid production (Branduardi et al., 2014) due to its extreme tolerance to weak acid preservatives, such as acetic acid (Zuehlke et al., 2013). The biodiversity of nature can also be used to gain insight into unique physiological traits that can then be transferred to traditional microbial cell factories by genetic engineering. However, even when the molecular mechanisms for the specific trait have been characterized, it is often difficult to transfer the observed phenotype into traditionally used microbial cell factories due to the high degree of complexity of most cellular processes, and the physiological differences between organisms. In addition, it is a challenge in many cases to couple phenotypic behavior with detailed molecular traits. In my research, *Z. bailii* was characterized to understand the traits that are important for its high acetic acid tolerance, and to identify features that could be engineered into in *S. cerevisiae* (**Papers I** & **II)**. In addition to its high acetic acid tolerance, *Z. bailii* was studied due to its relatively close relationship with *S. cerevisiae*, perhaps making it easier to transfer critical molecular traits.

2.2 Stress on the production organism

Microorganisms used as microbial cell factories are exposed to compounds and process conditions that have negative effects on cell physiology. Lignocellulose is a challenging substrate for microbial conversion as, in addition to fermentable sugars, it contains a range of inhibitory compounds (Almeida et al., 2007). Furthermore, many of the biofuels and biochemicals to be produced by microbial conversion inhibit critical cellular functions (Nicolaou et al., 2010). As the present research was focused on membrane engineering as a tool in cell factory design, this section discusses compounds causing cell inhibition, with special emphasis on compounds whose inhibitory mechanism relates to the physiochemical properties of the cell membrane, as described in Box 1.

2.2.1 Lignocellulosic inhibitors

Lignocellulosic inhibitors can be divided into three subclasses, furans, weak acids, and phenolics (Almeida et al., 2007; Jönsson & Martín, 2016). An overview of the composition of lignocellulosic biomass and its inhibitors is presented in Figure 2. Before using lignocellulose as a raw material in microbial production processes, it must be broken down by pretreatment and hydrolysis. During these processes, fermentable sugars are released from the carbohydrate polymers cellulose and hemicellulose. Depending on the harshness of the pretreatment method, sugars may be degraded into the inhibitory furans, hydroxymethylfurfural (HMF) and furfural, which may in turn be further degraded into the inhibitory weak acids, levulinic acid and formic acid. The

inhibitory weak acid, acetic acid is released from acetyl groups on the hemicellulose polymer even under relatively mild pretreatment conditions. The degradation of lignin generates a diverse mixture of inhibitory phenolic compounds.

Box 1: Two types of compounds whose mechanism of inhibition relates to the physiochemical properties of the cell membrane

Type 1: Hydrophobic molecules that partition in the lipid bilayer and inhibit cells by affecting the membrane structure and properties. Membrane engineering intended to prevent inhibition by type 1 inhibitors aims at restoring the original membrane properties, and the challenge is to identify the membrane lipid composition that restores membrane function.

Type 2: Hydrophilic molecules with sufficient hydrophobicity to penetrate the lipid bilayer and inhibit vital functions once inside the cell. Membrane engineering intended to prevent inhibition by type 2 inhibitors aims at increasing membrane rigidity to reduce the permeability of the membrane to the specific inhibitor. Note that reduced membrane permeability will only be efficient if cells have the capacity to balance inhibitor inflow with mechanisms for inhibitor removal.

The two types are defined to ease the discussion on membrane-related inhibitors but compounds may belong to both types.

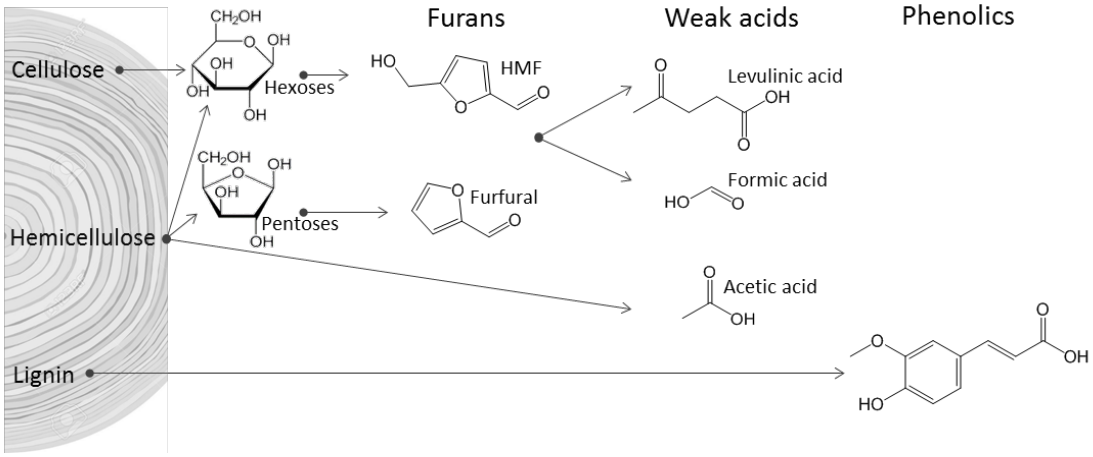


Figure 2: Illustration of the composition of lignocellulosic biomass and its inhibitory degradation products. The exact composition depends on the raw material and the pretreatment method. Hexoses and pentoses are used as carbon sources by the microorganism, while furans, weak acids, and phenolics are inhibitory to most microbial cell factories, and are commonly defined as lignocellulose-derived inhibitors.

The mechanisms of cell inhibition by lignocellulose-derived inhibitors are diverse and only partly understood. Weak acids enter the cell by passive diffusion across the cell membrane (Casal et al., 2008; Warth, 1989), and inhibition due to these is therefore indirectly related to the cell membrane lipid composition, best described as type 2 membrane-related inhibitors (Box 1, Table I). In this work, the reduction of weak acid membrane permeability was proposed as a means of reducing cell inhibition, and

attention was focused on acetic acid as this is the most abundant weak acid present in lignocellulosic raw material. Information on the effect of furans and phenolics on the cell membrane is scarce in the literature. Most of these compounds are relatively hydrophobic, which likely enable them to diffuse into the cell across the cell membrane. However, these compounds affect cell growth at relatively low concentrations (Adeboye et al., 2014; Almeida et al., 2007; Ask et al., 2013a), so it is likely that the cell inhibition by furans and phenolics is mainly due to their interaction with specific cellular functions rather than membrane-dependent inhibition.

Table I: Some of the inhibitory compounds encountered in biofuel and biochemical production from lignocellulose.

Inhibitor group	Membrane related inhibitor	References
Substrates		
Weak acids	Type 2	**Paper I**; Guo & Olsson, 2014
Furans	Unknown	-
Phenolics	Unknown	-
Products		
Alcohols	Type 1	Huffer et al., 2011, Stanley et al., 2010
Fatty acid derived products	Type 1	Bothun et al., 2016; Sheng & Feng, 2015
Carboxylic acids	Type 1/2	Jarboe et al., 2013; Sauer et al., 2008

Type 1: Hydrophobic compounds which partition in the cell membrane and inhibit cellular physiology by affecting the membrane structure and function. Type 2: Hydrophilic compounds with sufficient hydrophobicity to penetrate the cell membrane and inhibit vital functions inside the cell. Inhibition by type 2 compounds is indirectly related to the lipid composition of the cell membrane, as reduced membrane permeability can reduce the accumulation of the inhibitory compounds in the cell.

2.2.2 Inhibitory products

Many products are toxic to the microorganism at the titers required for commercial production (Keasling, 2010). Alcohols such as ethanol and butanol inhibit the growth and productivity of microorganisms due to their partition in the lipid bilayer of the cell membrane, causing altered membrane properties (Paper IV; (Huffer et al., 2011; Stanley et al., 2010). Therefore, they are classified as type 1 membrane-related inhibitors (Box 1, Table I). Products derived from fatty acids, such as free fatty acids, fatty acid methyl esters, fatty alcohols, and alkanes also inhibit microorganisms by affecting membrane structure (Bothun et al., 2016; Sheng & Feng, 2015), and are also classified as type 1 membrane-related inhibitors. Carboxylic acids produced from renewable resources are attractive building blocks for the chemical industry (Sauer et al., 2008). Carboxylic acids with shorter chain lengths inhibit cells in a similar way to lignocellulose-derived weak acids (Jarboe et al., 2013), and are thus classified as type 2 membrane-related inhibitors.

Carboxylic acids with longer chain lengths are defined as fatty acids, and are more belonging to type 1 membrane-related inhibitors. The overall aim of my research was define concepts that will pave the way for membrane engineering targeting both type 1 and type 2 membrane-related inhibitors, although the focus of my research was mainly on type 2 inhibitors. It should be noted that dividing inhibitors into two types are done to clarify the different modes of cell inhibition related to the physiochemical properties of the cell membrane, but compounds may belong to both types.

2.2.3 Inhibitory process conditions

In addition to inhibitory compounds, industrial cell factories must also tolerate process-related stresses such as suboptimal temperature, inhomogeneous mixing, shear stress and variations in the raw material composition (Koppram et al., 2014). High temperature increases the fluidity of membrane lipids, but this is compensated for to a certain extent by changes in membrane lipid composition in response to temperature change (Klose et al., 2012; Turk et al., 2011). The desire for processes with high substrate loadings, called high-gravity processes, further increases stresses related to mixing, substrate inhibition, and product inhibition (Koppram et al., 2014; Xiros et al., 2017).

2.2.4 Acetic acid inhibition in S. cerevisiae

S. cerevisiae produces small amounts of acetic acid during growth on glucose, and metabolizes acetic acid after the diauxic shift. In the presence of glucose, acetic acid consumption is repressed and under such condition, acetic acid enters the cell in its undissociated form, either by passive diffusion across the lipid bilayer, or by facilitated diffusion through the aquaglyceroporin channel Fps1 (Casal et al., 2008; Mollapour & Piper, 2007; Warth, 1989). In my research, attention was focused on ways of reducing the passive diffusion of acetic acid though the lipid bilayer of the cell membrane, and did not consider on the possible facilitated diffusion through Fps1. This decision was made based on the fact that Fps1 is actively degraded in the presence of acetic acid (Mollapour & Piper, 2007), and therefore less industrially relevant, as cell propagation is almost always combined with adaptation to the relevant inhibitors. Moreover, the main function of Fps1 is in osmoregulation by controlling the release of intracellular glycerol (Ahmadpour et al., 2014; Tamás et al., 1999). Acetic acid diffusion through Fps1 is therefore dependent on the cells need to release glycerol. In my experiments, Fps1 did not contribute to the rate of acetic acid diffusion into *S. cerevisiae* (**Paper IV**), as it did in a previous study (Mollapour & Piper, 2007).

Cell inhibition by acetic acid occurs primary once the acid is inside the cell, thus inhibition is greatest under the conditions resulting in the highest intracellular acetic

acid concentration. Figure 3 summarizes the mechanisms determining the concentration of acetic acid inside the cell, and the effects on the cell at high levels of intracellular acetic acid. The distribution of undissociated and dissociated acetic acid depends on the pH, and its pKa value of 4.8. Industrial processes involving yeast are often performed at around pH 5, to avoid contamination by bacteria, which prefers a higher pH (Albers et al., 2011). Once inside the cell, acetic acid dissociates as the cytosolic pH of *S. cerevisiae* is close to neutral, causing acetate accumulation and a reduction in intracellular pH (Russell, 1992). The amount of acetate accumulated and the decrease in pH depend on the capacity of the cell to remove acetate and protons from the cell. It has been reported that the efflux of the weak acid anions occurs through the ABC transporter Pdr12 (Piper et al., 2001), but it was later shown that Pdr12 is only responsible for the efflux of longer weak acids and not for shorter weak acids such as acetic acid and formic acid (Nygård et al., 2014). Instead, the paralogs Tpo2 and Tpo3 have been proposed to transport acetate ions out of the cell, based on the fact that they are multidrug transporters localized in the cell membrane, and are upregulated in the presence of acetic acid (Mira et al., 2010a). The proposed role of Tpo3 in acetate efflux was further supported by the finding that a *tpo3*⊿ mutant exhibited a prolonged acetic-acid-induced lag phase, as well as increased intracellular acetic acid accumulation compared to the wild type (Fernandes et al., 2005). Proton accumulation and the decrease in intracellular pH are counteracted in *S. cerevisiae* by the activity of Pma1, a cell membrane ATPase actively pumping protons out of the cell (Serrano et al., 1986). Cells able to grow in the presence of acetic acid are probably able to balance the rate of acetic acid influx, with the rate of acetate and proton efflux.

Cell inhibition by acetic acid is observed on an increased ATP demand, as efflux is ATP dependent, leading to a reduction in ATP pools (Pampulha & Loureiro-Dias, 2000; Ullah et al., 2013) and lower biomass yield (**Paper I**). High acetic acid concentrations also reduce the maximum specific growth rate (**Papers I** & **IV**). If the rate of acetic acid influx is higher than the rate of efflux, acetate will accumulate and the intracellular pH will be reduced. This causes secondary effects, such as reduced enzyme activity (Pampulha & Loureiro-Dias, 1990; Zhao et al., 2008), and changes in the membrane's electrochemical gradient (Orij et al., 2011). Acetic acid stress also generates reactive oxygen species (ROS), which damage vital cellular functions, and if the acetic acid dose is high, the cell undergoes apoptosis (Ludovico et al., 2001) through a ROS-mediated mechanism (Giannattasio et al., 2005). An increase in lag phase was observed experimentally with intracellular acetic acid accumulation (**Paper I**). Cells exposed to acetic acid also exhibited difficulties in starting to grow after the diauxic shift. This second lag phase could be explained as a consequence of glucose depletion, and the cells having less energy to use for acetate and proton efflux, again leading to acetate accumulation, requiring time for the cell to recover (**Paper I**). The high acetic acid

tolerance of *Z. bailii* compared to *S. cerevisiae* is a combined effect of the low acetic acid diffusion rate in *Z. bailii* (**Paper II**), and its ability to consume acetic acid in the presence of glucose (Rodrigues et al., 2012; Sousa et al., 1998), thereby providing an efficient mechanism to counteract intracellular acetate accumulation, further discussed in **Section 5.2.1**.

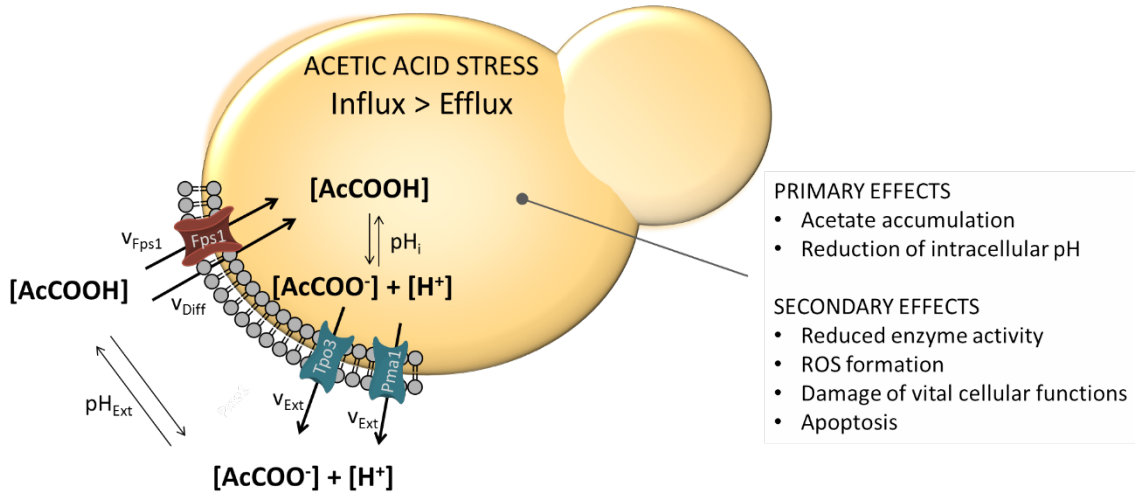


Figure 3: Overview of mechanisms determining the intracellular concentration of acetic acid in *S. cerevisiae*, and the cellular effects of acetic acid exposure. The pH determines the distribution between the undissociated and the dissociated forms of acetic acid, based on the pKa value, which is 4.8 for acetic acid. Mainly undissociated acetic acid enters the cell, either by passive diffusion across the lipid bilayer (v_{Diff}), or by facilitated diffusion through the Fps1 aquaglyceroporin channel (v_{Fps1}). The inhibitory effect of acetic acid is therefore highly dependent on cultivation pH, and cell inhibition by acetic acid occurs mainly when the intracellular pH (pH_i) is higher than the extracellular pH (pH_{Ext}). Undissociated acetic acid enters the cell until equilibrium is reached between the concentrations inside and outside the cell. Undissociated acetic acid is simultaneously in equilibrium with its dissociated form, and at an intracellular pH above 6, only a small fraction of the acid is undissociated, while a much larger fraction is dissociated. The difference in pH inside and outside the cell leads to the primary effects of acetic acid, which are acetate accumulation and a reduction of intracellular pH. However, *S. cerevisiae* counteracts these primary effects by acetate efflux (v_{Ext}) presumably through Tpo3 and proton efflux through Pma1 (v_{Ext}).

Recently, the long lag phase of *S. cerevisiae* observed upon exposure to high concentrations of acetic acid has been attributed to population heterogeneity in terms of intracellular pH (Fernández-Niño et al., 2015; Swinnen et al., 2014). Cells with a lower intracellular pH suffer from less acetate and proton accumulation after acetic acid exposure, and can therefore extrude the accumulated acetate and protons more quickly and resume growth. Intracellular pH is consequently a critical determinant of the cells' ability to resume growth after acetic acid exposure. The lag phase observed after acetic acid stress is thus a combination of the time required for the cells to adapt to the acetic acid, and a measure of the number of cells with sufficiently low intracellular pH to resume growth (González-Ramos et al., 2016). The increased tolerance of cells preadapted to acetic acid (Sànchez i Nogué et al., 2013), is probably a combined effect

of increased expression of genes related to acetic acid stress (Lee et al., 2015) and the lower intracellular pH of preadapted cells (Fernández-Niño et al., 2015; Stratford et al., 2013).

On the genetic level, weak acid stress activates the Haa1 and War1 transcription factors specifically involved in weak acid stress, as well as Msn2 and Msn4, the transcription factors involved in the general stress response of cells (Fernandes et al., 2005; Mira et al., 2010a; Mira et al., 2010b; Schüller et al., 2004). Upon activation, the transcription factor binds to the promotor region of the genes under its control, and regulates gene expression. A subset of genes under the control of these transcription factors has been confirmed to improve weak acid tolerance (Fernandes et al., 2005; Mira et al., 2010a; Schüller et al., 2004).

Chapter 3

Strategies for strain engineering

Strains can be engineered to improve their tolerance to a specific stress by random approaches without any information on the molecular mechanisms involved in dealing with the stress. If information is available on the molecular mechanisms involved, targeted strain engineering can instead be applied. In my research, I investigated the molecular mechanisms underlying the high acetic acid tolerance of *Z. bailii* (**Papers I** & **II**), and defined a targeted strain engineering approach with the aim of reducing the rate of acetic acid diffusion into the cell (**Paper III**). In this chapter, strategies for stain engineering are discussed based on the findings reported in the literature regarding improved acetic acid tolerance. The results of each strategy are given in terms of growth, viability or ethanol production. However, the results are highly dependent on the specific conditions used, so the values given should not be used to compare the efficiency of the different strategies presented. The various strategies are summarized in Table II.

3.1 Random approaches

Random approaches for strain engineering have the advantage that improved strains can be generated without any knowledge of the molecular mechanisms dealing with the stress. Furthermore, known complex physiological mechanisms can be targeted by random approaches in an easier setup than if the mechanisms would have been addressed by targeted strategies. If properly evaluated, random approaches also have the potential to provide physiological information on the specific stress. This section describes random strain engineering strategies based on a survey of studies available in the literature on improved acetic acid tolerance. Details of the strategies are listed in Table II.

Table II: Literature review of strategies used to improve acetic acid tolerance of *S. cerevisiae*

Modification	Target	Effect	Reference
Random approaches			
Random mutagenesis and genome shuffling	No predefined target	60% shorter lag phase	Zheng et al., 2011
Evolutionary engineering	No predefined target	75% increase in specific xylose consumption rate, but the evolved phenotype was lost after growth without selective pressure	Wright et al., 2011
Random mutagenesis and evolutionary engineering	No predefined target	1000-fold increase in survival (CFU count[1])	González-Ramos et al., 2016
Single gene deletions, by screening of yeast deletion collection	No predefined target	A list of candidate genes involved in acetic acid tolerance	Sousa et al., 2013; Teng et al., 2011; Kawahata et al., 2006; Mira et al., 2010b
Evaluation of cells' transcriptional response after stress exposure	No predefined target	A list of genes up- or down-regulated in response to acetic acid	Abbott et al., 2007; Bajwa et al., 2013; Kawahata et al., 2006; Lee et al., 2015; Mira et al., 2010a
Screening of library with histone point mutations	No predefined target	45% shorter lag phase	Liu et al., 2014
Screening of mutant alleles of the TATA-binding transcription factor *SPT15*	No predefined target	Improved growth (Drop test[2])	An et al., 2015
Screening of library with artificial transcription factors	No predefined target	Shorter lag phase and higher specific growth rate	Ma et al., 2015
Targeted approaches			
Increase in sphingolipid fraction by overexpression of *ELO3* and *AUR1*, and deletion of *ORM1* and *ORM2*	Reduced acetic acid inflow	Unaltered or decreased sphingolipid membrane fraction. Effect on acetic acid tolerance not evaluated	**Paper III**
Increase in oleic acid content in membrane lipids by *ELO1* overexpression	Reduced acetic acid inflow	45% increase in survival (CFU count[1])	Zheng et al., 2013

Prevention of acetic acid inflow through *FPS1* by *fps1Δ*	Reduced acetic acid inflow	Improved growth (Drop test[2])	Mollapour and Piper, 2007; Zheng et al., 2011., Zhang et al., 2011
Acetate conversion into ethanol	Reduced acetate accumulation and increased ethanol yield	15% higher ethanol yield	Wei et al., 2013
Pumping protons into the vacuole by overexpression of the V-ATPase *PEP3*	Reduced intracellular pH decrease	75% shorter lag phase	Ding et al., 2015
Increase flux through the pentose phosphate pathway by overexpression of *TAL1* and *TKL1*	Compensation for reduced enzyme activity due to intracellular pH decrease	40% higher ethanol yield	Hasunuma et al., 2011
Cell flocculation by *FLO1* expression	Enhanced general stress tolerance	80% increased ethanol production rate	Du et al., 2015
Increased expression of genes involved in acetic acid tolerance by overexpression of *HAA1*	Enhanced acetic acid stress response	Improved growth (Drop test[2])	Tankara et al., 2012
Increase redox capacity by overexpression of glutaredoxin *GRX5*	Reduced oxidative damage	30% increase in ethanol productivity	Fang et al., 2015
L-ascorbic acid production	Reduced oxidative damage	4-fold increase in survival (CFU count[1])	Martani et al., 2013
Spermidine accumulation by overexpression of *SPE1*, *SPE2*, *SPE3*	Enhanced general stress tolerance	35% shorter lag phase	Kim et al., 2015
Proline accumulation by deletion of *PUT1*	Enhanced general stress tolerance	85% increase in cell viability	Greetham et al., 2014
Trehalose accumulation by deletion of *NTH1* and *NTH2*	Enhanced general stress tolerance	Improvement in growth (Drop test[2])	Yoshiyama et al., 2015

Targeted approaches for improving acetic acid tolerance of *S. cerevisiae* are illustrated Figure 4. [1] Counting the number of colony forming units on agar plates. [2] Dilution series of cells dropped onto agar plates to evaluate cell growth.

Microorganisms have traditionally been improved by an iterative process of mutagenesis (using radiation or chemicals) and screening to select for strains with the phenotype of interest (Crook & Alper, 2012). This classical approach was later combined with methods such as genome shuffling and evolutionary engineering. Genome shuffling is a genome-scale recombination method in which the genomes of two strains recombine, for example, by mating or protoplast fusion, to create strains with mixed genomes (Biot-Pelletier & Martin, 2014). Genome shuffling has been proven effective in reducing the lag phase of *S. cerevisiae* cells exposed to acetic acid (Zheng et al., 2011).

Evolutionary engineering, also called adaptive laboratory evolution, is a method of evolving cells towards a specific phenotype (Dragosits & Mattanovich, 2013). Cells are often mutagenized to increase diversity, and then cultured in either batch or continuous cultures for several hundred generations under increasing selective pressure. This method is simple, and improved strains are almost always generated, however, the selective pressure must be carefully designed so that other important physiological traits are not lost (Hahn-Hägerdal et al., 2005). *S. cerevisiae* cells with the capacity to consume xylose in the presence of 6 g/L acetic acid (pH < 4), have been developed by evolutionary engineering (Wright et al., 2011). However, the xylose consumption capacity was lost after growth without selective pressure, and could only be induced by preadapting the cells to acetic acid. To improve the initial response of cells to acetic acid stress, rather than bring about adaptation, González-Ramos et al. designed a new evolutionary engineering approach, employing batch cultures of evolving cells at increasing concentrations of acetic acid, alternated with batch cultures without selective pressure (González-Ramos et al., 2016). This technique resulted a 1000-fold increase in the survival of *S. cerevisiae* cells grown on agar plates containing 9 g/L acetic acid at pH 4.5.

Random mutagenesis and evolutionary engineering are frequently used methods in strain development. This has in my experience resulted in little scientific progress in terms of elucidating molecular mechanisms, due to poor characterization of the strains developed. However, the increased access to whole-genome sequencing, and techniques for the characterization of the molecular mechanisms underlying tolerant phenotypes, have increased the potential of random approaches, not only to develop improved strains, but also to elucidate the mechanisms involved in a specific phenotype (Caspeta et al., 2014; González-Ramos et al., 2016).

Another random approach used to identify targets for strain engineering involves the utilization of commercial collections of *S. cerevisiae* strains with single gene deletions of each of the non-essential genes in the genome (Winzeler et al., 1999). These deletion collections have been generally available for more than a decade, and are frequently

used by researchers to quickly identify the genes that are important in a specific phenotype. Acetic acid stress has been studied by screening for mutants exhibiting affected growth in the presence of acetic acid (Kawahata et al., 2006; Mira et al., 2010b; Teng et al., 2011), and mutants showing reduced cell survival after exposure to acetic acid (Sousa et al., 2013). Increased acetic acid sensitivity has been observed in 213 (Kawahata et al., 2006), 640 (Mira et al., 2010b), 1642 (Teng et al., 2011), and 488 (Sousa et al., 2013) of the strains in which one gene had been deleted. Common categories of the identified genes were "carbohydrate metabolism", "transcription", "intracellular trafficking", "ion transport", "biogenesis of mitochondria", "ribosome" and "vacuole". Lipid metabolism has not been reported as a common category, but Mira et al. and Kawahata et al. highlighted a list of genes involved in membrane lipid biosynthesis.

Major disadvantages of screening yeast deletion collections are that only single gene deletions can be evaluated, which makes it difficult to identify important traits requiring multiple gene alterations, and the deletion of a gene responsible for sensitivity to a particular treatment does not directly imply that the specific gene is important for the tolerance of the cells to the treatment. It may also be difficult to extract the essence in all the data generated and the gene categories identified as being important for acetic acid tolerance, provide little understanding of the physiology underlying the mechanisms associated with the tolerance to acetic acid stress. Furthermore, the genes identified in different studies show surprisingly poor agreement: only 19%, 16%, and 11% of the genes identified in the studies by Kawahata et al., Mira et al., and Teng et al., were the same as the genes identified in the study by Sousa et al. (Sousa et al., 2013). This poor agreement suggests that the genes identified depend strongly on the experimental setup, and demonstrates that it is important to compare data from several studies to accurately identify the genes that are important for specific phenotypes.

The genes involved in tolerance to a particular kind of stress can also be identified by investigating the transcriptional response of the cells after exposure to stress. Genome-wide transcriptome analysis of cells exposed to acetic acid has led to the identification of upregulated genes within categories such as "stress response", "metal metabolism" and "cell wall architecture", and downregulated genes in "metabolism" and "protein synthesis" (Abbott et al., 2007; Bajwa et al., 2013; Kawahata et al., 2006; Lee et al., 2015; Mira et al., 2010a). The cell membrane proton pump *PMA1*, the putative acetate efflux pumps *TPO2* and *TPO3*, fatty acid desaturase *OLE1*, glycerophospholipid regulator *INO1*, and ergosterol biosynthesis genes are specific examples of upregulated genes in response to acetic acid exposure. As in the case of genome-wide deletion collection screening, transcriptome studies also show a high degree of inconsistency. A comparison of four transcriptome studies revealed between 87 and 192 genes with significantly altered expression after acetic acid exposure, but only 19 of these genes were significantly up- or downregulated in all four studies (Bajwa et al., 2013; Kawahata

et al., 2006; Lee et al., 2015; Mira et al., 2010a). The relevance of transcriptome studies has also been debated, as protein activity determines cell physiology, and mRNA levels are an unreliable indicator of protein activity (Evans, 2015). Nonetheless, randomly changing the transcription of genes by modifying the transcriptional machinery, using histone or transcription factor point mutations, and the introduction of artificial transcription factors, have led to the development of strains with improved acetic acid tolerance (An et al., 2015; Liu et al., 2014; Ma et al., 2015). However, such approaches, targeting the transcriptional machinery, are completely random, and do not provide any knowledge on the mechanisms involved in acetic acid stress.

A general limitation of genome-wide random approaches is the inherent difficulty in identifying complex phenotypes, as random mutations do not follow a strategic plan, and do not allow for "intermediate mutants" on the way to a "superior mutant". For example, changing the lipid composition of the cell membrane, which was the aim of my research, is not often achieved by a few random mutations, due to the tightly controlled membrane lipid homeostasis (see **Section 4.6** for details). In addition, random approaches only reveal information on how the intrinsic capacity of cells to tolerate a specific stress can be increased. To achieve the extreme tolerance often required in industrial processes, I would recommend to complement these strategies with novel targeted approaches, perhaps inspired from other organisms.

3.2 Targeted approaches

Targeted approaches can be used in strain engineering when information is available on the molecular mechanisms of a specific stress (Dunlop, 2011). Acetic acid stress has been extensively studied, but attempts of rational strain engineering to obtain acetic acid tolerance are still relatively rare, perhaps due to the complexity of many physiological traits. In my research, I aimed to increase the tolerance of *S. cerevisiae* to acetic acid by reducing the inflow of acetic acid into the cell (**Papers I-III**). This work provided me a glimpse into the challenges associated with targeted strain engineering, as will be discussed in particular in **Chapter 5**.

Improved tolerance of cells to a particular stress can be targeted either by reducing the damage caused by the stress, or by preventing the damage from occurring. Targeted approaches can also be used to improve the general stress tolerance of cells. The following sections describe targeted approaches, with examples of strain engineering intended to increase acetic acid tolerance. Figure 4 provides an overview of the targeted approaches used to increase acetic acid tolerance in *S. cerevisiae* and the details of each strategy are given in Table II.

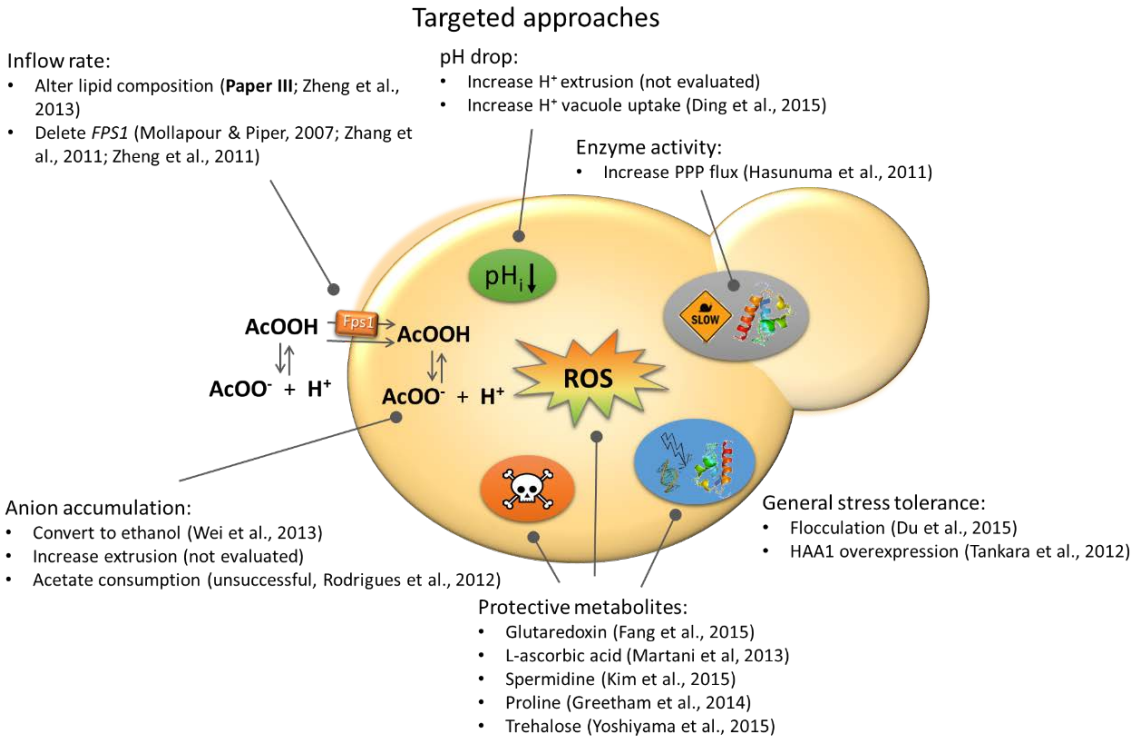


Figure 4: Targeted approaches for improving the acetic acid tolerance of *S. cerevisiae*. The details concerning each approach are given in Table II.

3.2.1 Preventing damage

An efficient strategy to prevent damage caused by a specific compound inside the cell is to prevent its entry into the cell. However, this has been the subject of surprisingly few studies, perhaps because this is often a complex task. In my research, I aimed at reducing the inflow of acetic acid into *S. cerevisiae* cells by engineering the lipid composition of the cell membrane so as to reduce the rate of acetic acid diffusion (**Papers I-III**). It was not possible for me to implement the desired lipid alterations in *S. cerevisiae*, and the feasibility of this approach could therefore not be demonstrated (**Paper III**). However, Zheng et al. were able to show that changing the lipid composition of the cell membrane resulted in increased acetic acid tolerance (Zheng et al., 2013). By overexpression of the fatty acid elongase *ELO1*, they increased the fraction of long C_{18} fatty acids by 18%, resulting in a 40% increase in the survival of *S. cerevisiae* grown with 10 g/L acetic acid at pH 4. However, as the authors only analyzed part of the yeast lipidome, and did not evaluate the acetic acid inflow rate, so it cannot be ruled out that the observed effect was due to other lipid alterations not analyzed in their study, or that other underlying mechanisms of *ELO1* were involved in the observed increase in tolerance to acetic acid. The few examples found in the literature in which membrane engineering has been used to improved tolerance to specific stresses are discussed in **Section 5.3**.

The diffusion of acetic acid into the cell has also been described by facilitated diffusion through the aquaglyceroporin channel Fps1 (Mollapour & Piper, 2007)). Deletion of *FPS1* has been proven successful in reducing intracellular acetic acid accumulation, and improving growth in the presence of acetic acid (Mollapour & Piper, 2007; Zhang et al., 2011; Zheng et al., 2011). However, in my work, deletion of *FPS1* did not change the rate of acetic acid diffusion in *S. cerevisiae* (**Paper IV**). The major role of Fps1 is to control the cells' osmotic pressure, though the controlled release of glycerol (Tamás et al., 1999). The reported importance of Fps1 in acetic acid tolerance therefore depends on the cells' need to release glycerol, which depends on the specific experimental conditions.

3.2.2 Reducing damage

Common strategies for a cell to reduce the damage caused by an inhibitory compound is to remove the molecule from the cell by efflux or consumption, or by conversion into less inhibitory compounds. These strategies to remove inhibitory compounds from the cell are often adopted in strain engineering. Extrusion of the acetate anion has been suggested to occur through the membrane proteins Tpo2 and Tpo3 (Fernandes et al., 2005), but they have not yet been targeted in strain engineering. Acetic acid can only be consumed in *S. cerevisiae* by respiration in the absence of glucose. Attempts to engineer *S. cerevisiae* so that it can consume acetic acid also in the presence of glucose have so far been unsuccessful (Rodrigues et al., 2012). *S. cerevisiae* has no known mechanism(s) for the detoxification of acetic acid, but the benefit of detoxification has been demonstrated by introducing heterologous genes to convert acetate into ethanol inside the cell, resulting in an approximately 15% increase in ethanol yield (Wei et al., 2013). This appears to be a promising strategy in ethanol production from lignocellulosic raw material, as acetic acid can then be used as a carbon source, instead of the cells having to expend energy on acetate and proton efflux and damage repair. Furthermore, the pathway introduced generates NAD^+, which is necessary in xylose consumption, so the driving force of the cells to convert acetic acid is NADH oxidation. It is generally advisable in strain engineering to try to couple the pathway of interest with a pathway critical for cell growth or survival.

To counteract the decrease in intracellular pH, the cell pumps protons out of the cell through the ATPase Pma1 (Serrano et al., 1986). This pump has not yet been targeted in strain engineering. An alternative way to reduce cytosolic acidification is to pump protons into the vacuole. Overexpression of *PEP3,* encoding vacuolar proton ATPase, has been shown to reduce the lag phase by approximately 75% in *S. cerevisiae* cells exposed to acetic acid (Ding et al., 2015). A reduction in intracellular pH affects the activity of many critical enzymes such as the glycolytic enzymes (Pampulha & Loureiro-Dias, 1990) and the NADH dehydrogenase (Zhao et al., 2008). Overexpression of the

genes *TAL1* and *TLK1* encoding transaldolase and transketolase in the pentose phosphate pathway has been shown to increase ethanol productivity by approximately 40% in cells under acetic acid stress (Hasunuma et al., 2011). The role of intracellular pH is complicated in acetic acid stress, as its reduction, resulting in a smaller difference between intracellular and extracellular pH, will result in lower anion accumulation (Figure 3). At the same time, cell growth is significantly impaired at low intracellular pH so, it is often necessary for the cell to increase the pH.

Increased production of protective metabolites can improve the general stress tolerance of cells and enhance their ability to repair damage. Glutathione is an antioxidant that acts as an intracellular redox buffer, neutralizing ROS and protecting the cysteine residues in proteins against damage (Grant, 2001). The protective effect of overproduction of glutathione by cells has been demonstrated in acetic-acid-rich lignocellulose fermentation, where a 42% increase in glutathione levels resulted in a 70% increase in ethanol yield (Ask et al., 2013b). Overexpression of the glutathione-dependent oxidoreductase *GRX5*, involved in the repair of oxidatively damaged proteins (Grant, 2001), has been reported to result in approximately a 30% increase in ethanol productivity (Fang et al., 2015). The production of L-ascorbic acid, popularly known as vitamin C, involved in ROS scavenging in plants and vertebrates, has been found to increase the survival of *S. cerevisiae* cells treated with acetic acid 4-fold (Martani et al., 2013). Increased production of spermidine, a polyamine important for tolerance to various environmental stresses, including nonoptimal temperature and oxygen levels (Balasundaram et al., 1993; Balasundaram et al., 1996; Bouchereau et al., 1999), has been reported to reduce the lag phase of *S. cerevisiae* cells exposed to acetic acid by approximately 30% (Kim et al., 2015). Proline, in addition of being an amino acid and nitrogen source, has been shown to have the ability to improve the order of disordered macromolecular systems (Cray et al., 2015), and to increase tolerance to freezing, desiccation, and oxidative stress (Sasano et al., 2012). Proline accumulation, by the deletion of the *PUT1* gene, converting proline to glutamate, has been reported to increase the viability of *S. cerevisiae* cells exposed to acetic acid by approximately 85% (Greetham et al., 2014). Trehalose is another protective metabolite that orders disordered macromolecular systems (Cray et al., 2015), and has been shown to reverse the increase in membrane permeability induced by ethanol (Mansure et al., 1994). Increasing the trehalose content in *S. cerevisiae*, by the deletion of *NTH1* and *NTH2* involved in trehalose degradation, has been shown to improve growth in the presence of acetic acid (Yoshiyama et al., 2015). Protective metabolites thus appear to be an efficient complement to more targeted strain engineering strategies, and their major advantage is that they offer protection against multiple stresses, a common situation in industrial processes. For example, in **Paper IV**, I highlight the combinatory effect of acetic acid and alcohols on cell performance (for details see **Section 5.2.2**).

Cell flocculation has been used to reduce cell damage by inhibitory compounds by creating a population of cells acting together (Westman et al., 2014). Expression of the flocculation gene *FLO1* in a non-flocculent industrial strain of *S. cerevisiae* has been reported to result in approximately an 80% increase in ethanol productivity in the presence of acetic acid (Du et al., 2015). The transcription factor *HAA1* regulates the expression of a range of genes associated with weak acid stress (Fernandes et al., 2005), and overexpression of *HAA1* has been shown to improve the growth of *S. cerevisiae* cells exposed to acetic acid (Tanaka et al., 2012). This strategy has a clear target, but does not contribute to our understanding of acetic acid tolerance.

The advantages of targeted strain engineering approaches over random approaches are in my opinion that the research has a clear aim, and is based on hypotheses that can be proven or disproven, providing further knowledge on the mechanisms involved. However, a major disadvantage, and perhaps the reason why relatively few targeted approaches have been described in the literature, is that it can be difficult to define a suitable strain engineering target, and it is often very difficult to perform the desired genetic alterations without affecting other molecular mechanisms in the cell. To avoid any undesirable effects of the modifications introduced, I suggest the combination of targeted strain engineering with a random approach, such as evolutionary engineering, to adapt the cell for optimal performance under its new physiological conditions.

Chapter 4

The biology of membranes

All living organisms have a membrane that surrounds the cell and separates it from its surroundings. The evolutionary conserved cell membrane, also called the plasma membrane, is composed of membrane proteins embedded in a lipid bilayer consisting of a large variety different lipid species. The organelles of eukaryotes are also surrounded by membranes. The membrane is a vital physical barrier controlling the exchange of compounds into and out of the cell. The membrane lipids and the membrane proteins determines the physiochemical properties of the membrane. Membrane lipids are also important for a number of cellular processes. This chapter presents a broad description of the biology of membranes, providing the background needed to understand my research on membrane engineering, and the discussion on membrane engineering in **Chapter 5**.

4.1 Membrane organization

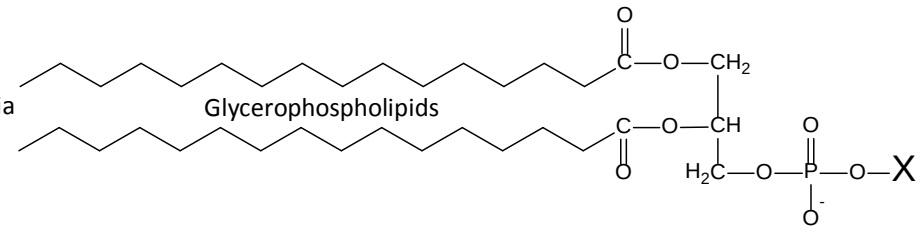
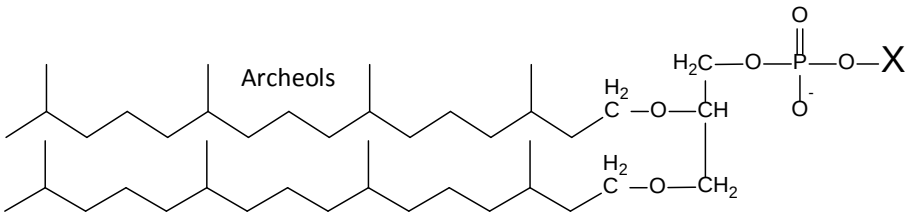
The cell membrane and organelle membranes have a heterogeneous distribution of lipids and proteins, with lipids asymmetrically distributed between the two leaflets of the lipid bilayer. It has been proposed that membrane lipids and proteins are organized in lateral domains, but the extent to which this lateral organization occurs in living organisms, or whether it is an artefact only observed *in vitro*, is a subject of debate. Two classes of lipids, sphingolipids and sterols (described in detail in **Section 4.3**), have been suggested to interact to form ordered "lipid rafts" in the cell membrane. A suggested driving force has been that the bulky sterol rings pack better next to saturated acyl chains, and are shielded from the aqueous environment by the large sphingolipid head groups (Gulati et al., 2010). Lipid rafts of sphingolipids and sterols has been demonstrated *in vitro* with yeast lipids (Klose et al., 2010), but its significance in yeast cells *in vivo* is unclear. In studies on mammalian cells, rafts have been described as small and short-lived (Eggeling et al., 2009). The presence of highly ordered sphingolipid rafts lacking ergosterol has also been suggested (Aresta-Branco et al., 2011). In addition

to the rafts formed by lipid interactions, protein scaffolds can also generate membrane domains. Three such domains have been described in yeast: the membrane compartment containing the arginine permease Can1 (MCC), the membrane compartment containing the kinase complex TORC2 (MCT), and the membrane compartment containing the cell membrane proton pump Pma1 (MCP) (Ziółkowska et al., 2012). MCC has been shown to co-localize with a peripheral membrane protein complex referred to as eisosome, and forms a long furrow in the membrane. MCP has been shown to form a network between the MCC and the MCT domains. Membrane proteins attached to the cell wall or the actin-based membrane skeleton constitute another barrier for lateral membrane diffusion, further separating the membrane into different domains. They way in which membrane organization influences the permeability of membranes to specific compounds is currently unclear to me. For example, I demonstrated in my research that an increase in the fraction of sphingolipids in the membrane reduced the rate of acetic acid diffusion, and that, on average, a more dense membrane was created (**Paper II**). However, membrane density is likely not equal throughout the cell membrane. A change in the diffusion rate of acetic acid may therefore depend on both a reduction in the number of regions where diffusion is more favorable, and an overall increase in the energetics for diffusion.

4.2 Membrane lipids

The general structure of membrane lipids has been conserved throughout evolution (Table III). The most abundant membrane lipids in bacteria and eukaryotes are glycerophospholipids (Lin & Weibel, 2016; Van der Rest et al., 1995). These lipids have a glycerol backbone linking two fatty acyl chains and a phosphate group through ester bonds. A range of different head groups can then be connected to the phosphate group. The most abundant membrane lipids in archaea are archeols (Koga & Morii, 2006; Matsumi et al., 2011). These lipids also have a glycerol backbone and a phosphate group with head groups linked to the glycerol. However, instead of ester-linked fatty acyl chains, archeols have ether-linked isoprenoid tails, and their head groups differ from those in bacteria and eukaryotes. The special phospholipid structure of the archeols in archaea is one physiological explanations of their ability to survive in extreme environments (Matsumi et al., 2011). The cell membranes in eukaryotes are more complex than bacterial membranes due to the existence of sphingolipids and sterols, found mainly in the cell membrane (Hannich et al., 2011).

Table III: General structure of the major class of lipids in bacteria, archaea and eukaryotes

Domain	General lipid structure
Eukaryotes & bacteria	
Archaea	

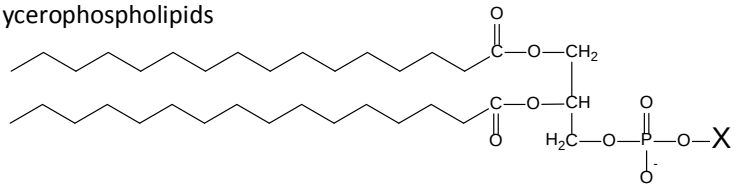
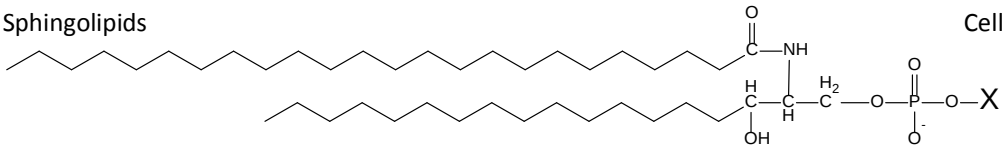
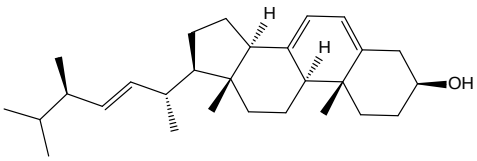
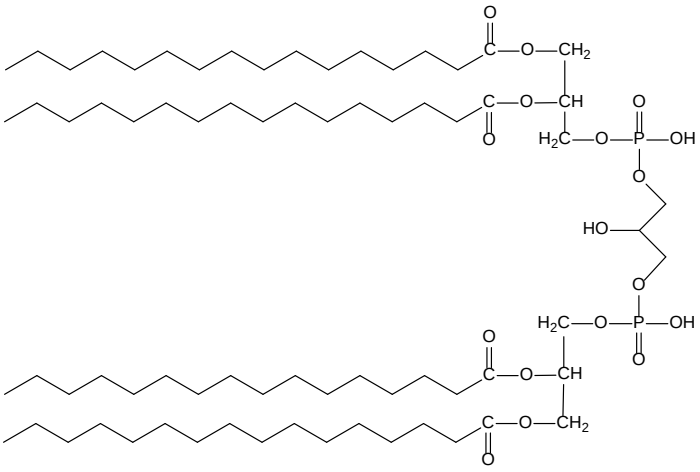
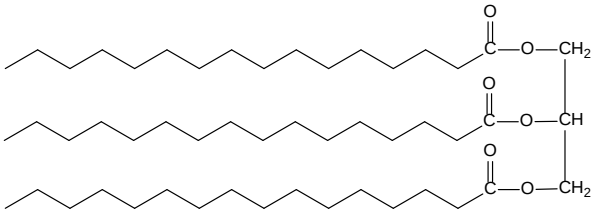
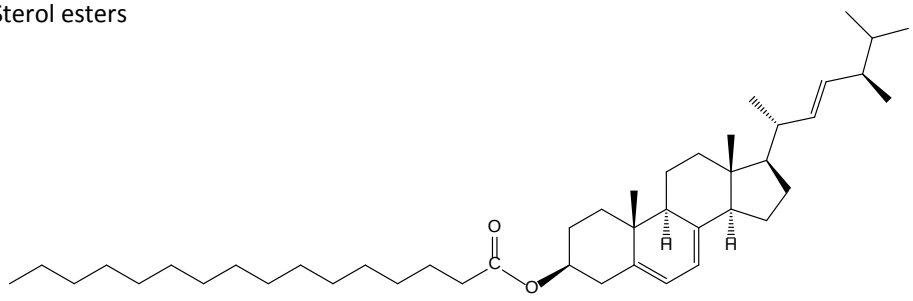
X denotes the lipid head group. Head groups of glycerophospholipids are listed in Table V, and the diverse head groups of archaea lipids are discussed by Koga & Mori (Koga & Morii, 2006).

4.3 Lipids in yeast

The lipidome of *S. cerevisiae* consists of cell membrane lipids, organelle membrane lipids, storage lipids and lipid intermediates. The cell membrane is composed of the major lipid classes, glycerophospholipids (approximately 70%), sphingolipids (approximately 15%), and sterols (approximately 15%) (Klose et al., 2012). Glycerophospholipids are the main constituent of organelle membranes (Daum et al., 1998), although mitochondria contain up to 15% of the glycerophospholipid dimer cardiolipin (Ploier et al., 2014). Lipids are stored in lipid bodies in the form of triacylglycerol (TAG), and sterol esters (Czabany et al., 2007). The general structures of the major classes of lipids in *S. cerevisiae* are given in Table IV.

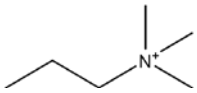
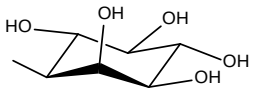
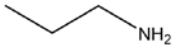
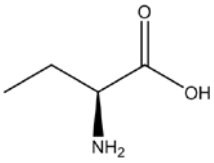
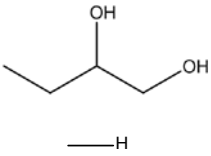
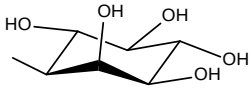
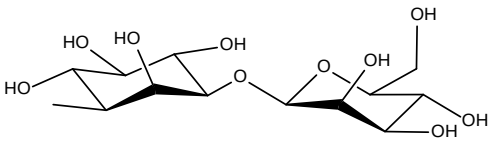
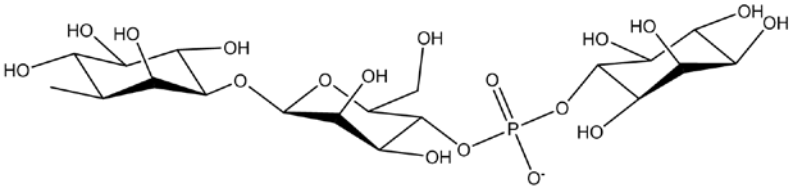
Glycerophospholipids and sphingolipids have similar general structures, but differ in their backbone, acyl chains and head groups (Klug & Daum, 2014; Van der Rest et al., 1995). Glycerophospholipids have a glycerol backbone, connecting the fatty acyl chains by an ester linkage. Sphingolipids instead use serine to connect their fatty acyl chains by an amide linkage. The fatty acyl chains in glycerophospholipids are commonly C_{16}-C_{18}, while sphingolipids have the long chain base (LCB) originating from C_{16}-C_{18} fatty acids combined with a very long chain fatty acid (VLCFA), commonly C_{24}-C_{26}. The acyl chains of glycerophospholipids can be unsaturated at the Δ9 position, while the acyl chains of sphingolipids are completely saturated, but can instead be hydroxylated (Megyeri et al., 2016). Glycerophospholipids are further divided according to their head group into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidyl-inositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidic acid (PA). Sphingolipids are classified by their head group into inositol phosphoryl ceramide

Table IV: Main classes of lipids in *S. cerevisiae*

Lipid class	Main location
Glycerophospholipids 	All membranes
Sphingolipids 	Cell membrane
Sterols (Ergosterol) 	Cell membrane
Cardiolipin 	Mitochondria
Triacylglycerol 	Lipid droplets
Sterol esters 	Lipid droplets

X denotes one of the head groups listed in Table V. Variation in head group composition, fatty acyl chain length and unsaturation generates several hundred unique lipid species in *S. cerevisiae* (**Paper I**, (Ejsing et al., 2009)).

Table V: Head groups of glycerophospholipids and sphingolipids in *S. cerevisiae*

Lipid subclass and head group structure[1]	**Main location**
Glycerophospholipids	
Phosphatidylcholine (PC) 	All membranes
Phosphatidylinositol (PI) 	All membranes
Phosphatidylethanolamine (PE) 	All membranes
Phosphatidylserine (PS) 	All membranes
Phosphatidylglycerol (PG) 	Intermediate
Phosphatidic acid (PA)	Intermediate
Sphingolipids	
Ceramide (Cer)[2]	Intermediate
Inositol phosphoryl ceramide (IPC) 	Cell membrane
Mannosyl-inositol phosphoryl ceramide (MIPC) 	Cell membrane
Mannosyl-di-inositol phosphoryl ceramide (MIP2C) 	Cell membrane

[1]The specific lipid subclass is formed when the indicated head group is attached to the general molecular structure illustrated in Table IV. [2]The head group of ceramides is bound directly to the lipid backbone without a phosphate group.

(IPC), mannosyl-inositol phosphoryl ceramide (MIPC) and mannosyl-di-inositol phosphoryl ceramide (MIP_2C). Sphingolipids in higher eukaryotes are more diverse, differing in chain length, saturation, and head groups (Coskun & Simons, 2011). The storage lipid TAG has a glycerol backbone, which is linked to three fatty acyl chains (Klug & Daum, 2014). Ergosterol is the main sterol in yeasts, while the main sterol in mammalian cells is cholesterol (Hannich et al., 2011). The general structure of sterols is a hydroxyl head group, an acyl side chain, and a four-ring structure (Klug & Daum, 2014). Sterols are stored as steryl esters constituting a sterol linked to a C_{16} or C_{18} fatty acid.

The lipid species found in *S. cerevisiae* are also found in other yeasts, but with minor modifications. Several yeast species, including *Z. bailii*, have, for example, fatty acyl chains that are saturated at both position 9 and position 12, while *S. cerevisiae* only has a desaturase active at position 9 (**Paper I**). In addition, many yeasts, including *Komagataella pastoris*, have glucosylceramides in addition to the inositol sphingolipids present in *S. cerevisiae* (Ternes et al., 2011).

4.4 Yeast lipid biosynthesis and regulation

Lipid biosynthesis starts with acetyl-CoA, which either enters the sterol biosynthesis pathway, or acts as a building block for the fatty acids used in the biosynthesis of glycerophospholipids, triacylglycerol or sphingolipids (Ploier et al., 2014). The synthesis of each class of lipids in *S. cerevisiae* is described in the following sections. The general characteristics are discussed, while minor additional lipids or pathways have been omitted. Since my research focused on the cell membrane, special emphasis is placed on lipids in the cell membrane. An overview of lipid metabolism, including fatty acid biosynthesis, is illustrated in Figure 5.

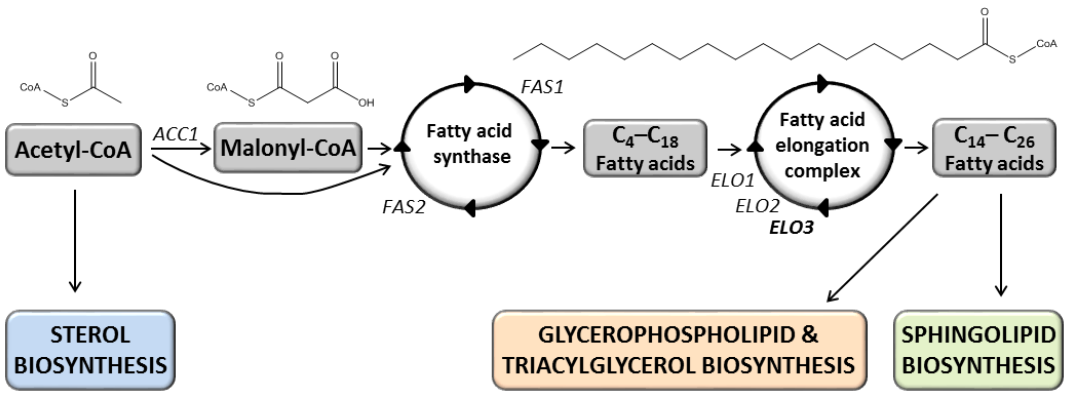


Figure 5: Illustration of lipid metabolism in *S. cerevisiae*. Genes discussed in this thesis are included in the figure, while other genes have been omitted for clarity. Genes discussed in **Paper III** are given in bold face.

4.4.1 Fatty acid biosynthesis

Fatty acyl chains in membrane lipids originate from *de novo* synthesis, endogenous lipid turnover, or exogenous supply (Tehlivets et al., 2007). *De novo* synthesis occurs in the cytosol as well as in the mitochondria, and further elongation and desaturation occur in the endoplasmic reticulum (ER). Acetyl-CoA is first carboxylated to malonyl-CoA by acetyl CoA-carboxylase, Acc1. Fatty acyl chains are then formed by the fatty acid synthase which consists of Fas1 and Fas2. Starting from acetyl-CoA, a fatty acyl chain is formed by the addition of two carbon atoms per cycle from malonyl-CoA. Fatty acyl chains up to C_{18} are formed by the cytosolic fatty acid synthase, while C_8 acyl chains are the main product of the fatty acid synthase in mitochondria (Hiltunen et al., 2010). Further elongation to form fatty acyl chains up to C_{26} is catalyzed by the fatty acid elongation complex in the ER, utilizing malonyl-CoA as a building block (Tehlivets et al., 2007). The length of the acyl chains depends on the activity of the three elongases Elo1, Elo2, and Elo3. Elo1 cannot elongate acyl chains longer than C_{16}, while Elo2 elongates acyl chains up to C_{24}, and Elo3 is required for the formation of C_{26} acyl chains (Leonard et al., 2004). C_{24} and C_{26} fatty acyl chains are mainly used for sphingolipid synthesis (Dickson et al., 2006). The exact mechanisms regulating the distribution of acyl chains of different lengths is unknown, but the distribution between C_{16} and C_{18} acyl chains has recently been shown to be regulated by Acc1, the enzyme catalyzing the formation of malonyl-CoA (Hofbauer et al., 2014). In my research, *ELO3* was overexpressed as part of a strategy intended to increase the fraction of sphingolipids in *S. cerevisiae* (**Section 5.3**, **Paper III**). No major lipidome alteration was observed as a result of 1.9-fold increased overexpression of *ELO3*, suggesting that fatty acid elongation could be regulated on a post-translational level.

The desaturation of fatty acyl chains in glycerophospholipid synthesis is catalyzed by the Ole1 desaturase localized in the ER. This enzyme introduces a double bond at the Δ9 position of the fatty acyl chain (Martin et al., 2007). Ole1 is regulated on a transcriptional level by the transcription factors Mga2 and Spt23, which in turn seem to be activated by altered fluidity of the ER membrane (Ballweg & Ernst, 2017). Mga2 is also involved in sterol biosynthesis through the activation of *ERG1*. The fatty acids involved in sphingolipid synthesis are fully saturated (Megyeri et al., 2016).

4.4.2 Glycerophospholipid and triacylglycerol biosynthesis

Glycerophospholipid and triacylglycerol synthesis starts in the ER with the formation of PA, an intermediate glycerophospholipid with low abundance in membranes, (De Kroon et al., 2013). Membrane-associated glycerophospholipids are then synthesized either through the CDP-DAG pathway or the Kennedy pathway (Figure 6). In the CDP-DAG pathway, PA is converted into cytidine diphosphate diacylglycerol (CDP-DAG), which is further converted to PI or PS in the ER, or transported to the

mitochondria and converted into cardiolipin with PG as intermediate. PS can then be decarboxylated to form PE which in turn can be methylated into PC. However, the PS decarboxylases are mainly localized in the mitochondria, so if the CDP-DAG pathway is used for PE and PC synthesis, PS must be transported to the mitochondria, and the PE produced must be transported back to the ER, in order to be used as a precursor for PC synthesis. Alternatively, PE and PC can be synthesized in the ER from PA with diacylglycerol (DAG) as an intermediate through the Kennedy pathway. Linkage of an additional fatty acyl chain to DAG forms the storage lipid TAG. This reaction takes place in the ER, and either fatty acyl-CoA or a glycerophospholipid is used as the acyl chain donor. The composition of the glycerophospholipids may change after synthesis through the action of enzymes catalyzing the exchange of one or both fatty acyl chains (Renne et al., 2015).

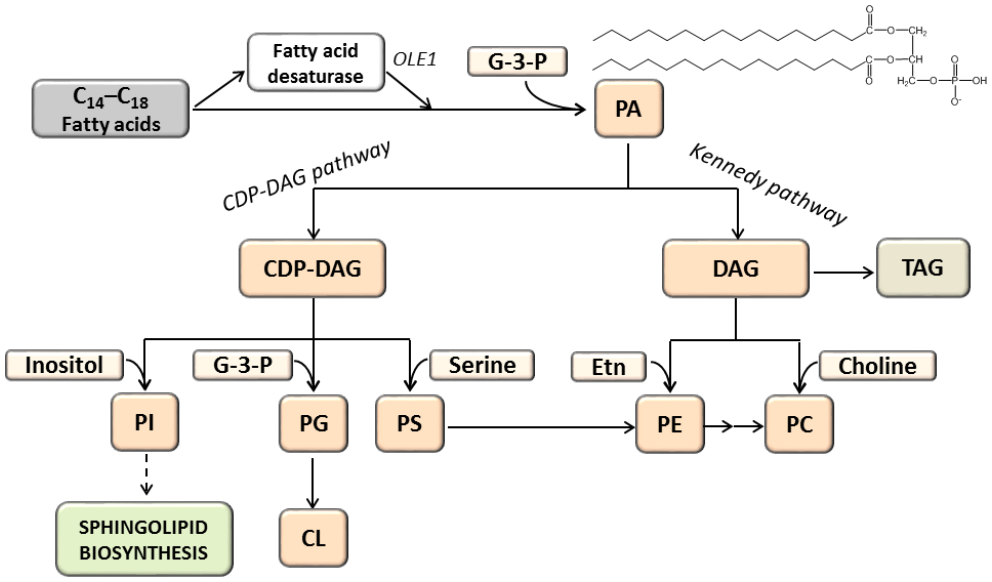


Figure 6: Glycerophospholipid and triacylglycerol biosynthesis in *S. cerevisiae*. Genes discussed in this thesis are included in the figure, while other genes have been omitted for clarity. Abbreviations: G-3-P, glycerol-3-phosphate; PA, phosphatidic acid; CDP-DAG, cytidine diphosphate diacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol; Etn, ethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL, cardiolipin.

Glycerophospholipid and TAG synthesis are to a large extent regulated on a transcriptional level by the inositol-responsive element (UAS_{INO}), present in a large number of phospholipid biosynthetic genes (Carman & Han, 2011). Gene expression is controlled by the transcription factors Ino1 and Ino2, and the suppressor Opi1, binding to the UAS_{INO} element. PA regulates the transcription of UAS_{INO} genes by binding to Opi1 and preventing its nuclear localization. The binding efficiency depends on the protonation state of PA, showing that intracellular pH regulates glycerophospholipid abundance (Young et al., 2010). PA levels are in turn regulated by inositol, choline, and zinc (Greenberg & Lopes, 1996; Han et al., 2005). Cytidine triphosphate is a glycerophospholipid precursor with regulatory function, and PC

synthesis is regulated by S-adenosyl-L-homocysteine (De Kroon et al., 2013). Glycerophospholipid synthesis is to some extent also regulated by phosphorylation (Da Silveira Dos Santos et al., 2014; De Kroon et al., 2013).

4.4.3 Sphingolipid biosynthesis

Sphingolipid synthesis differs from glycerophospholipid synthesis in the condensation of fatty acyl-CoA with serine to form LCB, and in the formation of VLCFA in the elongation cycle, both steps occurring in the ER (Figure 7). The serine palmitoyltransferase (SPT) complex, consisting of the two main enzymes Lcb1 and Lcb2, together with the smaller subunit Tsc3 (Gable et al., 2000; Pinto et al., 1992), catalyzes the formation of LCB. A C_{16} fatty acid will form a C_{18} LCB by condensation with serine. The activity of the SPT complex is negatively regulated by the two paralogs Orm1 and Orm2, through physical interaction, as well as by Sac1, negatively regulating the complex through an unknown mechanism (Breslow et al., 2010). The SPT complex together with its regulators is termed the SPOTS complex. LCB and VLCFA are combined in the ER into ceramides, the simplest form of sphingolipids, by the action of ceramide synthase consisting of one of the paralogs Lag1 or Lac1 together with Lip1 (Dickson, 2008). Sphingolipid synthesis then proceeds in the Golgi apparatus, where IPC is formed through the attachment of an inositol phosphate group to the ceramide moiety by the enzyme Aur1 (Cowart & Obeid, 2007). The inositol phosphate group originates from phosphatidylinositol 4-phosphate (PI_4P), which must be converted to PI by Sac1 before it can be used by Aur1 (Brice et al., 2009). It should be noted that Sac1 has a dual role and location as a regulator of LCB synthesis in the ER and supporting IPC production in the Golgi apparatus. A secondary function has also been assigned to the Orm proteins, namely stimulating the synthesis of complex sphingolipids in the Golgi by an unknown mechanism, possibly though the activation of Sac1 (Shimobayashi et al., 2013). IPC is then converted to MIPC by the addition of mannose to the inositol unit. Finally, MIP_2C is formed by adding a second inositol phosphate to MIPC. Ceramide act as precursor with low abundance in the cell, while IPC, MIPC, and MIP_2C are the sphingolipids occurring in the cell membrane, and are collectively called complex sphingolipids (Megyeri et al., 2016). The degradation of complex sphingolipids into ceramides is catalyzed by Isc1 (Sawai et al., 2000).

The regulation of sphingolipid biosynthesis is still poorly understood (Dickson, 2010; Olson et al., 2016), apart from a few recent discoveries. As described above, the Orm proteins and Sac1 have important regulatory functions in sphingolipid biosynthesis. In addition, many enzymes in the sphingolipid synthesis pathway have been shown to be regulated by phosphorylation (Da Silveira Dos Santos et al., 2014). Localization is another complex mode of regulation: both lipid intermediates and enzymes have been shown to have dual localization (Megyeri et al., 2016). The enzyme Nvj2 has recently

been found to be an important regulator of sphingolipid biosynthesis by transporting ceramides from their site of synthesis in the ER, to the Golgi, for conversion into complex sphingolipids (Liu et al., 2016a). The breakdown of complex sphingolipids into ceramides by Isc1 is regulated by the compartmental localization of Isc1 (Matmati & Hannun, 2008). In **Paper III**, I attempted to increase the fraction of sphingolipids in the cell membrane of *S. cerevisiae* by overexpressing the fatty acid elongase *ELO3*, and the IPC synthase *AUR1* genes, together with the deletion of the regulators *ORM1* and *ORM2* to suppress LCB formation. However, instead of increased sphingolipid synthesis, *ELO3* and *AUR1* overexpression resulted in unaltered sphingolipid synthesis, while strains in which *ORM1* and *ORM2* were deleted showed reduced sphingolipid synthesis and reduced levels of PI. These results indicate that *ELO3* and *AUR1* are regulated on a level beyond mRNA quantities, and that the absence of *ORM1* and *ORM2* creates a dysfunctional Sac1, incapable of catalyzing the release of PI in the Golgi. However, further work is needed to confirm this. The details of this study are discussed in **Section 5.3**.

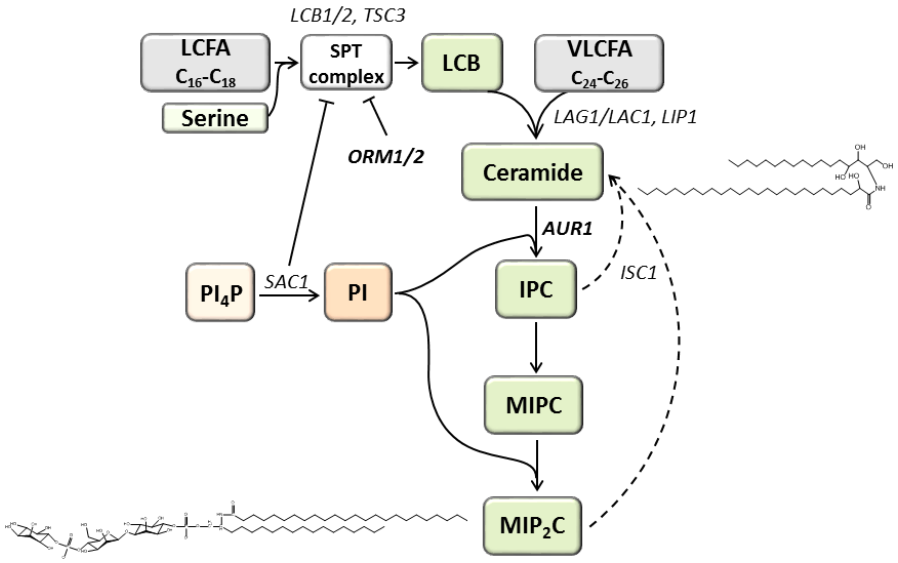


Figure 7: Sphingolipid biosynthesis in *S. cerevisiae*. Genes discussed in this thesis are included in the figure, while other genes have been omitted for clarity. Genes discussed in **Paper III** are shown in boldface. Abbreviations: LCFA, long-chain fatty acid; LCB, long-chain base; VLCFA, very-long-chain fatty acid; PI_4P, phosphatidylinositol 4-phosphate; PI, phosphatidylinositol; IPC, inositol phosphoryl ceramide; MIPC, mannosyl-inositol phosphoryl ceramide; MIP_2C, mannosyl-di-inositol phosphoryl ceramide.

4.4.4 Sterol biosynthesis

Sterol synthesis is a complex process involving almost 30 enzymes, mostly localized in the ER (Klug & Daum, 2014). Initially, three acetyl-CoA molecules are consumed to form HMG-CoA, which is further reduced to mevalonate. Thereafter, many steps are required to form the final sterol, ergosterol, which is the major sterol in yeast (Figure 8).

The pathway requires oxygen, but under oxygen-limited conditions, sterols can be incorporated into the cell membrane by exogenous supply (Jacquier & Schneiter, 2012). Sterols are synthesized in excess and stored in lipid droplets in the form of sterol esters (Ploier et al., 2014).

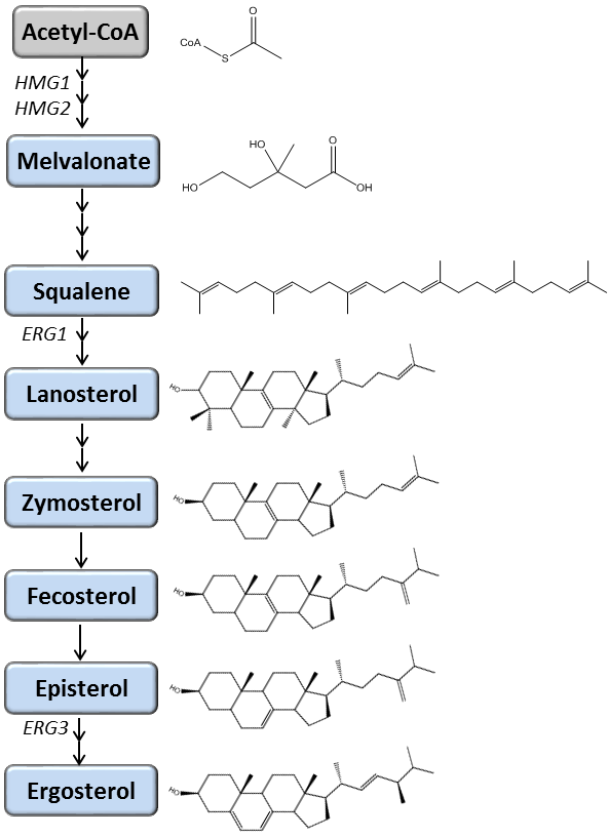


Figure 8: Sterol biosynthesis in *S. cerevisiae*. Genes discussed in this thesis are included in the figure, while other genes have been omitted for clarity.

Sterol biosynthesis is regulated by ergosterol abundance, membrane fluidity, and internal transport. Free ergosterol inhibits the HMG-CoA reductases Hmg1 and Hmg2, catalyzing an early step in the sterol biosynthesis pathway (Ploier et al., 2014). Transcription of *ERG1*, catalyzing the first step converting squalene to lanosterol, is regulated by the transcription factor Mga2, which also regulates the transcription of fatty acid desaturase *OLE1* (Ballweg & Ernst, 2017). Changes in membrane fluidity are believed to activate Mga2. Ergosterol increases throughout the secretory pathway, its main location being the cell membrane (Ploier et al., 2014). Internal transport by vesicular and non-vesicular transport is a major regulator of sterol abundance in the cell membrane. Sterol biosynthesis appears not to be regulated by phosphorylation to any large extent, as screening of kinase and phosphatase mutants has revealed very small variations in the abundance of sterols (Da Silveira Dos Santos et al., 2014).

4.5 Lipids in cell biology

Membrane lipids are often described as bulk lipids organized in bilayers to form physical barriers. In reality, the situation is more complex, and many lipids in membranes, as well as their precursors, have vital regulatory functions in the cell, affording them a fundamental role in cell biology (Dowhan, 1997; Hannich et al., 2011). Lipids play a crucial role in intracellular trafficking, for example, sphingolipids and sterols are involved in the localization of membrane proteins to the cell membrane (Santos & Riezman, 2012). In addition, the activity of many membrane proteins is dependent on their proximity to specific membrane lipids (Coskun & Simons, 2011), such as the cell membrane proton pump, Pma1 which requires sphingolipids for its proper function (Wang & Chang, 2002). Many lipids are also involved in cell signaling. The glycerophospholipid PI is a precursor for the phosphoinositides, a group of second messengers involved in many cellular functions (Strahl & Thorner, 2007). Intermediates of the sphingolipid synthesis are also important second messengers (Epstein & Riezman, 2013). LCBs have been shown to activate Pkh kinases involved in endocytosis and actin organization (Friant et al., 2001). Phosphorylated LCBs have been connected to calcium signaling (Birchwood et al., 2001), the induction of heat shock proteins (Skrzypek et al., 1999), and activation of the unfolded protein response (Han et al., 2010). Ceramides have been shown to be involved in heat shock response (Wells et al., 1998), growth arrest (Nickels & Broach, 1996), and as a target for the kinase complex TORC2 (Aronova et al., 2008). High levels of ceramides have also been detected in apoptotic cells (Rego et al., 2014). Complex sphingolipids have so far not been described as second messengers, but ceramides can be rapidly generated from complex sphingolipids by Isc1-mediated breakdown, therefore indirectly involved in cell signaling (Epstein & Riezman, 2013). Sterols have an indirect regulatory role in forming liquid-ordered membrane states needed for processes such as signal transduction, cytoskeleton reorganization and asymmetric growth (Dufourc, 2008).

4.6 Membrane lipid homeostasis and flexibility

The vital function of membranes, in combination with the role many lipids play in cell biology, illustrates the importance of tightly regulated lipid metabolism. The connection between lipid imbalance and many diseases further demonstrates the significance of membrane lipid homeostasis (Stordeur et al., 2014). In addition to transcriptional regulation altering mRNA levels and post-translational regulation altering enzyme activity (Da Silveira Dos Santos et al., 2014), lipid synthesis is highly regulated by subcellular localization and transport (Natter et al., 2005; Ploier et al., 2014). Pathways within the lipid metabolism are also closely connected with cross-talk between different lipid species (Henry et al., 2012; Nohturfft & Shao, 2009; Shevchenko & Simons, 2010). The sterol and sphingolipid synthesis pathways, for example, show strong coordination

(Eisenkolb et al., 2002; Guan et al., 2009; Gulati et al., 2010), with 12 sphingolipid biosynthetic genes showing genetic interactions with nine sterol biosynthetic genes (Fügi et al., 2015). Fatty acid desaturation and ergosterol biosynthesis are also linked by the transcription factor Mga2, which is believed to respond to changes in membrane fluidity and to activate the transcription of lipid biosynthesis genes such as fatty acid desaturase *OLE1,* and the sterol biosynthesis gene *ERG1*. The tight regulation and high interconnection of lipid metabolism make it difficult to predict the outcome of modifications in lipid synthesis (Stordeur et al., 2014), and hamper studies on membrane engineering for cell factory design. This challenge will be further discussed in the next chapter.

Membrane lipid composition is remarkably flexible, despite, or perhaps, thanks to, its tight regulation. Cells have a unique ability to adapt and alter their membrane lipid composition in response to external stimuli. Stresses such as weak acids (**Paper I**), solvents (Huffer et al., 2011), temperature (Henderson et al., 2013; Klose et al., 2012), and pH (Young et al., 2010), altering the physiochemical properties of the membrane, have been shown to induce rapid changes in the cell lipid profile, further discussed in **Section 5.1.2**. Factors not obviously interfering with the membrane, such as medium composition and nutrient availability, also have considerable impact on the lipid profile (Janssen et al., 2000; Klose et al., 2012). The storage of ergosterol intermediates and enzymes for ergosterol synthesis in liquid droplets is one example of how cells can rapidly change their lipid composition (Natter et al., 2005). The physiological driving force behind alteration in lipid profile after exposure to stresses affecting membrane properties is believed to be compensatory, in an attempt to restore membrane function. The mechanisms behind changes in lipid profile in response to stimuli not obviously interacting with the cell membrane are less well understood. Changes in the lipid profile may simply be an indirect effect of global metabolic alterations, without consequences on the overall membrane properties, as similar membrane properties can be achieved with a range of lipid profiles. To better understand the effect of a specific membrane lipid composition on the physiochemical properties of the cell membrane, I would recommend more studies on the relation between the global lipid profile and the physiochemical properties of the membrane. For example, in my research, I combined lipidomic profiling of the major lipid species in yeast, with molecular dynamics simulations of lipid bilayers designed to resemble the measured lipid composition (**Papers I** & **II**).

4.7 Methods in membrane research

A key concept in membrane engineering is the correlation between the lipid composition of a membrane and the membrane properties. This section briefly

describes how membrane lipids can be accurately analyzed, and the use of *in vitro* and *in silico* model membranes to investigate the physiochemical properties of membranes.

4.7.1 Lipidomics

Analysis of the complete lipid profile before and after membrane engineering is important to obtain knowledge on the effects of the strategy employed. Lipid analysis has traditionally been a tedious process requiring several different quantification techniques (Wenk, 2005). Within the past decade, high throughput methods based on mass spectrometry (MS) have been developed, allowing quick analysis of most lipid species within the cell (Ejsing et al., 2009; Guan et al., 2009; Klose et al., 2012). Rapid analysis of the major lipid species in a cell is referred to as lipidomics. In my research presented in **Papers I & III**, I used advanced lipidomics to analyze all the major membrane lipid species in *S. cerevisiae* and *Z. bailii*, using the workflow illustrated in Figure 9.

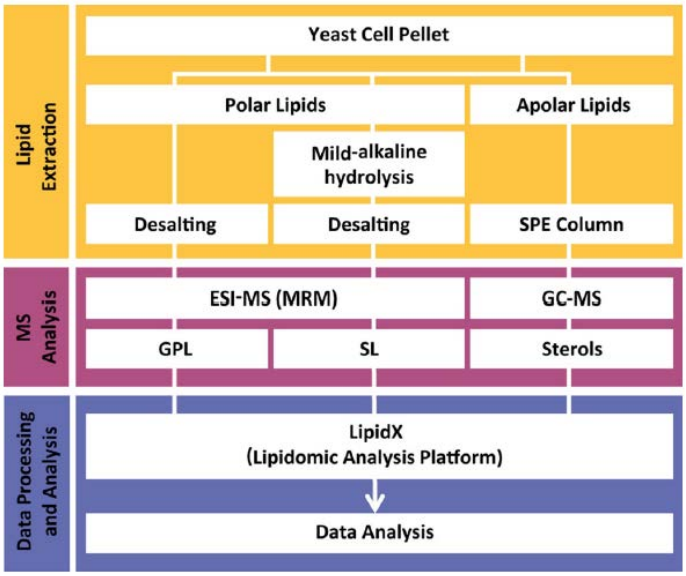


Figure 9: Lipidomics platform used for lipid analysis in Papers I & III. Abbreviations: SPE, solid-phase extraction; ESI-MS, electrospray ionization mass spectrometry; GC-MS, gas chromatography mass spectrometry; GPL, glycerophospholipids; SL, sphingolipids. (Da Silveira Dos Santos et al., 2014)

The first step in lipid analysis is the extraction of the lipids from the sample of interest. Different extraction methods are available, and the method of choice will affect the quantities of the specific lipids extracted (Carrasco-Pancorbo et al., 2009), which stresses the importance of including appropriate internal standards during sample preparation (Guan et al., 2010). Due to the different molecular properties of specific lipid species, and to avoid underestimation of specific lipid species resulting from ion suppression, lipidome analysis is often preformed in a few different lipid fractions (Carrasco-Pancorbo et al., 2009; Ejsing et al., 2009). In my work, the three different

classes of lipids, glycerophospholipids, sphingolipids, and sterols, were analyzed in separate fractions.

Glycerophospholipid and sterol quantification are possible with appropriate internal standards and robust analytical methods. Sphingolipid analysis, on the other hand, is less reliable due to the lack of commercially available internal standards for yeast sphingolipids. Therefore, sphingolipid abundances are best expressed in a comparative manner within species (Megyeri et al., 2016). Furthermore, sphingolipid analysis suffers from ion suppression by glycerophospholipids (Hannich et al., 2011), and thus a mild-alkaline hydrolysis step was included to remove glycerophospholipids from the sphingolipid sample before analysis. The lack of internal standards for yeast sphingolipids has resulted in disagreement within the scientific community regarding which sphingolipids are most abundant in *S. cerevisiae*, with both IPC (Megyeri et al., 2016) and MIP_2C (Ejsing et al., 2009) being proposed. Inconsistency also occurs between different studies presenting sphingolipid data. Taking the deletion of *ORM1* and *ORM2* as an example, both an increase (Breslow et al., 2010), and a decrease (**Paper III**, (Shimobayashi et al., 2013), in sphingolipids have been reported. The sphingolipid analysis in my work encountered large variations in biological and technical replicates when analyzing low levels of sphingolipids. This was discovered in a later re-analysis of the sphingolipids in *S. cerevisiae*, which prompted us to publish a correction withdrawing the findings regarding increased sphingolipid abundance in *S. cerevisiae* (**Paper I correction**). No such problems were encountered in the sphingolipid analysis of the *Z. bailii* samples, which showed reproducible results. Careful experimental design is important when evaluating the lipid profile of *S. cerevisiae*, as the cultivation conditions have a considerable impact on the lipid profile (Klose et al., 2012), and well-defined cultivation conditions are therefore recommended. For example, in my work, cells were harvested for lipid analysis when 50% of the carbon source had been depleted, instead of at a specific optical density (OD) (**Papers I** & **III**), as differences in cell physiology affect the distribution between respiration and fermentation when grown on glucose, affecting the final OD. Sphingolipids have traditionally been analyzed using thin-layer chromatography (TLC). This technique could be an alternative to MS when quantification in terms of trends is sufficient due to its simple setup and robust performance (Fuchs et al., 2011).

4.7.2 Membrane models in vitro

The physiochemical properties of lipid bilayers can be determined using model membranes constructed from commercially available lipids. Lipid monolayers, supported lipid bilayers, or membrane vesicles are commonly used membrane models (Lopes et al., 2017). A description of potential applications of membrane models *in vitro* has been included here, as it in my opinion represents an important tool in membrane

engineering. However, as none of these methods were used in my own research, this section only contains a brief overview.

Systems with flat surfaces, such as lipid monolayers and supported lipid bilayers, can be useful in microscopy studies. Supported lipid bilayers, for example, have been used to study the behavior of PC membranes in the presence of ethanol using atomic force microscopy and fluorescence microscopy (Vanegas et al., 2012). Membrane vesicles, also called liposomes, are circular membrane models useful in a range of techniques. Giant unilamellar vesicles (GUVs) have been used to study the mechanical properties of PC membranes in the presence of alcohols using micropipette aspiration (Ly & Longo, 2004). In this system, measurements are performed directly on the membrane vesicle. Smaller membrane vesicles, such as large unilamellar vesicles (LUVs) and small unilamellar vesicles, (SUVs) are instead used in techniques where the results are obtained by measuring the average over many membrane vesicles. The partition coefficient of butanol in PG membranes has been determined in large unilamellar vesicles using both differential scanning calorimetry and high pressure liquid chromatography (Kurniawan et al., 2013). Liposomes and proteoliposomes have also been used to measure osmotic membrane permeability and aquaporin activity, using a microfluidic technique called stopped flow (Frick et al., 2013). With this technique, liposomes prepared with a fluorescent dye are rapidly mixed with a solute of interest, and the diffusion rate can be determined by monitoring the fluorescence as the solute enters the liposome. This technique could potentially be useful in measuring the diffusion rate of acetic acid through the membrane, if the vesicles are prepared with a pH-sensitive fluorescent dye, since acetic acid inflow will reduce the pH inside the vesicles.

A major limitation in the use of *in vitro* model membranes is that the membrane complexity achievable depends on the lipids available. For example, it would have been interesting in my research to study the effect of yeast sphingolipids on model membranes, but this was not possible as none are commercially available. Membrane lipids extracted from *S. cerevisiae* and *Z. bailii* could perhaps have been used, but there is a risk that different impurities in the two lipid extracts could have influenced the measured permeability of the model membrane to acetic acid. Studies are therefore usually performed on very simple model membranes consisting of only one or two classes of glycerophospholipids, which reduces the physiological relevance of the findings obtained with these techniques.

4.7.3 Molecular dynamics simulations

Molecular dynamics (MD) simulations have attracted increasing attention over recent years in studies of the molecular structure and dynamics of biological membranes at

atomistic resolution (Pluhackova & Böckmann, 2015). Physiochemical membrane properties, lipid–protein interactions, and membrane pore formation are examples of membrane features that can be studied using MD simulations. In my research, MD simulations were used to predict the effect of a high fraction of sphingolipids in the cell membrane (**Section 5.1.3**, **Paper II**), and to evaluate the influence of the alcohols ethanol and n-butanol on the membrane properties (**Section 5.2.2**, **Paper IV**). Simulations have been used previously to predict the membrane properties of bilayers enriched, for example, in cardiolipin (Róg et al., 2009) and bolalipids originating from archaea (Bulacu et al., 2012).

A model membrane is simulated using the molecular structures of the lipids and the water molecules, together with a description of the interactions between atoms (Figure 10). The interactions are described in terms of force fields, and consist of the potential contributions from intermolecular interactions such as electrostatic interactions and van der Waals forces, as well as intramolecular interactions such as bond stretching, bending, and rotation potentials (Pluhackova & Böckmann, 2015). Different lipid force fields have been reported in the literature, such as the Berger lipid force field, the CHARMM lipid force field, and the Stockholm lipid (Slipid) force field. Lipid force fields are available in Lipidbook, a public database for force-field parameters (Domański et al., 2010). The Slipid force field (Jämbeck & Lyubartsev, 2013) was used in the simulations in my research.

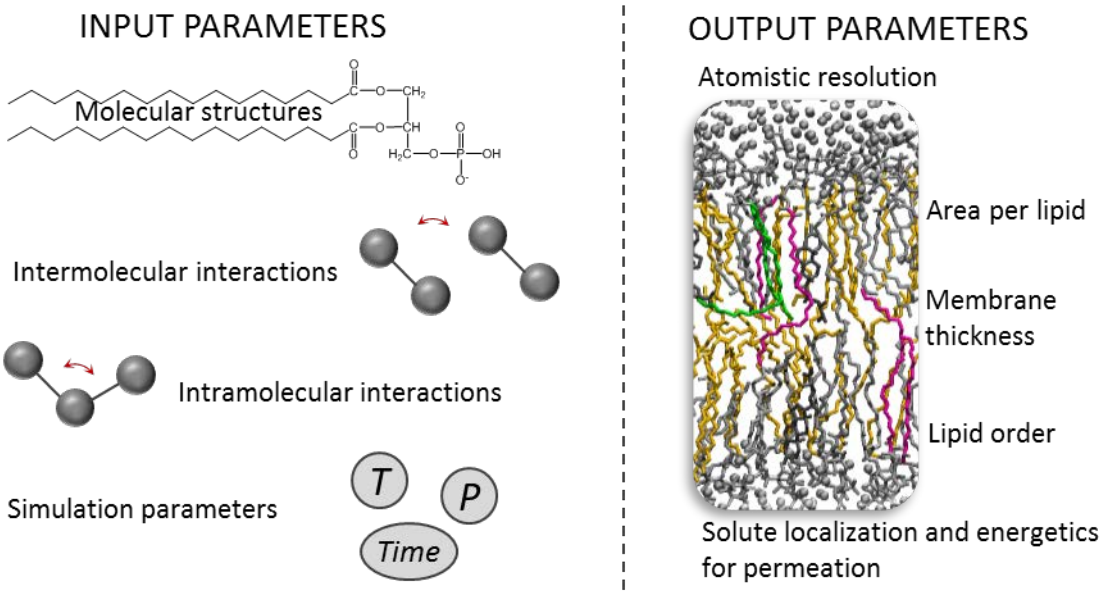


Figure 10: Overview of input and output parameters in molecular dynamics simulations. Molecular structures, interactions between atoms (force fields), temperature (T), pressure (P), and time are input parameters. Newton's law of motion (force = mass × acceleration) is then used to determine the position of each atom as a function of time. MD simulations provide atomistic resolution of the simulated systems, and generate parameters such as the area per lipid, membrane thickness, and lipid order. If the membrane is simulated together with a solute in addition to water, solute localization and the energetics for diffusion can be obtained.

Molecular structures and force fields are then combined into lipid bilayers using, for example, the CHARMM membrane builder (Wu et al., 2014). Before running simulations, the bilayer formed requires time to equilibrate, i.e., to allow the lipids to become organized in an energetically favorable manner. Newton's law of motion (force = mass × acceleration) is then used in the simulations to determine the position of each atom as a function of time. MD simulations thus create a "movie" of the behavior of the simulated system, using software such as Gromacs (Hess et al., 2008). Membrane parameters such as the area per lipid, membrane thickness, and lipid order can be calculated from the simulation. If the membrane is simulated together with water and another solute, localization and energetics for diffusion of the particular solute can also be obtained.

Two kinds of MD simulations are commonly used. Atomistic simulations describing lipids atom by atom provide a means of investigating membrane interactions with high resolution. However, these simulations are limited to 100 nm scale and a millisecond time frame, due to the high computational demand. (Dror et al., 2012). In my research, the behavior of a lipid bilayer with an approximate area of 36 nm^2 (128 lipids) was predicted over a time of 100 ns using atomistic simulations. An alternative to atomistic simulations, which increases the possible size and time scale, is coarse-grained simulations, where groups of atoms are lumped together into pseudo-particles or beads (Venturoli et al., 2006). For example, PC lipids of 130 atoms have been simplified into 12 beads using the Martini coarse-grained force field (Pluhackova & Böckmann, 2015). The drawback of coarse-grained simulations is reduced accuracy, for example, it is difficult to simulate membrane electrostatics, resulting in a dipole potential of the lipid bilayer in the opposite direction to that known from experimental studies (Yu et al., 2012).

The major advantage of MD simulations is that they provide atomistic resolution of the system of interest, which enables the elucidation of mechanisms that are very difficult to measure experimentally. For example, I have in my research obtained detailed information on the partitioning and location of ethanol and n-butanol in the lipid bilayer (**Paper IV**). The ability of MD simulations to describe the partitioning and location of drug candidates interacting with the lipid bilayer is also important in pharmaceutical research (Lopes et al., 2017). However, the disadvantage is that MD simulations are limited in scale and time due to the high computational demand. In addition, most MD simulations have been performed using very simple membrane models, as in the *in vitro* studies, which limits the biological relevance of these studies. The reason for the use of simple membrane systems is probably that only a few lipid force fields have hitherto been available, and establishing new force fields is a tedious process. However, more lipid force fields have recently become available as researchers have realized the importance of membrane complexity (Monje-Galvan & Klauda, 2015;

Zhuang et al., 2016). The diversity of the force fields available was also increased by my research, as this was to the best of my knowledge the first time force fields were described for yeast sphingolipids (**Paper II**).

Chapter 5

The concept of membrane engineering

Using microorganisms as cell factories for production of renewable fuels and chemicals, exposes cells to various stresses among which some are detrimental to the cell due to their interactions with the cell membrane (**Section 2.2**). The aim of my research was to evaluate the feasibility of engineering the lipid composition of the cell membrane in *S. cerevisiae* so as to alter its physiochemical membrane properties. This was done with the objective to increase the tolerance of the cells to compounds that inhibit cell performance directly by interfering with the cell membrane, or indirectly by their diffusion through the membrane. The concept of developing microbial cell factories that can withstand membrane-related stresses by targeting the membrane lipid composition has been envisioned for quite some time (Keasling, 2010), but membrane engineering studies have only started to appear in the literature during the past few years, and the first two review papers related to membrane engineering have only recently been published (Jezierska & van Bogaert, 2016; Sandoval & Papoutsakis, 2016). Jezierska and van Bogaert focused on ways of increasing the capacity of microorganisms to produce compounds which act as membrane-related inhibitors by modifying cell membrane integrity, or by increasing the efflux of produced compounds via membrane protein or vesicle transport. Sandoval and Papoutsakis instead focused on ways of adapting the bacterial membrane and cell wall to solubilizing compounds. This chapter provides insight into important considerations in membrane engineering, together with the opportunities and challenges encountered. Examples and lessons learned from my research on membrane engineering regarding the reduction of the acetic acid diffusion rate in *S. cerevisiae* (**Paper I-IV**) will be discussed together with examples from the literature.

Two distinct strategies can be used in membrane engineering to alter the physiochemical properties of the cell membrane, as summarized in Box 2. Physiochemical membrane properties can be altered either by changing the abundance of the cell's own lipid species, or by introducing novel lipid species from other

organisms into the selected microorganism. Many different terms are used to define physiochemical membrane properties; illustrated in Figure 11. Membrane rigidity is often used as an antonym to fluidity, and high rigidity often implies low membrane permeability (Endo et al., 2011). High membrane thickness, lipid packing, and lipid order, together with a small membrane area, are also parameters describing a rigid membrane with low permeability. The term used often depends on the specific method used to determine membrane properties.

Box 2: Two strategies in membrane engineering that can be used to alter the physiochemical properties of the cell membrane

Changing the abundance of the microorganism's own lipid species: This can be achieved by engineering the intrinsic lipid biosynthesis pathways of the selected microorganism. The flux can be changed either by targeting the cell's own enzymes, or by introducing genes from related organisms encoding enzymes with similar functions, but with altered activity/specificity.

Introducing novel lipid species from other organisms: Heterologous lipid species with interesting properties can be identified by studying other microorganisms. Membrane engineering will then be used to introduce a biosynthetic pathway for this particular lipid into the microorganism of interest, and to ensure that it is localized in the cell membrane.

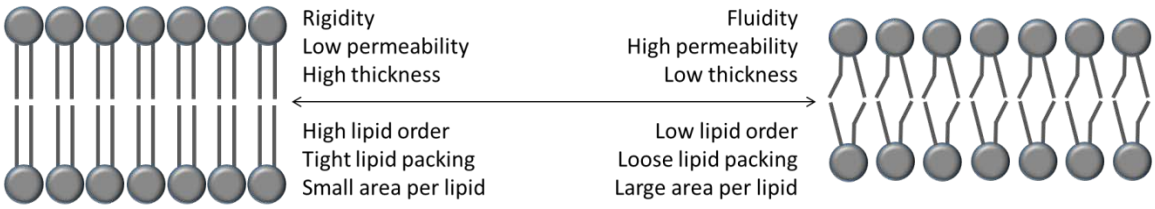


Figure 11: Illustration of the terms used to describe physiochemical membrane properties. Membrane rigidity is often used as an antonym to fluidity, and high rigidity often implies low membrane permeability. High membrane thickness, lipid packing, and lipid order, together with a small membrane area, are parameters describing a rigid membrane with low permeability.

5.1 Identifying which lipids to target in membrane engineering

Cell membrane lipid composition is highly diverse, as discussed in **Chapter 4**. It can therefore be difficult to predict which membrane lipid composition that will give a specific physiochemical membrane property, which is desirable to know in membrane engineering. The molecular structure of the different lipid species has traditionally been used to predict how each lipid species will influence the physiochemical properties of the membrane. The specific features of the membrane lipids in *S. cerevisiae* are illustrated in Figure 12. Long, saturated fatty acyl chains, and a high ratio of cylindrical-to-cone-

shaped glycerophospholipids ((PC+PI)/PE), as well as a high sterol and sphingolipid membrane fraction have all been reported to cause high membrane rigidity (**Paper II**; (Flis et al., 2015; Gulati et al., 2010; Van der Rest et al., 1995). Long fatty acyl chains increase membrane rigidity by increasing the membrane thickness and lipid packing, as the van der Waals force between fatty acyl chains increases with length (De Kroon et al., 2013; Kučerka et al., 2011). Saturated fatty acyl chains increase lipid packing and membrane thickness due to their straight conformation, in comparison to unsaturated fatty acyl chains, which have a bent conformation. A high (PC+PI)/PE ratio increases lipid packing as cylindrically shaped PC and PI pack tighter together than cone-shaped PE. Sphingolipids increase membrane lipid packing and membrane thickness due to their long and fully saturated fatty acyl chains, combined with the amide group in the backbone, facilitating tighter lipid packing by creating hydrogen bonds to adjacent lipids (**Paper II**; (Levine et al., 2000; Slotte, 2016). Sterols increase membrane rigidity by ordering the fatty acyl chains, thus allowing tighter lipid packing (Caron et al., 2014; Dufourc, 2008; Holthuis & Menon, 2014). At high membrane rigidity, sterols have the opposite effect and instead reduce membrane rigidity. However, describing the effect of specific lipid species on the membrane properties in this way is a simplification, and in reality, when lipids interact in a highly heterogeneous membrane, the situation is much more complex. Learning from solutions already invented in nature, evaluating the lipid profile in response to the specific stress, and computer simulations to predict membrane properties, are three strategies that can be used to design the membrane lipid composition for a specific purpose in a more complex setting. These will be discussed in the following three sections.

5.1.1 Solutions in nature

Scientists have realized the potential in the enormous biodiversity created by nature, and the field of biomimicry, i.e., mimicking nature, is growing rapidly (Hunter, 2017). Many of the desirable properties of a cell factory have already been developed through evolution, as organisms have adapted to diverse habitats. Evaluating these naturally tolerant organisms offers a means of obtaining guidance and inspiration for membrane engineering. Naturally tolerant organisms can be studied for two distinct purposes. A tolerant organism can be studied to identify novel lipid species with interesting properties not present in the targeted microbial cell factory. Membrane engineering will then involve introducing a biosynthetic pathway for this heterologous lipid species. A tolerant organism can also be studied to identify the changes in membrane lipid abundance that can be achieved by engineering the intrinsic lipid biosynthesis pathways of the selected microorganism. It is thus beneficial to consider which kind of membrane engineering is to be used before selecting a model organism.

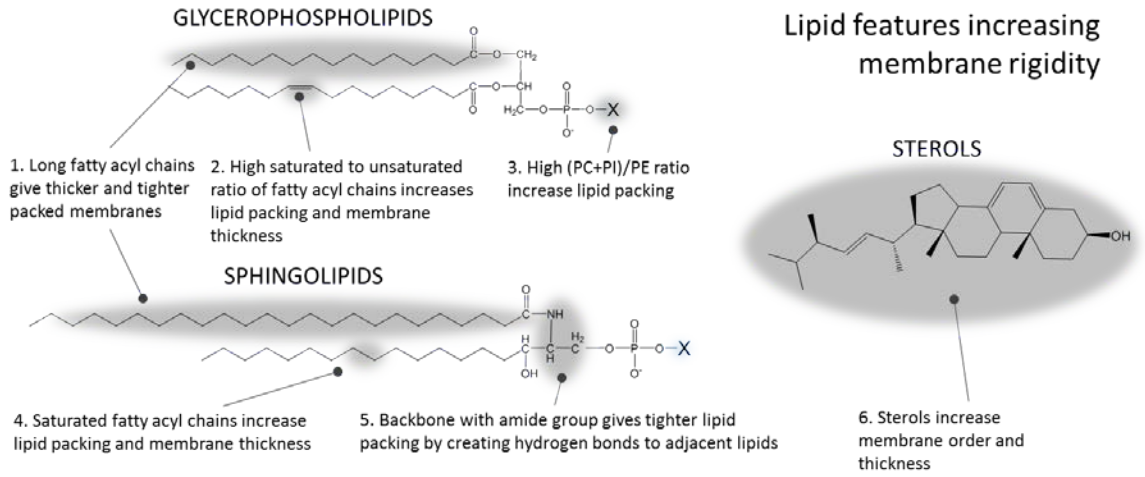


Figure 12: General features of the lipids in *S. cerevisiae* that contribute to increased membrane rigidity. 1. Stronger van der Waal forces between long lipid chains facilitate tighter lipid packing. 2. The double bond introduces a bent lipid tail with poorer packing properties than saturated lipid tails. 3. Cylindrically shaped PC and PI pack tighter than cone-shaped PE. 4. Sphingolipids in yeast have fully saturated fatty acyl chains. 5. The glycerol backbone of glycerophospholipids cannot form hydrogen bonds. 6. Sterols organize and straighten up lipid tails resulting in thicker, more tightly packed membranes. However, at high membrane rigidity, sterols have the opposite effect and instead reduce membrane rigidity.

The aim of my research was to improve acetic acid tolerance in *S. cerevisiae* by reducing the passive diffusion of acetic acid into the cell through the lipid bilayer of the cell membrane by membrane engineering (**Papers I-III**). Acetic-acid-tolerant microorganisms have evolved over time due to their use in the production of vinegar (Mas et al., 2014), and the use of weak organic acids as food preservatives (Piper, 2011). In my work, I intended to engineer the intrinsic lipid metabolism of *S. cerevisiae*, and the closely related spoilage yeast *Z. bailii* was therefore chosen as the model organism. Acetic acid bacteria were not used, as bacteria have a cell membrane consisting mainly of proteins and glycerophospholipids, while eukaryotes have a more complex membrane composed of proteins, glycerophospholipids, sphingolipids and sterols (Hannich et al., 2011; Kaiser et al., 2011). The cell membranes in the two domains have a similar degree of order, but the order in prokaryotes arises from membrane proteins, while in eukaryotes sphingolipids and sterols obtain membrane order (Kaiser et al., 2011). If the aim is instead to discover unique lipid features, a model organism distant from yeast is probably a better choice.

Interesting lipid species that are novel to *S. cerevisiae*, originating from other microorganisms are summarized in Table VI. The ether-linked isoprenoid lipid tails of the phospholipids found in archaea distinguish them from the phospholipids found in bacteria and eukaryotes (Table III, **Section 4.2**). These create a rigid membrane with low ion permeability, important for survival under extreme conditions such as high temperature or salinity (Oger & Cario, 2013). A membrane engineering project is ongoing to introduce ether-linked isoprenoid lipids into *E. coli* (Caforio et al., 2015).

Bolalipids such as tetraether lipids are isoprenoid lipids in archaea with sufficiently long lipid tails to span both leaflets of the lipid bilayer, with a head group on each side of the membrane (Oger & Cario, 2013). Bolalipids can also bend and localize in only one of the leaflets. It has been suggested that bolalipids can increase membrane rigidity, and they have been found to offer protection *in silico*, to membranes exposed to butanol (Bulacu et al., 2012). Cyclopropane ring formation of lipid tails occurs in both bacteria and archaea, and this has been demonstrated, for example, to increase membrane rigidity, improving membrane stability under high temperature stress, in both bacteria and archaea (Grogan & Cronan Jr, 1997; Oger & Cario, 2013). Hopanoids are a group of molecules present in certain bacteria with a ring structure resembling that of sterols in eukaryotes (Caron et al., 2014). They have been shown to have a similar function to sterols in controlling membrane rigidity, and have been suggested to be important in the high temperature and alcohol tolerance of *Zymomonas mobilis* (Caron et al., 2014; Schmidt et al., 1986). Isomerization of the commonly occurring bent fatty acids in *cis*-conformation to straight fatty acids in *trans*-conformation is a mechanism used by *Pseudomonas* and *Vibro* species to increase membrane rigidity in response to temperature and solvent stress (Heipieper et al., 2003). This mechanism has proven successful in membrane engineering to increase the tolerance of *E. coli* cells to octanoic acid, as well as other membrane-related inhibitors such as acetic acid and butanol (Tan et al., 2016). The membrane engineering targets presented here will be further discussed in **Section 5.4**.

Table VI: Lipid species novel to *S. cerevisiae* with interesting properties

Lipid	Organism	Reference
Ether-linked isoprenoid lipids	Archaea	Caforio et al., 2015; Oger & Cario, 2013
Bolalipids	Archaea	Bulacu et al., 2012; Oger & Cario, 2013
Cyclopropane ring formation	Bacteria, Archaea	Grogan & Cronan, 1997; Oger & Cario, 2013
Hopanoids	*Zymomonas mobilis*	Caron et al., 2014; Schmidt et al., 1986
Trans-unsaturated fatty acids	*Pseudomonas* & *Vibro* species	Tan et al., 2016

In the search for interesting profiles of lipid species occurring naturally in *S. cerevisiae*, most studies have compared lipid profiles of tolerant and non-tolerant *S. cerevisiae* strains (Aguilera et al., 2006; Chi & Arneborg, 1999; Zheng et al., 2013). These comparisons within the same species showed only minor differences in lipid profiles, making it difficult to draw general conclusions. The lack of greater differences in lipid profiles is probably due to the fact that the tolerance of the tolerant *S. cerevisiae* strains has many other physiological explanations apart from membrane properties. It is therefore desirable to have a hypothesis regarding the cause of the differences in tolerance of the strains, before investigating membrane lipid composition. In my research, *Z. bailii* was used as a model for high acetic acid tolerance, and it was

hypothesized that one mechanism underlying its high tolerance was a membrane lipid composition that affords a rigid membrane, leading to a low acetic acid diffusion rate. This hypothesis was based on the previous observation that *Z. bailii* retains its intracellular pH and plasma membrane integrity upon exposure to acetic acid, better than *S. cerevisiae* (Arneborg et al., 2000; Prudêncio et al., 1998). To test this hypothesis, detailed lipidomic profiling of *S. cerevisiae* and *Z. bailii* was performed (**Paper I**). The most striking finding was that the membrane of *Z. bailii* was highly enriched in sphingolipids at the expense of sterols, compared to *S. cerevisiae* (Figure 13). *Z. bailii* also exhibited increased glycerophospholipid chain length, reduced glycerophospholipid saturation, and increased (PC+PI)/PE ratio, compared to *S. cerevisiae*. Interpreting these differences in terms of overall membrane characteristics is difficult. The sphingolipid abundance, glycerophospholipid chain length, and (PC+PI)/PE ratio of *Z. bailii* indicate a more rigid membrane than in *S. cerevisiae*, while sterol abundance and glycerophospholipid saturation indicate the opposite (Figure 12). However, glycerophospholipid saturation is not a good measure of the overall membrane saturation, as *Z. bailii* contains a high fraction of sphingolipids whose fatty acyl chains are completely saturated. The relatively lower amount of sterol is then the only factor that does not indicate increased rigidity of the *Z. bailii* membrane, compared to *S. cerevisiae*. To learn more, the changes in membrane lipid profile caused in *Z. bailii* and *S. cerevisiae* by exposure to acetic acid were investigated (**Section 5.1.2**, **Paper I**).

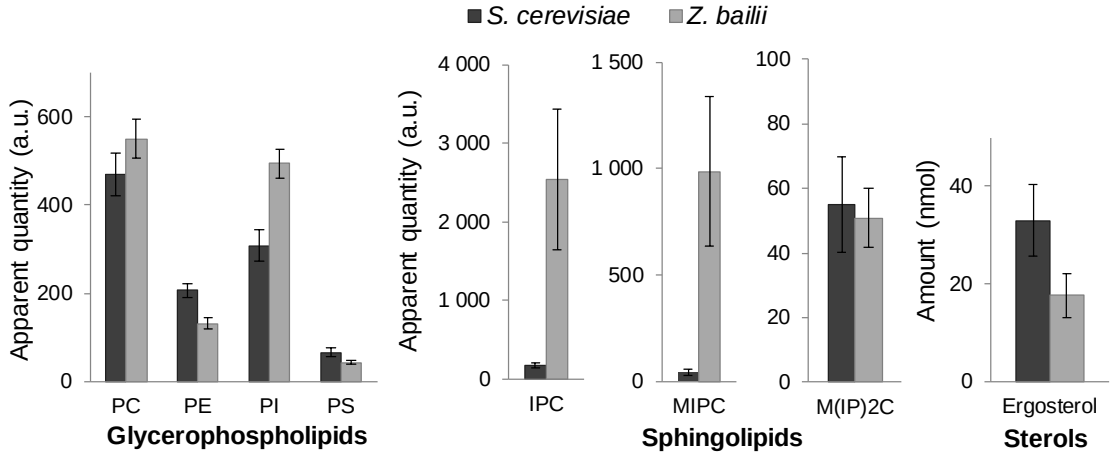


Figure 13: Membrane lipid profiles of *S. cerevisiae* and *Z. bailii*. Cells used for lipid analysis were cultured in minimal medium at pH 5 and harvested during exponential growth. Lipids were analyzed using ESI-MS and GC-MS. Apparent quantities were calculated relative to the appropriate internal standard, and normalized to the total amount of phosphate in each sample. (For experimental details, see Paper I.) Each bar represents the mean of four biological replicates ± standard deviation. Abbreviations: PC, phosphatidylinositol; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; IPC, inositol phosphoryl ceramide; MIPC, mannosyl-inositol phosphoryl ceramide; MIP_2C, mannosyl-di-inositol phosphoryl ceramide.

5.1.2 Lipid profile changes in response to membrane-related inhibitors

Cells change their membrane lipid profile in response to a wide range of external stimuli (**Section 4.6**, (Huffer et al., 2011; Klose et al., 2012). This section describes how cells adapt their lipid profile when exposed to membrane-related inhibitors, and how this information can be used in membrane engineering. In my research (**Paper I**), the lipid profiles of *S. cerevisiae* and *Z. bailii* were analyzed in cells grown in the presence of acetic acid at a concentration causing a 50% reduction in maximum specific growth rate (9 g/L and 24 g/L at pH 5, for *S. cerevisiae* and *Z. bailii*, respectively). In spite of a similar level of inhibition in terms of growth reduction, the more tolerant *Z. bailii* was able to redesign its membrane lipid composition to a greater extent than *S. cerevisiae* upon acetic exposure (Figure 2 and Figure 3 in **Paper I**). In fact, as extreme rearrangement of the lipid profile as that seen in *Z. bailii* (Figure 14) is, to my knowledge, rarely reported in the literature. Sphingolipids, which were already higher in *Z. bailii* than in *S. cerevisiae*,

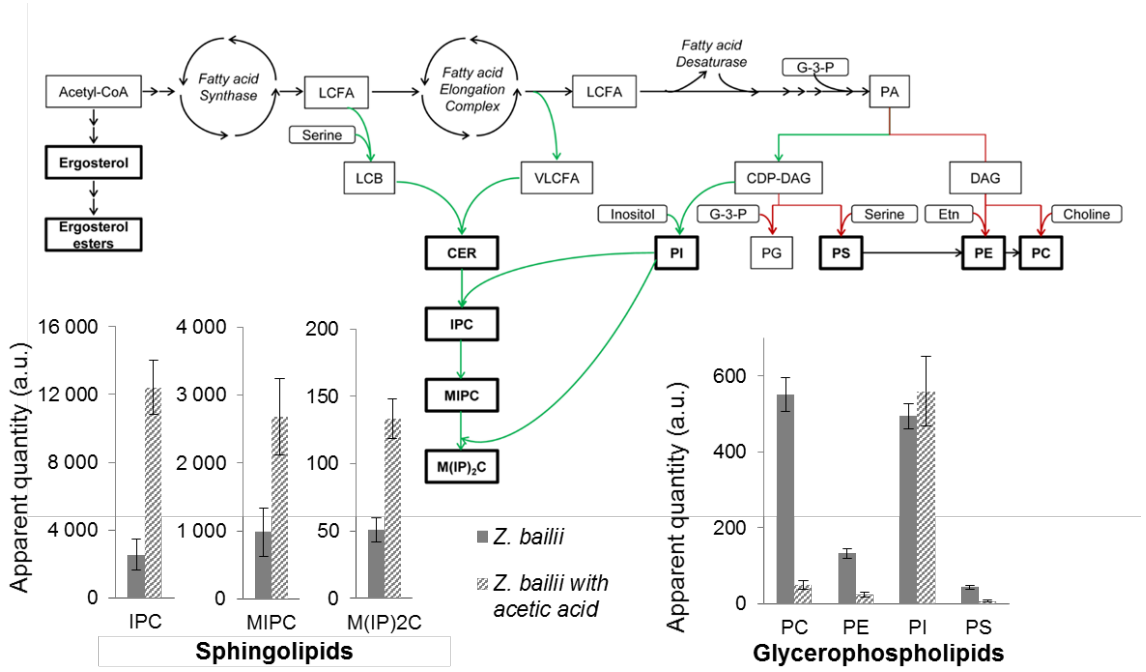


Figure 14: Changes in lipid profile in *Z. bailii* when cultured in the absence and presence of acetic acid. A simplified illustration of lipid metabolism is shown together with the quantities of sphingolipids and glycerophospholipids detected in *Z. bailii* grown at pH 5, without and with 24 g/L acetic acid. Green/red arrows indicate increased/reduced quantities upon exposure to acetic acid. Boxes in bold indicate the lipid classes analyzed (**Paper I**), but all the classes are not shown in this figure. (For experimental details, see **Paper I**.) Each bar represents the mean of four biological replicates ± standard deviation. Abbreviations: LCFA, long-chain fatty acid; LCB, long-chain base; VLCFA, very-long-chain fatty acid; CER, ceramide; IPC, inositol phosphoryl ceramide; MIPC, mannosyl-inositol phosphoryl ceramide; MIP_2C, mannosyl-di-inositol phosphoryl ceramide; G-3-P, glycerol-3-phosphate; PA, phosphatidic acid; CDP-DAG, cytidine diphosphate diacylglycerol; DAG, diacylglycerol; Etn, ethanolamine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PS, phosphatidylserine; PE, phosphatidyl-ethanolamine; PC, phosphatidylinositol.

increased by about 5-fold upon exposure to acetic acid. Furthermore, glycerophospholipids, which are normally the most abundant class of lipids in yeast, decreased to very low levels, with the exception of PI, the concentration of which remained high, probably because it also acts as a precursor for sphingolipids.

Initially, it appeared that the abundance of sphingolipids increased also in *S. cerevisiae* cultured with acetic acid (**Paper I**). However, this increase could not be confirmed in a later re-analysis of sphingolipid quantities due to large variations between biological and technical replicates (**Paper I correction**). However, the sphingolipid profile of *Z. bailii*, showed consistent results, probably because the quantities were higher in this yeast, and therefore less influenced by variations. The glycerophospholipid profile of *S. cerevisiae* was largely unaffected by exposure to acetic acid, while the concentration of ergosterol decreased (Figure 2 and Figure 3 in **Paper I**). A reduction in ergosterol was not expected as it is thought that this would lead to a reduction in membrane rigidity (Caron et al., 2014; Holthuis & Menon, 2014). In general, I consider that cells would strive towards a more rigid membrane in response to membrane-related inhibitors which either solubilize the membrane, or depend on passive diffusion through the cell membrane for their inhibition. In an attempt to validate this hypothesis, the literature on changes in membrane lipid profile in *S. cerevisiae* upon exposure to membrane-related inhibitors was surveyed, and the results presented in Table VII.

Table VII: Membrane lipid composition of *S. cerevisiae* exposed to membrane-related inhibitors

Stress	Log *P*	Sterols	Sphingolipids	Glycerophospholipids			Reference
				(PC+PI)/PE	Chain length	Saturation	
Acetic acid	-0.31	↓	N/A[1]	↑	→	→	**Paper I**
Acetic acid[2]	-0.31	→	↑	↑	→	↑	**Paper I**
H_2O_2	-0.09	→	↓[3]	↑	→	↑	Pedroso et al., 2009
Ethanol	0.07	↓	N/A	N/A	N/A	↓	Alexandre et al., 1994
Ethanol	0.07	N/A	N/A	N/A	↑	↓	Yazawa et al., 2011
Ethanol	0.07	N/A	N/A	N/A	N/A	↑	Huffer et al., 2011
Butanol	0.97	N/A	N/A	N/A	N/A	↑	Huffer et al., 2011
Isobutanol	0.95	N/A	N/A	N/A	N/A	↑	Huffer et al., 2011
Octanoic acid	2.43	N/A	N/A	N/A	↑	↑	Liu et al., 2013
D-limonene	3.01	↓	N/A	N/A	↑	↑	Liu et al., 2012
Temperature	-	↑	↑	↑	↑	↑	Klose et al., 2012

All the lipid parameters presented have previously reported to increase membrane rigidity. ↑= Increase, ↓ = decrease, → = no or minor change, N/A = No data available. [1]Retracted from the study due to non-reproducible data. [2]In *Z. bailii*. [3]Sphingolipids were quantified by the total level of very-long-chain fatty acids.

Lipid profiles in Table VII are described in terms of the parameters reported to increase membrane rigidity. Unfortunately, it is difficult to draw any general conclusions from the findings presented in the table, as complete lipid profiling was not performed in

many of the studies, and the magnitude of the changes are also important when estimating the overall effect. However, a few general trends can be observed. The fatty acyl saturation index was evaluated in all the studies, and increased degree of saturation was observed in the majority (Huffer et al., 2011; Klose et al., 2012; Liu et al., 2012; Liu et al., 2013; Pedroso et al., 2009), except for in two of the studies investigating the effect of ethanol (Alexandre et al., 1994; Yazawa et al., 2007). The fatty acyl chain length and (PC+PI)/PE ratio of glycerophospholipids were either increased or unchanged in all the studies where this lipid parameter was analyzed. Sterol levels decreased in response to exposure to acetic acid, ethanol and D-limonene, while they increased with increasing temperature. This indicates that cells perhaps do not use sterols to increase membrane rigidity in response to membrane-related stresses, but a larger set of studies are required to confirm this. Sphingolipids were only evaluated in three studies making it difficult to draw any general conclusions. However, several findings in a recent publication indicate the need for sphingolipids in tolerance to acetic acid also in *S. cerevisiae* (Guerreiro et al., 2016).

5.1.3 Prediction of membrane properties using molecular dynamics simulations

MD simulations of model membranes is useful in identifying which lipids that should be targeted in membrane engineering to achieve a specific membrane property (method described in **Section 4.7.3)**, and to obtain an indication of the physiochemical properties of a membrane composed of the membrane lipids detected in a particular organism. In my research, MD simulations were performed based on the findings of the lipidomic profiling performed on *S. cerevisiae* and *Z. bailii* (**Paper II**). In particular, the effect on the membrane of an increased fraction of sphingolipids at the expense of glycerophospholipids, as observed in *Z. bailii* cultured with acetic acid, was investigated. Model membranes consisting of the two major glycerophospholipid classes PC and PI, the major sphingolipid class IPC, and the sterol ergosterol were constructed in collaboration with a computational chemist (for details see Table I in **Paper II**). From these simulations it was concluded that a high fraction of sphingolipids increased lipid packing and membrane thickness, resulting in an increase in the free energy barrier for acetic acid diffusion though the membrane. To further confirm the results obtained from MD simulations, I used myriocin, a specific inhibitor of sphingolipid biosynthesis (Wadsworth et al., 2013), to gradually decrease the sphingolipid fraction in *Z. bailii* (**Paper II**). This experiment demonstrated that a high fraction of sphingolipids is required in *Z. bailii* to maintain its low rate of acetic acid diffusion and its high acetic acid tolerance. Furthermore, I have used MD simulations to predict the effect of ethanol and n-butanol on membrane properties, discussed in **Section 5.2** (**Paper IV**).

MD simulations have also been used to predict the effects of interesting lipid species that are novel to *S. cerevisiae*. The effects of bolalipids, a lipid species spanning both

membrane leaflets present in archaea, have been predicted in model membranes with saturated PC lipids (Bulacu et al., 2012). Bolalipids were shown to increase membrane density, and reduce the increase in membrane area caused by butanol. MD simulations have also been used to predict the effects on the membrane of hopanoids, a sterol-like structure present in some bacterial species, showing that hopanoids, in similarity to sterols, increase membrane rigidity at elevated temperatures (Caron et al., 2014). MD simulations may also prove to be useful in the planning of membrane engineering aimed at introducing novel lipid species from other organisms into *S. cerevisiae*. Such simulations may provide indications of the titers of the heterologous lipid species required to obtain a specific membrane effect, giving information on the plausibility of achieving this titer by metabolic engineering.

5.2 Additional considerations in membrane engineering

A number of factors must be taken into consideration when planning a membrane engineering strategy to significantly improve the tolerance of cells to membrane-related inhibitors. In this section, the connection between a low rate of inflow of membrane-related inhibitors and an improved tolerance are discussed, together with synergism between various membrane-related inhibitors.

5.2.1 The effect of reduced membrane permeability on inhibitor tolerance

This section discusses the conditions under which changes in membrane lipid composition will have a significant effect on the tolerance to type 2 membrane-related inhibitors (Box 1). I consider that the effect of changing the physiochemical properties of the membrane depends on the molecular structure of the inhibitory compound diffusing through the cell membrane. More specifically: compounds that have a relatively low rate of permeation, such as acetic acid, will be influenced to a larger extent by a change in lipid profile, than more hydrophobic compounds, which diffuse through the membrane at a higher rate. This conclusion was partly based on my finding that reduced sphingolipid abundance in *Z. bailii* decreased its tolerance to the less hydrophobic weak acids, acetic acid, formic acid, and lactic acid, while no effect was seen on its tolerance to the more hydrophobic weak acids, sorbic acid and benzoic acid (Figure 5 in **Paper II**). Furthermore, low concentrations of benzoic acid and sorbic acid (1-10 mM) affected cell growth, indicating that they inhibit cells by a specific target. In contrast, acetic acid, formic acid, and lactic acid, which affected cell growth at much higher concentrations (0.1-1 M), have instead been found to have general effects as a result of reduced intracellular pH and changed intracellular environment (Ullah et al., 2012).

In membrane engineering aimed at increasing the tolerance to inhibitors that enter the cell by passive diffusion, it is important to bear in mind that the diffusion rate is only one parameter influencing the cells' tolerance. For example, if the membrane lipid composition of the *S. cerevisiae* cell membrane is designed to resemble that of *Z. bailii*, implying that the rate of acetic acid diffusion will be similar in the two yeasts, they will still not exhibit the same tolerance to acetic acid, as the tolerance also depends on the capacity of the specific organism to remove intracellular acetic acid (Figure 15).

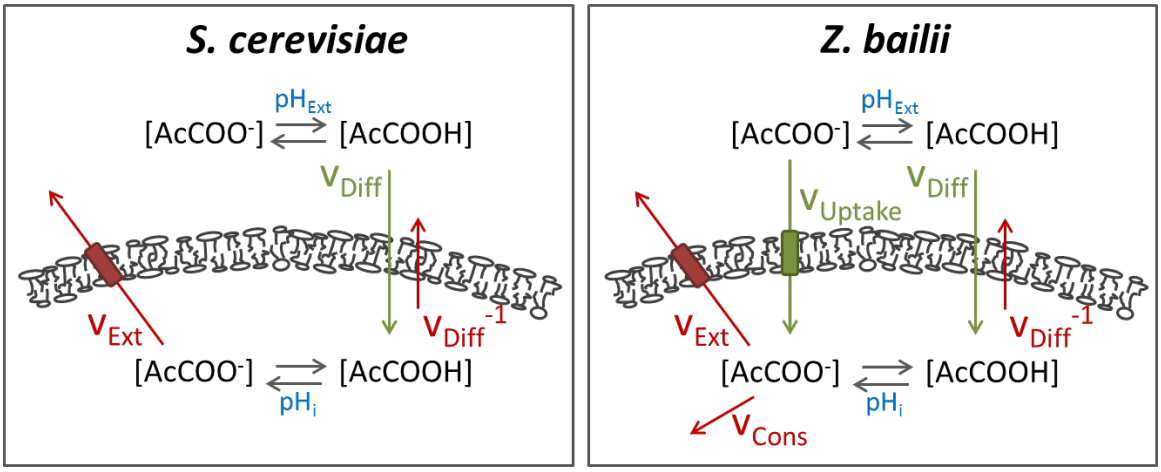


Figure 15: Illustration of the mechanisms determining the intracellular acetic acid concentration in *S. cerevisiae* and *Z. bailii*. The pH determines the distribution between the undissociated and the dissociated forms of acetic acid, based on the pKa value, which is 4.8 for acetic acid. In *S. cerevisiae*, undissociated acetic acid enters the cell mainly by passive diffusion across the lipid bilayer (v_{Diff}). (Facilitated diffusion through the Fps1 channel has been omitted from this illustration due to the degradation of Fps1 upon exposure to acetic acid; (Mollapour et al., 2008). In *Z. bailii*, undissociated acetic acid enters the cell by passive diffusion across the lipid bilayer, in similarity with *S. cerevisiae*, but at a lower rate than in *S. cerevisiae* (**Paper II**). In addition, *Z. bailii* has a facilitated transport system for the uptake of dissociated acetic acid (v_{Uptake}), which is lacking in *S. cerevisiae*. The accumulation of acetic acid in *S. cerevisiae* is counteracted by acetate and proton efflux (v_{Ext}), while acetate consumption is the major mechanism of acetic acid removal in *Z. bailii* (v_{Cons}). Proteins for acetate and proton efflux (v_{Ext}) are also present in *Z. bailii*, but their significance is unclear (Piper et al., 2001). The combination of all these rates and the extracellular pH (pH_{Ext}) and intracellular pH (pH_i) determine the acetic acid concentration in the cell.

In *S. cerevisiae*, acetic acid influx is counteracted by acetate and proton efflux (Fernandes et al., 2005; Piper, 2011; Serrano, 1984). Acetic acid influx in *Z. bailii* is, in addition to the mechanisms in *S. cerevisiae*, also counteracted by its ability to consume intracellular acetate in the presence of glucose (Sousa et al., 1996). This provides *Z. bailii* with an efficient method of removing intracellular acetic acid. Another physiological difference between *S. cerevisiae* and *Z. bailii* is that *Z. bailii* has a facilitated transport system for the uptake of dissociated acetic acid from the extracellular environment (Sousa et al., 1996; Sousa et al., 1998). Combining membrane engineering for reduced inflow of type 2 membrane-related inhibitors (Box 1), with engineering to increase the rate of inhibitor removal, would therefore be extra effective.

5.2.2 Synergism between membrane-related inhibitors

One of the purposes of membrane engineering is to equip microbial cell factories with a membrane lipid composition that affords optimal membrane properties, such as fluidity, under the process conditions to be used in a specific industrial application. In my research, attention was focused on the membrane-related inhibitor acetic acid, but in an industrial process, it is likely that several membrane-related inhibitors will be present, as discussed in **Section 2.2**. Few studies have been carried out on the synergistic effects of inhibitors in the context of the cell membrane. In order to obtain information on the extent to which membrane-related inhibitors that partition in the lipid bilayer affect the rate of acetic acid diffusion, studies were performed on the synergistic effects of acetic acid and ethanol or n-butanol (**Paper IV**). Ethanol and n-butanol were chosen as both are renewable fuels that can be produced from lignocellulosic raw material, often rich in acetic acid. This study revealed that ethanol and n-butanol have strong effects on the acetic acid diffusion rate in *S. cerevisiae*; n-butanol being the alcohol that increased the acetic diffusion rate most. By measuring the diffusion of ^{14}C-labeled acetic acid into *S. cerevisiae*, it was concluded that concentrations of 40 g/L ethanol and of 8 g/L n-butanol both caused a 65% increase in the acetic acid diffusion rate (Figure 3 in **Paper IV**). This ethanol concentration is industrially relevant as 40 g/L has been described as the minimal titer for an economically feasible process, as the energy demand for alcohol distillation in the downstream processing is significantly increased at lower titers (Galbe et al., 2007). However, an n-butanol concentration of 8 g/L is below than what is industrially feasible. MD simulations revealed that the effect of n-butanol is stronger than the effect of ethanol because its longer hydrocarbon chain penetrates deeper into the membrane, thereby increasing the membrane area, and the number of water molecules in the membrane interior to a larger extent than ethanol (Figure 6 in **Paper IV**). Furthermore, it was concluded that the more hydrophobic alcohol n-butanol is preferentially accumulated in the membrane, rather than in the water phase, while ethanol was relativity equally distributed between the two phases (Table II in **Paper IV**). The n-butanol concentration in the cell membrane is thus likely to be higher than the 8 g/L which was initially added to the cells, further explaining the stronger effect of n-butanol than ethanol on the rate of acetic acid diffusion. The findings of this study underline the importance of considering the interaction between compounds interacting with the cell membrane.

Ethanol and high temperature constitutes another combination that probably enhances the level of stress needed for membrane disruption, as both are known to cause increased membrane fluidity (Guyot et al., 2015; Stanley et al., 2010). In contrast to alcohols, the microbial production of carotenoids suffers from increased membrane rigidity (Liu et al., 2016b). An interesting approach would be to investigate the

combined production of compounds increasing membrane rigidity and compounds increasing membrane fluidity. However, such an approach may cause too large increase in membrane area, as both compounds would likely be located in the membrane. Another approach would be to add or overproduce compounds that increase membrane stability by interacting with the head groups of membrane lipids, such as the non-reducing disaccharide trehalose (Kapla et al., 2013). Trehalose is known to provide protection against various environmental stresses in *S. cerevisiae*, including acetic acid and ethanol (Mansure et al., 1994; Yoshiyama et al., 2015).

5.3 Membrane engineering – what has been done so far?

Membrane engineering has only been used successfully in a few studies to develop microbial cell factories with maintained performance in the presence of membrane-related inhibitors. However, membrane engineering is commonly suggested as a potential engineering strategy (Dunlop, 2011). This section describes my research carried out to increase the sphingolipid membrane fraction in *S. cerevisiae* (**Paper III**), together with membrane engineering strategies reported in the literature. The lessons learned from these strategies are then discussed in the following section on challenges and opportunities in membrane engineering.

Steps exhibiting the highest flux control of the sphingolipid biosynthesis pathway should be targeted to increase the fraction of sphingolipids occurring in the membrane. In addition, it is important to ensure that the effect of increased sphingolipid production is not lost due to increased degradation. If the pathway regulation is well characterized, relieving flux control at the rate determining steps, by engineering the regulatory mechanisms, may be a viable option. If this is not possible, attempts could be made to reduce the flux control of a particular step by overexpressing the genes encoding the enzymes involved in that particular step. Furthermore, if the pathway contains toxic intermediates or intermediates with a second cellular function, the accumulation of these intermediates must be avoided. The structural enzymes of the sphingolipid biosynthesis pathway are well characterized, while the regulatory mechanisms and transport of lipids between cellular compartments are still poorly understood (Olson et al., 2016). LCBs, phosphorylated LCBs, and ceramides are sphingolipid intermediates with essential cell functions besides the formation of structural lipids (Epstein & Riezman, 2013). Poorly understood regulation and the requirement not to accumulate LCBs and ceramides are therefore important considerations when designing a metabolic engineering strategy. In my work, I decided to use a combined strategy, targeting both pathway regulation and mRNA quantities of structural enzymes (Figure 16). The idea was to shunt the C_{16} and C_{18} fatty acids produced into sphingolipid production, rather than glycerophospholipid production,

by targeting the SPT complex, which catalyzes the condensation of C_{16} and C_{18} fatty acids and serine to form LCBs (Breslow et al., 2010). An attempt was made to increase the flux though the SPT complex by deleting the *ORM1* and *ORM2* genes, encoding negative regulators of the complex. Furthermore, to increase the flux through the sphingolipid biosynthesis pathway, the fraction of VLCFA, a lipid species unique to sphingolipids, was targeted by overexpression of the *ELO3* gene, encoding a protein in the fatty acid elongation complex, required for the production of the longest fatty acyl chains (Choi & Martin, 1999). In addition, to ensure that increased flux through the sphingolipid synthesis pathway resulted in the accumulation of membrane sphingolipids rather than the toxic precursor ceramide, the gene catalyzing the addition of a phosphatidylinositol head group to the ceramide moiety, *AUR1*, was over-expressed.

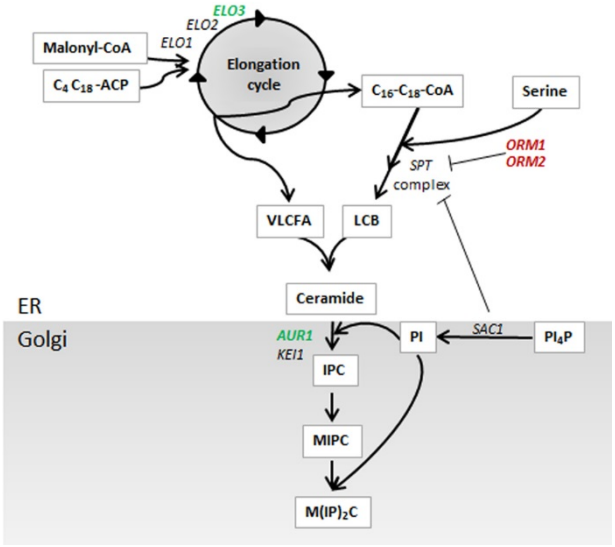


Figure 16: Metabolic engineering strategy used to increase the sphingolipid abundance in *S. cerevisiae*. Illustration of sphingolipid metabolism. Genes overexpressed in this study are indicated in green and deleted genes are indicated in red. Abbreviations: VLCFA, very-long-chain fatty acid (C_{24}-C_{26}); LCB, long-chain base; PI, phosphatidylinositol; PI_4P, phosphatidylinositol 4-phosphate; IPC, inositol phosphoryl ceramide; MIPC, mannosyl-inositol phosphoryl ceramide; MIP_2C, mannosyl-di-inositol phosphoryl ceramide. (Reprinted from **Paper III**.)

The strategy designed to increase the sphingolipid membrane fraction in *S. cerevisiae* resulted in unaltered or reduced sphingolipid levels (Figure 2 in **Paper III**), which is contrary to the original hypothesis. Deletion of *ORM1* and *ORM2* led to a decrease in the fraction of complex sphingolipids, and overexpression of *ELO3* and *AUR1* had only minor effects on the lipid profile, compared to the wild type. The strain with all four mutations (*ELO3-AUR1-OE-orm1/2Δ*), showed reduced levels of sphingolipids, in similarity with the *orm1/2Δ* strain. The overall conclusion drawn from these results was that too little is known about the regulatory network for sphingolipid biosynthesis, and that targeting mRNA levels of structural enzymes is not a suitable approach to increase sphingolipid levels, as recent studies have demonstrated post-translational

regulation of sphingolipid biosynthesis (Aguilera-Romero et al., 2014; Da Silveira Dos Santos et al., 2014).

More than half of the published studies on membrane engineering have focused on increasing the fraction of glycerophospholipids with the fatty acid oleic acid ($C_{18:1}$) in the membrane, with the aim of improving cell tolerance to acetic acid, ethanol, and octanoic acid, as well as improving growth at low temperatures (Table VIII) (Kajiwara et al., 2000; Liu et al., 2013; Yazawa et al., 2011; You et al., 2003; Zheng et al., 2013). All the studies focused specifically on the beneficial effect of oleic acid, but it should be stressed that oleic acid has two district properties which have different effects on the membrane. Its chain length of 18 carbon atoms contributes to increased membrane thickness, as most glycerophospholipids consist of either 16 or 18 carbons. Also, its degree of unsaturation contributes to a reduction in membrane saturation, which instead increases membrane fluidity and decreases membrane thickness (as discussed in **Section 5.1**). To understand the effect of increased oleic acid content on the membrane, it is therefore important to consider the increase in oleic acid in relation the overall changes in fatty acyl chain length and saturation. Overexpression of the fatty acid desaturase *OLE1* in *S. cerevisiae* resulted in a 33% increase in the oleic acid content at the expense of palmitic acid ($C_{16:0}$), causing a 7% overall increase in unsaturated fatty acids (Kajiwara et al., 2000). This strain showed improved cell growth and ethanol productivity at low temperature (10 °C), which could be expected, as the changes in the lipid profile indicate a more fluid membrane, which is beneficial at low temperature, since membrane fluidity decreases with decreasing temperature (Aguilera et al., 2007). However, a high oleic acid content has also been reported to be important in tolerance to stress by acetic acid, ethanol, and octanoic acid (Liu et al., 2013; Yazawa et al., 2011; You et al., 2003; Zheng et al., 2013). All of the authors tried to increase the oleic acid content by increasing the degree of fatty acid desaturation, either by overexpressing fatty acid desaturase, *OLE1*, or by introducing desaturases from other organisms. This particular strategy, to reduce fatty acid saturation, seemed surprising to me as it reduces membrane rigidity (Figure 12), which is in contrast to my attempt to increase sphingolipid abundance, and thus membrane rigidity (**Paper III**). However, *OLE1* has indeed been found to be upregulated, for example, in the presence of acetic acid stress, but together with other changes in gene expression affecting the lipid profile (Kawahata et al., 2006; Lee et al., 2015). Introducing the fatty acid desaturase TniNPVE from *Trichoplusia ni* into *S. cerevisiae* led to a 2-fold increase in the oleic acid content and improved growth in 5% ethanol (w/v, or v/v not indicated) (You et al., 2003). However, it is also important to note that the $C_{18:1}$ fatty acyl chains also increased, with an even greater reduction in $C_{16:1}$ fatty acyl chains. Thus, the main change in membrane composition was an increase in fatty acyl chain length, and not in the degree of saturation. The same strategy, introducing TniNPVE from *Trichoplusia ni* into *S. cerevisiae*

was adopted by Liu et al. to improve its tolerance to octanoic acid. However, only a minor increase in oleic acid content was seen this time, and no improvement in octanoic acid tolerance (Liu et al., 2013). Attempts to increase the oleic acid content by *OLE1* overexpression have also been made to improve tolerance to ethanol and acetic acid, although no significant effects were seen on the oleic acid content (Yazawa et al., 2011; Zheng et al., 2013). The reason why *OLE1* overexpression increased the oleic acid content in the study by (Kajiwara et al., 2000) could be that the cells were grown at 10 °C, instead of 30 °C, since cells likely prefer to increase their membrane unsaturation at lower temperatures (Aguilera et al., 2007).

Increasing oleic acid content by targeting the fatty acid elongation complex enabled improved tolerance to ethanol and acetic acid. Yazawa and coworkers increased the oleic acid content by 44%, by introducing the fatty acid elongase *ELO2* originating from the rat (*rELO2*) into *S. cerevisiae*, resulting in prolonged survival time in ethanol (Yazawa et al., 2011). However, the increase in $C_{18:1}$ fatty acids was compensated for by a reduction in $C_{16:1}$ fatty acids, so the main change in fatty acids was an increase in chain length, similar to the results reported by (You et al., 2003). (Zheng et al., 2013) also increased the oleic acid content by targeting fatty acid elongation, specifically by the overexpression of fatty acid elongase *ELO1*. This change in mRNA levels caused an 18% increase in oleic acid content, resulting in a 44% increase in survival after acetic acid exposure. However, the increase in $C_{18:1}$ fatty acid was also compensated for by a reduction in $C_{16:1}$ fatty acids in this case. In conclusion, the ability of oleic acid to reduce membrane saturation appears to be important for the growth of *S. cerevisiae* at low temperatures (Kajiwara et al., 2000), while the ability of oleic acid to increase fatty acid chain length appears to be important for the tolerance of *S. cerevisiae* to acetic acid, ethanol, and octanoic acid (Yazawa et al., 2011; You et al., 2003; Zheng et al., 2013).

Increased membrane saturation was the explicit aim in two other studies (Table VIII). Tan et al. introduced a *cis-trans* isomerase from *Pseudomonas aeruginosa* into *E. coli*, to convert the 30-degree bent unsaturated fatty acids in *cis*-conformation, to straight, unsaturated fatty acids in *trans*-conformation, which do not occur naturally in *E. coli* (Tan et al., 2016). This strategy leads to a lipid conformation like that of tightly packed saturated lipids, but probably avoids the cells regulation of fatty acyl chain saturation. A ratio of *trans* to *cis* fatty acids of 0.078 appeared to be optimal, resulting in a 12% increase in the maximum specific growth rate of *E. coli* cells in the presence of octatonic acid, as well as other carboxylic acids and solvents. Another strategy that has been used to improve the viability of *E. coli* cells producing free fatty acids was to increase the saturation of fatty acyl chain membrane lipids (Lennen & Pfleger, 2013). This was achieved by engineering the free fatty acid production to use mainly unsaturated fatty acids, thereby reducing the cells' ability to incorporate them into membrane lipids.

Cyclopropane ring formation on fatty acyl chains takes place in many bacteria and archaea species, and it has been shown to decrease membrane permeability in *E. coli* (Grogan & Cronan Jr, 1997; Oger & Cario, 2013). Membrane engineering to produce cyclopropane fatty acids in *S. cerevisiae* successfully transformed 10% of the fatty acids into cyclopropanated fatty acids, but no increase in octanoic acid tolerance was observed (Liu et al., 2013). Through evolutionary engineering it was discovered that a point mutation in the *ERG3* gene, encoding a structural enzyme in the sterol biosynthesis pathway, lead to an increase in the growth of *S. cerevisiae* at high temperature (39.5 °C) (Caspeta et al., 2014). Lipid analysis revealed that this point mutation made the sterol precursor fecosterol the major sterol, constituting about 80% of the total sterols. Fecosterol forms a bent sterol structure, while ergosterol, usually the major sterol in yeast, has a straight sterol structure. This structural difference was suggested to be the reason for the increased thermotolerance, but there was no detailed discussion of the biophysical mechanisms.

A number of comprehensive membrane engineering projects are ongoing (Table VIII). De Kroon and coworkers are attempting to increase the tolerance of yeast to hydrophobic substances by membrane engineering (De Kroon, 2016). Kengen and coworkers have introduced ether-linked isoprenoid lipids originating from archaea into *E. coli*, but the fraction of archaea lipids in the membrane must be increased before a study on membrane robustness can be carried out (Caforio et al., 2015). A systematic study is also ongoing in an attempt to understand the functional basis of membrane composition in *E. coli*, by knocking out genes in the lipid metabolism and reintroducing the genes on plasmids with promotors for titratable gene expression (Budin, 2014).

Table VIII: Studies on membrane engineering to improve tolerance of microorganisms to membrane-related inhibitors

Target stress	Organism	Target lipid change	Strategy	Effect	Reference
Successful membrane engineering strategies					
Acetic acid	*S. cerevisiae*	Increased fraction of $C_{18:1}$ fatty acids (oleic acid)[1]	Overexpression of fatty acid elongase *ELO1*	44% increase in survival after acetic acid exposure	Zheng et al., 2013
Ethanol	*S. cerevisiae*	Increased fraction of $C_{18:1}$ fatty acids (oleic acid)[1]	Introduction of fatty acid elongase *rELO2* from the rat	Improved survival time in ethanol-rich medium	Yazawa et al., 2011
Ethanol	*S. cerevisiae*	Increased fraction of $C_{18:1}$ fatty acids (oleic acid)[1]	Introduction of fatty acid desaturase TniNPVE from *Trichoplusia ni*	Improved growth in ethanol-rich medium	You et al., 2003
Low temperature	*S. cerevisiae*	Increased fraction of $C_{18:1}$ fatty acids (oleic acid)[2]	Overexpression of fatty acid desaturase *OLE1*	Increased growth and ethanol productivity at 10 °C	Kajawara et al., 2000
High temperature	*S. cerevisiae*	Accumulation of the sterol precursor fecosterol	Point mutation inactivating *ERG3* in sterol biosynthesis[3]	50% increase in maximum specific growth rate at 39.5 °C	Caspeta et al., 2014
Octanoic acid	*E. coli*	Conversion of unsaturated fatty acids from *cis*- to *trans*-conformation	Introduction of fatty acid *cis-trans* isomerase from *Pseudomonas aeruginosa*	12% increase in maximum specific growth rate in the presence of octanoic acid	Tan et al., 2016
Free fatty acids	*E. coli*	Increased fraction of saturated fatty acids	Prevention of incorporation of unsaturated fatty acids in the membrane by their use in free fatty acid production	Improved viability of free fatty acid producing *E. coli*	Lennen & Pfleger, 2013

Membrane engineering strategies not showing the desired effect

Acetic acid	*S. cerevisiae*	Increased fraction of sphingolipids	Overexpression of *ELO3* and *AUR1*, deletion of *ORM1* and *ORM2*, all genes in sphingolipid biosynthesis	Unchanged or decreased fraction of sphingolipids	**Paper III**
Acetic acid	*S. cerevisiae*	Increased fraction of $C_{18:1}$ fatty acids (oleic acid)	Overexpression of fatty acid desaturase *OLE1*	No effect on oleic acid content	Zheng et al., 2013
Ethanol	*S. cerevisiae*	Increased fraction of $C_{18:1}$ fatty acids (oleic acid)	Overexpression of fatty acid desaturase *OLE1*	Small increase in oleic acid content, stress tolerance not evaluated	Yazawa et al., 2011
Octanoic acid	*S. cerevisiae*	Increased fraction of $C_{18:1}$ fatty acids (oleic acid)	Introduction of fatty acid desaturase TniNPVE from *Trichoplusia ni*	9% increase in oleic acid content but only 3% increase in oleic acid content in cells cultured with octanoic acid. No increase in octanoic acid tolerance	Liu et al., 2013
Octanoic acid	*S. cerevisiae*	Introduction of cyclopropane fatty acids	Introduction of cyclopropane fatty acyl phospholipid synthase from *E. coli*	10% of the fatty acids were cyclopropanated, but no increase in octanoic acid tolerance	Liu et al., 2013
Ongoing studies					
Hydrophobic substances	*S. cerevisiae*	Not specified	Not specified	No results reported yet	De Kroon, 2016
No specific	*E. coli*	Introduction of ether-linked isoprenoid lipids from archaea	Reconstruction of pathway for ether-linked isoprenoid lipids by introducing nine heterologous enzymes	Lipid titers are still too low, only proof of a functioning pathway	Caforio et al., 2015
No specific	*E. coli*	No specific - aim was to understand the functional basis of cell membrane composition	Deletion of lipid synthesis genes and their reintroduction on plasmids for titrable gene expression	No results reported yet	Budin, 2014

[1]Increased fraction of glycerophospholipids with $C_{18:1}$ fatty acids at the expense of $C_{16:1}$ fatty acids, so the overall effect on the membrane was increased fatty acyl chain length. [2]Increased fraction of glycerophospholipids with $C_{18:1}$ fatty acids at the expense of $C_{16:0}$ fatty acids, so the overall effect on the membrane was increased fatty acyl unsaturation and increased fatty acyl chain length. [3]Thermotolerance by mutation of the *ERG3* gene was discovered by evolutionary engineering coupled to whole-genome sequencing of the evolved strains.

5.4 Challenges and opportunities in membrane engineering

Membrane engineering as a tool to increase the tolerance of cells to membrane-related inhibitors has been demonstrated in only a few studies, in which the effect has been obtained by targeting one specific gene, and the strategies have, with a few exceptions, been focused on the fatty acyl chain composition of membrane lipids. The lack of successful comprehensive membrane engineering strategies is probably due to the fact that membrane engineering is a relatively new concept, and that significant rearrangement of the membrane lipid profile is challenging, as the cells constantly strive towards membrane lipid homeostasis. My attempt to increase the sphingolipid membrane fraction of *S. cerevisiae* (**Paper III**) exemplifies the difficulty in changing the abundance of the cells own lipid species. This kind of strain engineering suffers from an insufficient understanding of the regulatory mechanisms of lipid biosynthesis, cross-talk between lipid classes, and the fact that many lipids have dual functionality, being important for other cellular functions (as discussed in **Section 4.5-4.6**). In addition to that, I have the impression that the literature describing membrane lipid profile of specific gene alterations are not always consistent. For example, *orm1/2*Δ has been reported to result in an increase *and* a decrease in sphingolipids (**Paper III**, (Breslow et al., 2010; Shimobayashi et al., 2013). Reasons for this inconsistency may be that the lipid profile is sensitive to specific experimental conditions (Klose et al., 2012), and that the advanced analytical methods employed are sensitive to variations in sample preparation. Furthermore, studies in which only part of the lipidome is analyzed may be misleading, as the high degree of interconnection in lipid metabolism (Fügi et al., 2015; Shevchenko & Simons, 2010), may cause important changes in lipid species not quantified in the specific study.

My idea of reducing the rate of acetic acid diffusion into *S. cerevisiae* by increasing the fraction of sphingolipids in the membrane arose from studying *Z. bailii*, a yeast that has evolved naturally to tolerate high levels of acetic acid. This example shows that nature often excels in finding solutions, however, the solutions developed by nature may be challenging to mimic with genetic engineering. Changing the membrane lipid profile by genetic engineering is facilitated by a low degree of post-translational regulation, intermediates without other cellular functions, and robust analytical methods for lipid quantification. Attempting to change the abundance of sphingolipids in *S. cerevisiae* is thus fraught with difficulties. Sphingolipid regulation is not yet fully understood, but it is controlled to a high degree by post-translational modifications, and many of the intermediates in the pathway also act as signaling molecules (**Sections 4.4 & 4.5**). In addition, the mass spectrometry method, which is used to analyze sphingolipids, suffers from both ion suppression and the inability to accurately quantify sphingolipid species due to a lack of internal standards (Megyeri et al., 2016). Therefore, it would perhaps

be easier to focus on changing glycerophospholipid or sterol abundance as these have been studied traditionally, leading to robust analytical methods and better characterized regulation. In fact, engineering of the fatty acyl chain composition of glycerophospholipids has already been successfully demonstrated to increase the tolerance of microorganisms to membrane-related inhibitors (Yazawa et al., 2011; You et al., 2003; Zheng et al., 2013). In addition, changes in membrane properties, such as increased thermotolerance, have been achieved by the inactivation of *ERG3* in the sterol biosynthesis pathway, resulting in the accumulation of the sterol precursor fecosterol, instead of the final product ergosterol (Caspeta et al., 2014). However, predicting the outcome of a strategy designed to change the abundance of glycerophospholipids or sterols is still challenging due to extensive regulation and cross-talk between lipid species (Ballweg & Ernst, 2017; Fügi et al., 2015; Henry et al., 2012).

Another interesting approach would be to alter the physiochemical properties of the cell membrane by introducing novel lipid species from other organisms to the microorganism of interest. This strategy takes advantage of the full diversity of nature and avoids the cells' own regulatory system. However, in addition to having a functioning metabolic pathway for the specific lipid species, the cell must also have mechanisms to ensure that the lipids produced are incorporated into the cell membrane. Examples of interesting lipid species novel to *S. cerevisiae* are listed Table VI. Increasing membrane rigidity by converting fatty acids in the *cis*-conformation to the *trans*-conformation has been proven to increase the specific growth rate of *E. coli* exposed to a list of membrane-related inhibitors (Tan et al., 2016). Engineering of *S. cerevisiae* to produce cyclopropanated fatty acids, enabled cyclopropane ring formation on 10% of the fatty acyl chains, but with no effect on octanoic acid tolerance (Liu et al., 2013). However, it was not investigated whether the lack of effect could be due to mislocalization of the cyclopropanated fatty acids.

It is important in membrane engineering to ensure that the new lipid composition does not affect the activity of the membrane proteins. Specific lipid species have been shown to be important for the activity of several membrane proteins (Coskun & Simons, 2011), demonstrating the need for at least a small amount of the native lipid species in the membrane. However, as the cell rearranges its membrane lipid composition in response to environmental changes (Klose et al., 2012), so have membrane proteins probably evolved to tolerate fluctuations in the membrane lipid profile. Indeed, there was no obvious trade-off in *Z. bailii* after considerable changes in the lipid profile upon exposure to acetic acid (**Paper I**), suggesting that membrane engineering could be feasible without largely affecting the function of membrane proteins.

Chapter 6

Conclusions

Membrane engineering is an emerging tool in cell factory design. The method has great potential, but is highly complex as membrane lipids and membrane homeostasis are vital for many cellular functions. In this work, I have investigated the possibility of increasing the tolerance of the yeast *S. cerevisiae* to acetic acid by engineering the lipid composition of the cell membrane to reduce the rate of acetic acid diffusion into the cell. In the first study (**Paper I**), I discovered that the yeast *Z. bailii,* which is very tolerant to acetic acid, exhibited a considerable difference in membrane lipid composition compared to *S. cerevisiae,* and a unique ability to remodel its membrane lipid composition upon exposure to acetic acid. The cell membrane of *Z. bailii* contained much higher levels of sphingolipids, which is the third major class of lipids in *S. cerevisiae*, and upon exposure to acetic acid, the level of sphingolipids in *Z. bailii* increased to such an extent that the major class of lipids, glycerophospholipids, were reduced to very low levels. In the next step (**Paper II**), I designed a model membrane and concluded from MD simulations that an increased fraction of sphingolipids creates a thicker and more tightly packed membrane, with increased free energy barrier for the diffusion of acetic acid through the membrane. The importance of a high fraction of sphingolipids in the cell membrane of *Z. bailii* was further demonstrated by gradual inhibition of sphingolipid synthesis, causing a decrease in acetic acid tolerance and an increase in the diffusion rate of acetic acid. Based on these findings, I hypothesized that a high fraction of sphingolipids in the cell membrane would have the potential to reduce the rate of acetic acid diffusion in *S. cerevisiae*. However, in order to increase acetic acid tolerance, reduced diffusion must be balanced by intracellular removal of acetic acid, and it is the net effect of entry and removal that influences which concentrations of acetic acid that the cell can tolerate. In *S. cerevisiae*, the diffusion of acetic acid into the cell is balanced by acetate and proton efflux, while in *Z. bailii*, acetate consumption is the major mechanism counteracting the accumulation of intracellular acetic acid.

My aim of the study described in **Paper III** was to evaluate the possibility of increasing the fraction of sphingolipids in the cell membrane of *S. cerevisiae* by changing the

expression of the genes associated with sphingolipid biosynthesis. With the gene combinations investigated, I was unable to increase the fraction of sphingolipids in the cell membrane, and the strains instead showed unaltered or decreased levels of sphingolipids. Despite these unexpected results, this study demonstrated that much remains to be learnt on the regulation of sphingolipid biosynthesis, and contributed to unraveling some of the complex interactions involved.

To explore the synergistic effects of membrane-related inhibitors, I investigated the extent to which compounds that partition into the membrane affect the acetic acid diffusion rate, and focused on ethanol and n-butanol (**Paper IV**). This work revealed that ethanol and n-butanol have a strong effect on the acetic acid diffusion rate; n-butanol increasing the acetic diffusion rate more than ethanol. MD simulations revealed that the biophysical explanation was that the longer hydrocarbon chain in n-butanol penetrates deeper into the membrane, thereby increasing the membrane area, and the number of water molecules in the membrane, to a greater extent than ethanol. It was also concluded that the more hydrophobic alcohol n-butanol partitions in the membrane, rather than in the water phase, while ethanol was relatively equally distributed between the two phases, further explaining the stronger effect of n-butanol than ethanol on the acetic acid diffusion rate. This work highlights the value of a comprehensive view of the industrial conditions that can be expected before designing a specific membrane engineering strategy.

Chapter 7

Future perspectives

I envision that membrane engineering will be a well-established tool in cell factory design within 10 years. I also predict that research efforts within the field will increase steadily, not due to a high degree of success, but because studies on cell physiology will indicate the need to adapt cell membranes to the conditions used in industrial applications. Only a few attempts to employ membrane engineering have proved successful in increasing the tolerance of microorganisms to membrane-related inhibitors, largely due to the complexity of the technique. To remedy this, I suggest interdisciplinary collaborations as a first step, in which skilled researches in lipidomics, fundamental lipid research, membrane biophysics, and cell factory design work closely together. Moreover, a better understanding of how cells sense and change their membrane lipid composition is required.

Lipidomics has had a tremendous impact on lipid research and, thanks to advanced mass spectrometry, it is now possible to accurately quantify all the major lipid species in an organism. Traditionally, lipid analysis, and thus the identification of lipid species thought to be important with regard to a specific phenotype, has been based on only a subset of lipid species in the cell. Now with improved analytical techniques, I encourage complete lipidome analysis in membrane engineering research. Like the carbon balance in cell physiology, it is important to balance the membrane lipid species when evaluating the lipidome response. In my work, I discovered that enrichment of the sphingolipids in the cell membrane is an important mechanism by which *Z. bailii* gains its high acetic acid tolerance (**Papers I** & **II**). Without having performed extensive lipidomic profiling, this discovery would not have been made, as lipid analysis is often restricted to glycerophospholipids and sterols. I consider that caution should be exercised when evaluating the results of previous studies in which only a subset of the lipid species available was analyzed, as the lipid species not analyzed may also be important for the particular phenotype. For example, sphingolipids may be more important in stress tolerance than has been reported in the literature so far. I had the privilege to collaborate with skilled lipidomics researchers in the lab of Howard Riezman, at the

University of Geneva. In the ideal world, lipidome analysis would be available to all researchers, but as these analytical methods require considerable expertise, I suggest collaboration with specialized lipidomics labs, rather than the establishment of lipidomics platforms by each research group.

A fundamental understanding of lipid metabolism and its regulation is of the utmost importance in membrane engineering. During the course of my research, I have experienced the challenges associated with changing the sphingolipid abundance in membrane engineering, and do not consider that this group of lipids is sufficiently characterized to be targeted in membrane engineering. In designing a new strategy aimed at changing the abundance of lipid species already present in *S. cerevisiae,* I would instead try to alter the physiochemical properties of the cell membrane by targeting glycerophospholipids or sterols, as these classes of lipids have traditionally been studied in greater detail, and are likely better characterized. An interesting alternative to targeting the cells' own lipid species is to learn from other organisms in nature and introduce lipid species with interesting properties that are novel to the selected microorganism. This strategy has the advantage that it would probably avoid the cells' own regulatory system and the problems associated with lipids with secondary cellular functions.

Membrane biophysics is another key aspect of membrane engineering. I believe that investigating membrane properties using MD simulations will become more important in the future, along with more advanced membrane models. Most studies carried out to date have evaluated physiochemical membrane properties using model membranes consisting of only a few lipid species. However, the molecular dynamics of more complex membranes has been simulated recently, increasing the biological relevance of this technique (**Papers II & IV**; (Konas et al., 2015; Zhuang et al., 2016). Setting up new membranes is time-consuming, but once it has been done it is relatively easy to change the abundance of lipid species or to replace the water with a solute of interest to obtain biophysical data at atomistic resolution. Therefore, it will probably become increasingly easy to obtain valuable information on physiochemical membrane properties using MD simulations.

Engineering membrane lipid composition is a complex task, and to facilitate cell factory design, I suggest the potential of membrane engineering to be demonstrated using bacteria, which lack the compartmental division present in eukaryotes. Once the field has become more established, research can be expanded to yeasts and other microbial cell factories with more complex physiology.

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