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PHD THESIS

Producing food oils from wood in yeast

The human population is constantly growing. And so is its demand for food oils. However, an increase in food oil production currently requires the deforestation of mostly tropical rainforests to allow the plantation of oil crops. Because of the massive risks caused by climate change and increased awareness of biodiversity, we need to find alternative ways to produce food oils.

Help could come from the tiniest living beings on earth – microorganisms. Microorganisms have been isolated and cultivated to produce a variety of products. A very promising host organism is the yeast *Yarrowia lipolytica*. One of its characteristics is the ability to accumulate high amounts of fat, making it especially interesting to produce fatty acid-derived products, such as food oils.

This thesis explores four different approaches to advance Y. lipolytica as a platform organism for sustainable food oil production. By genetic engineering, we abolished filamentation, an unfavorable behavior in industrial applications. We then altered the fatty acid profile of *Y. lipolytica* by tinkering with proteins called desaturases to mimic a high-value food oil – cocoa butter. To increase the sustainability of the production, we further explored lignocellulosic hydrolysates (e.g. from wood) as an alternative to feed our yeast. By overexpressing native genes, Y. lipolytica utilized xylose as a carbon source. Additionally, we documented the tolerance of our yeast to inhibitors commonly found in hydrolysates. Finally, we compared urea to ammonium sulphate as an alternative nitrogen source using transcriptomic analysis.

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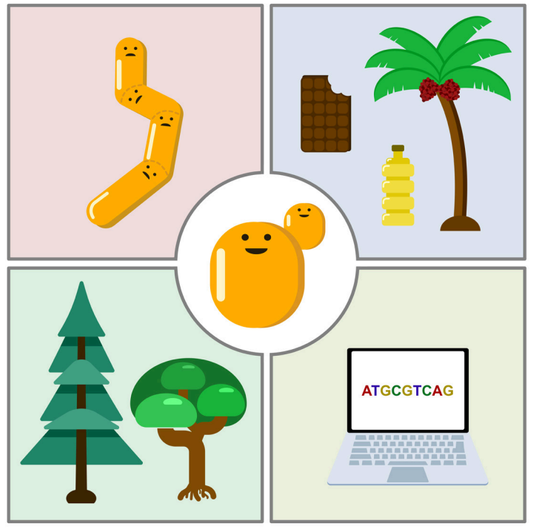
OLIVER KONZOCK

Genetic engineering of Yarrowia lipolytica for the sustainable production of food oils

2022



Genetic engineering of *Yarrowia lipolytica* for the sustainable production of food oils

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CHALMERS UNIVERSITY OF TECHNOLOGY
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THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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Schematic representation of the four different topics of this thesis.

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Abstract

The human population and its demand for food oils is constantly growing. However, increasing food oil production currently requires the deforestation of mostly tropical rainforests to allow the plantation of oil crops. Because of the massive risks caused by climate change and increased awareness for biodiversity, we need to find alternative ways to produce food oils.

Microbial cell factories can be such an alternative. A very promising host organism is the oleaginous yeast *Yarrowia lipolytica*. One of *Y. lipolytica's* characteristics is its ability to accumulate high amounts of lipids, making it especially interesting to produce fatty acid-derived products, such as triacylglycerides (TAGs).

To establish *Y. lipolytica* as a food oil production platform, we first abolished its ability to form filaments by deletion *MHY1.* As a proof of concept, we aimed to mimic cocoa butter as a high value product. We exchanged the Δ9 desaturase OLE1 with homologs from other species and altered the expression level of both Δ9 and Δ12 desaturase to mimic the fatty acid composition of cocoa butter.

To increase the sustainability, we engineered our strain to consume xylose as an alternative carbon source and investigated the effect of different inhibitors commonly found in hydrolysates on *Y. lipolytica*. Finally, we identified urea as an alternative nitrogen source by running chemostat cultivations and performing RNA sequencing.

Overall, this thesis achieved different relevant aspects to develop *Y. lipolytica* as a microbial cell factory for food oils.

Keywords: filamentation, cocoa butter, desaturase, triacyclglycerols, RNA-seq, hydrolysate, tolerance

Content

List of publications

This thesis is based on the work contained in the following papers and manuscripts:

I. **Konzock O**, Norbeck J. Deletion of MHY1 abolishes hyphae formation in *Yarrowia lipolytica* without negative effects on stress tolerance. PloS one. 2020 Apr 3;15(4):e0231161.

II. **Konzock O**, Matsushita Y, Zaghen S, Sako A, Norbeck J. Altering the fatty acid profile of *Yarrowia lipolytica* to mimic cocoa butter by genetic engineering of desaturases. Microbial cell factories. 2022 Dec;21(1):1-1.

III. **Konzock O***, Zaghen S*, Norbeck J. Tolerance of *Yarrowia lipolytica* to inhibitors commonly found in lignocellulosic hydrolysates. BMC microbiology. 2021 Dec;21(1):1-0.

IV. **Konzock O***, Zaghen S*, Fu J, Norbeck J, Kerkhoven E. Coherent transcriptional response of *Yarrowia lipolytica* to carbon and nitrogen limitation in urea and ammonium sulphate. *Manuscript*

Additional published papers not included in this thesis:

V. Blitzblau HG, Consiglio AL, Teixeira P, Crabtree DV, Chen S, **Konzock O**, Chifamba G, Su A, Kamineni A, MacEwen K, Hamilton M. Production of 10-methyl branched fatty acids in yeast. Biotechnology for Biofuels. 2021 Dec;14(1):1-7.

VI. Pandit S, **Konzock O**, Leistner K, Mokkapati VR, Merlo A, Sun J, Mijakovic I. Graphene coated magnetic nanoparticles facilitate the release of biofuels and oleochemicals from yeast cell factories. Scientific Reports. 2021 Oct 18;11(1):1-9.

VII. Tous Mohedano M*, **Konzock O***, Chen Y. Strategies to increase tolerance and robustness of industrial microorganisms. Synthetic and Systems Biotechnology. 2022 Mar 1;7(1):533-40.

* both authors contributed equally to the work

Contribution Summary

Paper I
Designed and performed the experiments, analysed results, and wrote the manuscript.

Paper II
Designed and performed or supervised all experiments, analysed most of the results and wrote the manuscript.

Paper III
Designed and performed or supervised the experiments, analysed results, and wrote the manuscript with the shared co-first author.

Paper IV
Designed and performed the experiments with the co-authors, performed some data analysis and wrote most of the manuscript.

Paper V
Designed and performed some of the experiments.

Paper VI
Designed and performed some of the experiments.

Paper VII
Shared the work of the review equally with the co-first author.

Preface

This dissertation serves as partial fulfilment of the requirements to obtain the degree of Doctor of Philosophy at the Department of Biology and Biological Engineering at Chalmers University of Technology. The PhD studies were carried out between February 2018 and June 2022 at the Division of Systems and Synthetic Biology (Sysbio) under the supervision of Joakim Norbeck and co-supervision of Eduard Kerkhoven. The thesis was examined by Jens Nielsen. The thesis was mainly funded by FORMAS (formas.se) grant number 2017-01281.

Oliver Konzock

May 2022

Abbreviations

ALE	Adaptive laboratory evolution
AMP	Adenosine monophosphate
AS	Ammonium sulphate
C16:0	Palmitic acid
C16:1	Palmitoleic acid
C18:0	Stearic acid
C18:1	Oleic acid
C18:2	Linoleic acid
CBE	Cocoa butter equivalents
CoA	Coenzyme A
Coniferyl aldehyde	4-hydroxy-3-methoxycinnamaldehyde
CRISPR	Clustered regularly interspaced short palindromic repeats
DE	Differentially expressed
ddNTPs	dideoxynucleosides
DGA	Diacylglycerol
DHAP	Dihydroxyacetone phosphate
dNTPs	deoxyribonucleotides
dsDNA	Double strand DNA
ER	Endoplasmic reticulum
FAME	Fatty acid methyl ester
FAS	Fatty acid synthesis
FFA	Free fatty acids
Furfural	2-furaldehyde
G3P	Glycerol-3-phosphate
GC-MS	Gas chromatography-mass spectrometry
GlcNAc	N-acetylglucosamine
HMF	5-hydroxymethylfurfural
HPLC	High performance liquid chromatography
LPA	Lysophosphatidic acid
LPU	Lipid production media with Urea as nitrogen source
MAG	Monoacylglycerol
NHEJ	Non-homologous end joining
NL	Neutral lipids
ORF	Open reading frame
PA	Phosphatidic acid
PCA	Principle component analysis
PL	Phospholipids
PPP	Pentose Phosphate Pathway
ROS	Reactive oxygen species
SPE	Solid-phase extraction
TAG	Triacylglyceride
TCA	Tricarboxylic acid cycle
X5P	D-xylulose-5-phosphate

How to read this thesis

"Always write with the reader in mind" is a commonly given advice before writing any kind of text. Naturally, I asked myself the same question before writing this thesis. Although the chances are high that this thesis will only be read by a few experts who will evaluate me during my graduation, I have the naïve hope that also non-experts from other fields or even non-scientists might stumble upon this book. Therefore, I made the decision to write this thesis in a style that is different from the usual impersonal and prosaic way of writing, which is used in scientific journal articles.

Instead of a report, I tried to write a story that I would enjoy reading myself. The story will present most of the results and discoveries I made during the four years of my PhD. The details on the methodology are kept to a minimum but are found in the attached papers at the end of the thesis.

I hope you will enjoy reading this thesis.[1]

[1] Additionally, I sprinkled footnotes all over this book. They are not essential for understanding the thesis but contain random facts, anecdotes, or personal comments that I hope you will find interesting.

An understanding of the natural world and what's in it is a source of not only great curiosity, but great fulfilment.

– Sir David Attenborough

Introduction

What do cookies, ice cream, cake and chocolate have in common? Besides being one of the favourite treats of most kids (and adults), they have a high content of fat in the form of food oils. Worldwide palm (35%), soybean (29%), rapeseed (14%) and sunflower seed oil (10%) are the most widely used food oils [1]. With a growing world population estimated to reach 8.5 billion by the year 2050 [2], the demand for food oils will keep increasing. However, the plantation of oil crops is consuming vast areas of land. Since some of the most effective oil crops (e.g. palm oil) grow in tropical climates, big areas of tropical rain forests are being destroyed for the plantation of monocultures. These monocultures have significantly lower biodiversity and store less carbon than the original, diverse forest, which increases the effects of climate change [3, 4].

It is apparent that we need an alternative way of supplying the world's need for food oils other than ravaging our planet's most valuable ecosystems. But how?

The history and potential of microbial cell factories

The rescue could come from some of the smallest living beings on earth – microorganisms. Microorganisms are single-cell organisms that have existed for at least 3.8 billion years and have evolved into an immense diversity [5]. They virtually flourish in every corner of our planet: from hot acid springs and black smokers on the bottom of the sea to your skin and intestines [6]. It is not surprising that microorganisms have been in contact with humanity and have even been exploited. The most famous example is the baker's yeast *Saccharomyces cerevisiae*, which ancient civilizations have already used to ferment beer, wine, and bread [7, 8]. However, back then, people were oblivious to the existence of yeast and microorganisms in general. It was not until the period of 1665-1683 that Robert Hooke and Antoni van Leeuwenhoek observed the first microorganisms [9]. It took almost another two centuries until Theodor Schwann identified yeast as the driving force for fermentation in 1837 [10]. Later, Louis Pasteur confirmed these findings and linked the contamination of microorganisms to the spoilage of food, which he showed can be countered by a process he called pasteurization [11, 12]. Since then, more and more microorganisms have been discovered, isolated, studied and finally put into the service of humanity. Cultivated in bioreactors, these microbial cell factories have been used to produce various compounds. But the application has not always been peaceful, such as the production of acetone by *Clostridium acetobutylicum*, which was largely used to produce explosives during the First World War [13, 14]. However, since most organisms only produce small amount of the desired product, scientists tinkered around with cultivation methods and media composition to increase the titres.

This approach changed drastically in the second half of the 20th century. First, Oswald Avery, Colin MacLeod, and Maclyn McCarty identified deoxyribonucleic acid (DNA) as the molecule

that encodes information in 1944 [15]. Then James Watson, Francis Crick, and Rosalind Franklin[2] demonstrated its double-helix structure in 1953 [17]. Now that the code of life was discovered, scientists started to engineer it. In 1972 Paul Berg and his colleagues inserted DNA pieces from *E. coli* into the DNA of virus SV40 [18]. Then Stanley Cohen and Herbert Boyer created the first genetically modified organism (GMO) and set the foundation for recombinant DNA technology by building a recombinant plasmid from DNA fragments of two different sources and then transforming it into *E. coli* [19]. Shortly after, in 1974, they showed that even genes from different kingdoms could be exchanged and expressed by integrating *Xenopus laevis* (African clawed frog) genes into a plasmid and transforming it into *E. coli* [20].

During the same time, different researchers tried to develop methods to sequence DNA [21]. In 1977, Frederick Sanger revolutionized DNA sequencing with his chain-termination method [22]. This method synthesizes DNA from deoxyribonucleotides (dNTPs), the building blocks of DNA. By adding building blocks that lack the 3' hydroxyl group (dideoxynucleosides, ddNTPs), which is essential for the elongation of the DNA, the chain extension is terminated. Radioactive labelling of the ddNTPs enabled the precise identification of the DNA sequence. Constant improvements in this method allowed the sequencing of more and more complex genomes and led to the sequencing of the human genome in 2001 [23]. Since this first-generation sequencing, new approaches have been developed that massively decreased the cost and time for sequencing. Today a human genome can be sequenced for under 1000 US$ [24].

DNA sequencing led to the development and production of recombinant proteins such as human insulin in *E. coli* by David Goeddel, which drastically decreased insulin prices and saved countless lives [25, 26]. The advances in DNA sequencing allowed identifying pathways of complex molecules, which could then be cloned in host organisms for large-scale production, such as the antimalarial drug artemisinin in *S. cerevisiae* by the group of Jay Keasling [27].

The latest revolution is marked by the discovery and utilization of the RNA-dependent *molecular scissor-enzyme* Cas9, for which Emmanuelle Charpentier and Jennifer A. Doudna received the Nobel Prize in Chemistry in 2020. *Clustered regularly interspaced short palindromic repeats* (CRISPR) were first found in *E. coli* in 1987, but their function was still unknown [28]. It took another 20 years and the findings of numerous researchers[3] until Philippe Horvath and his colleagues finally unravelled the biological function of CRISPR/Cas9 as a prokaryotic adaptive immune system [30]. Briefly, if a prokaryote is infected by a virus and manages to survive, the cell stores parts of the attacker's DNA in its genome. These target sequences are used to guide the CRISPR associated enzyme Cas9, the *molecular scissors*, to

[2] Although not listed as an author, Franklin's crystallographic evidence was key to the discovery of the DNA structure [16].
[3] The full discovery story would go beyond the scope of this thesis, but has been nicely reviewed in [29].

the genome of new invading viruses. Cas9 cuts the virus's genome, thereby preventing its integration into the cells genome and ultimately a new infection [29].

The modification of this CRISPR/Cas9 system has led to the development of a variety of genetic tools [31]. Cas9 can initiate dsDNA breaks mediating the genomic integration of desired fragments via homologous recombination. These repair fragments can knock out or integrate genes or manipulate their regulatory sequences. Additionally, mutated Cas9 can repress gene expression (CRISPRi) [32] or guide other enzymes to specific positions in the genome, such as the activation-induced cytidine deaminase (AID) for *in vivo* mutagenesis [33].

CRISPR/Cas9 tools allow the rapid and precise genetic manipulation of a constantly growing number of organisms, ranging from established model organisms such as *S. cerevisiae* [34] and *E. coli* [35], over unconventional organisms such as *Yarrowia lipolytica* [36] and cyanobacteria [37], to multicellular organisms such as plants [38] and mice [39]. CRISPR has even been used to modify the human germline. The announcement was followed by an outcry of ethicists and scientists [40] and initiated the World Health Organization to set guidelines for human gene editing [41].

Finding the right host organism

Given the enormous potential of microbial cell factories and the countless ways of engineering them, why not use them to produce food oils?

To use a microbial cell factory to produce food oils, we first need to decide which host organism to use. A microbial cell factory has to fulfil several criteria to be suitable for the development into a feasible production strain. The organisms should grow fast and in high cell density to ensure a sufficient product titre (product/volume) and production rate (product/time), which is essential for the feasibility of a process [42]. It should be robust for a wide range of pH and temperature to endure different production processes, and its natural metabolism should be in favour of the desired product. For its fast engineering, different tools and protocols should be established in the organism of choice. Additionally, it is beneficial if the organism can consume alternative carbon sources besides glucose, to establish sustainable processes based on lignocellulosic biomass or waste streams.

There are several types of potentially suitable microorganisms (yeasts, moulds, and algae) that have an inherent natural capability to accumulate high lipid contents. If an organism can accumulate more than 20% of its dry weight as lipids, it is considered an *oleaginous* organism and represents an ideal platform for developing lipid-derived products, such as food oils [43]. One widely used representative of oleaginous yeasts is *Yarrowia lipolytica*[4], which meets most

[4] The genus is named after David Yarrow for his contribution to yeast systematics [44] and the species after its ability to consume lipids.

of the above requirements. In recent years, many different tools for this yeast's fast and efficient engineering have been developed. For instance, toolboxes enable precise and rapid genetic engineering [45], CRISPR/Cas9 tools can be used to repress or active gene expression [36], and the characterization and engineering of promoters allow a wide range of protein expression levels [46]. With these and other tools, *Y. lipolytica* has successfully been engineered to produce a wide range of products from proteins (e.g. lipases [47]) over terpene products (e.g. limonene, squalene and β-carotene [48]) to organic acids (e.g. citric acid, itaconic acid [49]) and complex lipids (e.g. branched fatty acids [50], omega-3 eicosapentaenoic acid [51]). Additionally, *Y. lipolytica* can grow fast and reach high cell density and can naturally consume sugars such as glucose, fructose, mannose, and other carbon sources such as fatty acids, alkanes, and glycerol [52].

Furthermore, *Y. lipolytica* has obtained the *Generally Recognized as Safe* (GRAS) status for various applications, such as erythritol and eicosapentaenoic acid (EPA)-rich triglyceride oils production [53]. This status is especially important when considering the production of products for human consumption.

Aim of this thesis

The overall goal of this thesis was the development of *Yarrowia lipolytica* for the sustainable production of food oils. As described above, *Y. lipolytica* is an excellent organism for such an application. However, in the **first chapter (Paper I)**, I will introduce you to the dark side of *Y. lipolytica* – its dimorphic growth – and how we abolished that behaviour to ensure smooth engineering and production performance. In the **second chapter (Paper II)**, we will learn about the composition of food oils, the lipid metabolism of *Y. lipolytica* and how to engineer it to mimic a high-value product, cocoa butter. The **third chapter (paper III)** focuses on using hydrolysates as an alternative carbon source. We will see what hydrolysates are and which problems common inhibitors can pose to the cultivation process. In the **fourth chapter (Paper IV),** we move from the carbon to the nitrogen source and investigate the effect of urea compared to the widely used ammonium sulphate as a nitrogen source. Finally, I will finish this thesis with a summary and an outlook of future developments.

Chapter 1 – Filamentation in *Y. lipolytica*

One soul, two bodies

Y. lipolytica is well known for its lipid production capacities and is considered an oleaginous model organism. However, it is also known as a model organism for dimorphic growth[5]. Dimorphic growth refers to the ability to grow in different morphologies and occurs in many different species [55]. Although the term dimorphism might suggest two different morphologies, it also refers to species that can grow in more morphologies, such as *Candida albicans* with four different forms[6] [56]. *Y. lipolytica* can grow in three different forms: round spherical yeast cells and two elongated filamentous forms, pseudohyphae and septate hyphae [57, 58]. Since pseudohyphae and septate hyphae are very similar, I will refer to both as filaments.

A variety of different environmental factors can trigger filamentation [59]. One of the major factors for filamentation is the pH of the medium. Neutral pH induces filamentation, while lower pH values results in low to no filamentation [55]. Additionally, the carbon and nitrogen source can either trigger or inhibit filamentation. For example, hydrophilic sugars (e.g. glucose or N-acetylglucosamine (GlcNAc)) and ammonium salts favour filamentation, while glutamate or glutamine and hydrophobic carbon sources (e.g. castor oil) do not [55, 60]. It has also been shown that different stress factors such as heat shock, starvation, and anaerobic and oxidative stress can induce filamentation [55, 61, 62].

Furthermore, the aeration method in bioreactors can influence the morphology. It was observed that aeration with stirred tank bioreactors showed less compact, more elongated and irregular cells compared to airlift reactors [63]. Moreover, the morphological changes also depend on the strain background [58].

The round spherical form is usually preferred for biotechnological applications, resulting in better production titres [64]. Round cells can easily be cultivated in large bioreactors in high density while filaments clump together. Filamentation leads to bad aeration and supply of nutrients and can block tubing during bioreactor cultivation, potentially ruining an experiment or cultivation[7]. Because of these problems, the first objective of my work was to find a way to abolish filamentation.

A straightforward way to avoid filamentation is to avoid its potential triggers described above. However, the list of filamentation favouring conditions is long, strain-dependent, and there might still be unexplored triggers. An alternative approach is genetic engineering.

[5] *Y. lipolytica* is also a model for the mitochondrial complex I, which is present in human but not *S. cerevisiae* [54].
[6] David Kerridge criticized the terminology as “best confusing and at its worse potentially misleading” [56].
[7] Sadly, I can confirm this from first-hand experience.

Since every reaction of a cell is encoded in its genes, removing a gene (knock out) could also block its ability to filament. The most promising gene targets are genes with regulatory functions, i.e. genes that regulate the transcription of genes (transcription factors) or the activity of other proteins (protein kinases or phosphatases). Often, these genes regulate multiple other genes and work as components of signalling paths to respond to environmental changes [65].

Gene deletion to abolish filamentation

We sifted through the literature and picked three gene targets that have been reported to abolish filamentation upon deletion: *HOY1*, *CLA4* and *MHY1*.

Hoy1 is a homeobox-protein transcription factor. Its deletion was reported to abolish the hyphae formation [66], while its overexpression resulted in increased filamentation with reduced viability [67]. Cla4 is a protein kinase. Its deletion was reported to abolish filamentation and its ability to invade agar [68]. Mhy1 is a transcription factor containing a C2H2-zinc finger domain. Similar to *HOY1*, the deletion of *MHY1* has been shown to abolish filamentation [69, 70], while its overexpression led to an increased filamentation [69]. Additionally, there have been indications that the deletion of *MHY1* positively impacts lipid production [71, 72].

As mentioned in the introduction, CRISPR/Cas9 has become a very powerful tool to genetically engineer microorganisms. We constructed knockout strains with a CRISPR/Cas9 guided approach [45]. Our strains are based on ST6512 [73], a strain that has the gene encoding Cas9 integrated into the *KU70* locus. KU70 is a protein essential for non-homologous end joining (NHEJ), *Y. lipolytica*'s preferred way to repair double-strand DNA (dsDNA) breaks [74, 75]. With KU70 deleted, the cell is forced to use homologous recombination to repair the dsDNA break, a circumstance we exploit for gene integration and knockout.

To delete the three target genes, we designed guide RNAs (gRNA) that efficiently guide Cas9 to the ORFs of the chosen genes and simultaneously have minimal predicted off-targets [76]. The constitutively expressed Cas9 introduces dsDNA breaks in the target genes that the cell needs to repair to survive. For the gene knockout, we provided the cells with a repair fragment consisting of 50 bp up and 50 bp downstream of the open reading frame (ORF), which is integrated via homologous recombination, effectively deleting the ORF in between.

Testing of the deletion strains

To test which deletions reliably abolished filamentation, we first needed to find the condition in which our parent strain showed strong filamentation. An efficient way to trigger

filamentation is N-acetylglucosamine (GlcNAc) as a carbon source [77]. However, our strain only showed a low level of filamentation when the media was supplemented with GlcNAc.

Since the degree of filamentation was unsatisfying in liquid cultures, we decided to have a closer look at cultures grown on agar plates. We found that colonies grown on plates for a few days almost exclusively consisted of filamentous cells[8]. Therefore, we used cultivations on YPD and LB agar plates to measure the success of the filamentation deletions. YPD is a common complex medium that allows good growth of *Y. lipolytica*. LB media is often used to cultivate *E. coli* and has a high sodium chloride content (5%). *Y. lipolytica* has a high salt tolerance [78] and grows well on LB plates, but the salt still inflicts mild osmotic stress that can trigger additional filamentation.

When we tested our deletion strains, we found that only the mhy1Δ reliably abolished filamentation (**Figure 1**). The hoy1Δ still showed filamentation in both media and cla4Δ on LB plates. The different cellular morphology can also be predicted from the morphology of the colonies. Round yeast cells form smooth and homogenous looking colonies, while filamentous cells show irregular patterns.

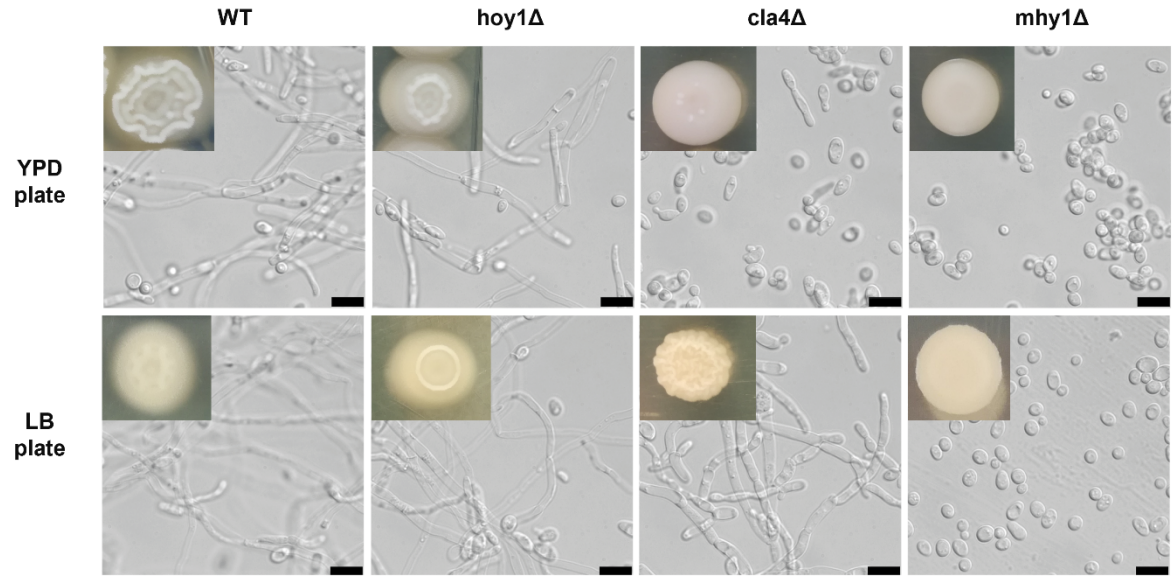


Figure 1: Cell morphology of wild type and deletion strains from plate cultivation. Microscope pictures were taken after 4 days of incubation at 30°C and the black scale equals 10 µm. Colony pictures were taken after 6 days of cultivation of a 10 µL drop of overnight culture on the corresponding plates. Adapted from [206] (Paper I).

Our results showed that the mhy1Δ is the most reliable modification to remove filamentation in our strain background. Additionally, these results are an example of the necessity to publish

[8] Round yeast cells have a creamy consistency on plates, which makes the sampling for experiment or inoculation very easy. Filamentous colonies have a consistency that compares to a chewing gum and is very unpleasant to work with in every-day lab work. Another good reason to abolish this behavior.

negative results (hoy1Δ and cla4Δ) that do not align with previous reports. For example, the hoy1Δ has been tested in the same strain background (W29) before and reported to be very efficient to block filamentation [66]. Yet, in our experimental setup with plate cultivation, we found strong filamentation.

Is there a price to pay for the loss of filamentation?

After identifying mhy1Δ as a suitable target to abolish filamentation, it is tempting to immediately jump into the production of food oils. However, given that Mhy1 is a transcription factor and considering the complexity of the yeast-to-hyphae regulation, there might be some unintended consequences from the genetic engineering we performed. Therefore, before we further engineered our strain to produce food oils, we investigated possible negative effects that might arise from the deletion of *MHY1*.

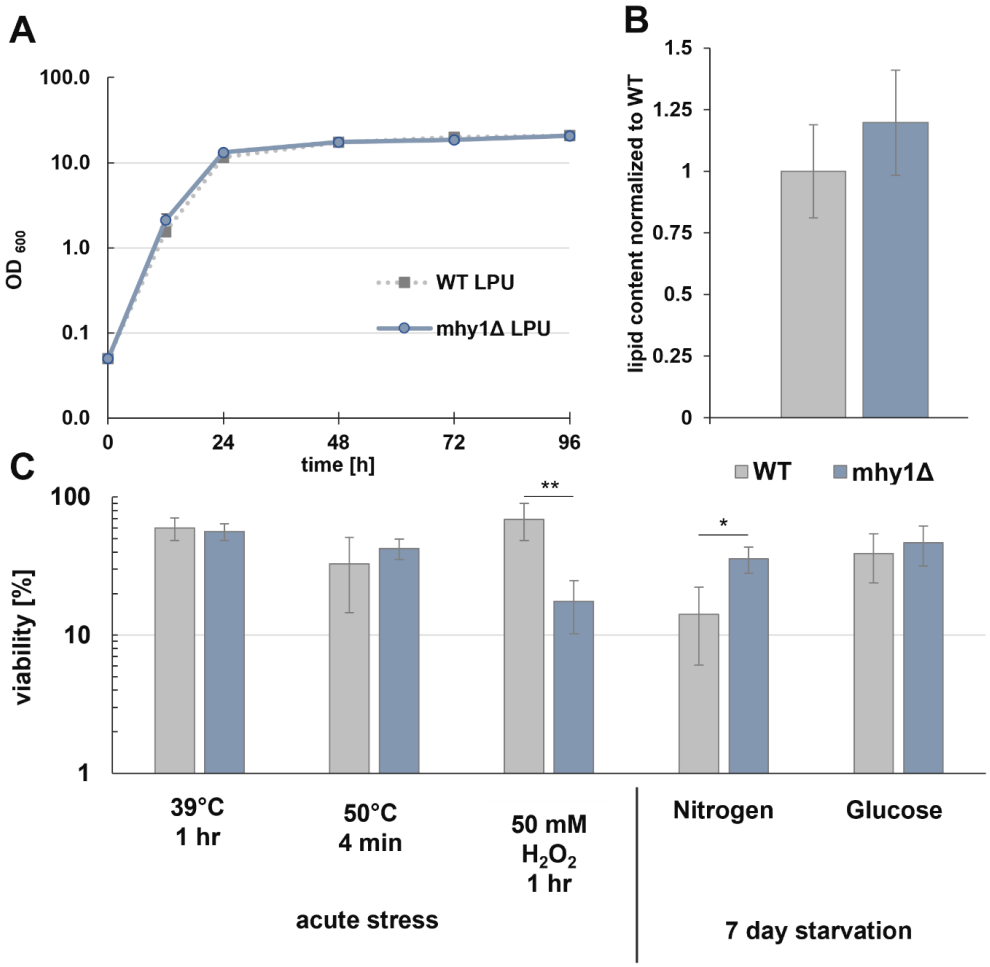


Figure 2: Impact of mhy1Δ on growth, lipid content and stress tolerance. A Growth curves of wild type (WT) and mhy1Δ strain over time. **B** Lipid content normalized to wild type after 4 days of cultivation. **C** Stress tolerance based on colony forming units compared to a non-treated control. Displayed is the mean and standard deviation of triplicates. Significance: * indicates a p-value < 0.05 and ** p-value < 0.01. Adapted from [205] (Paper I).

A negative effect that needs to be assessed is the impact on growth and lipid production. We evaluated the growth by measuring the optical density (OD_{600}) over time and found no difference between the parental and the mhy1Δ strain (**Figure 2A**). Additionally, we measured the total lipid content after four days of cultivation in lipid production media and found no significant difference between the two strains (**Figure 2B**).

In *S. cerevisiae,* the *MHY1* homologues (*ScMSN2* and *ScMSN4)* are important for the general transcriptional stress response [79], and their deletions increased sensitivity to heat, osmotic and oxidative stress [80]. The tolerance to these three stresses is important for large-scale cultivations, and an increased sensitivity could jeopardize future up-scaling attempts and product titres. Therefore, we tested the stress tolerance of the mhy1Δ strain to heat and oxidative stress. Additionally, we tested the long-term starvation over seven days without glucose or nitrogen source since lipid production is achieved during long time cultivation. We used two different heat shocks to test the heat tolerance: a mild heat shock of 39°C for 1 h, which is part of the transformation protocol for *Y. lipolytica*, and a harsh heat shock of 50°C for 4 minutes. Osmotic stress was induced by cultivating the cells in 50 mM hydrogen peroxide (H_2O_2) for one hour.

We found no significant difference between the wild type and mhy1Δ strain for heat tolerance and glucose starvation (**Figure 2C**). During nitrogen starvation, a key inducer for lipid production (for details, see chapter 2), we even observed a positive impact of the mhy1Δ. For the oxidative stress tolerance, we found a significant negative effect of the mhy1Δ. However, the inflicted stress of 50 mM H_2O_2 for one hour is very high. In regular cultivation, oxidative stress of this scale does not occur. Moreover, previous studies reported no effect on growth for mild oxidative stress (0.5mM H_2O_2) [81].

All in all, these results encouraged us to use the mhy1Δ as a background to engineer food oil production strains. We never observed filamentation with this strain throughout all following experiments and did not observe any other negative side effects that we could trace back to the mhy1Δ.

Now that we have tamed the filamentation of our host organism, we can move on to the production of food oils.

Chapter 2 – Mimicking cocoa butter

Vegetable oils have various applications, from food ingredients over cosmetics to biofuels. Last year the worldwide production of vegetable oils reached 206 million tons (**Figure 3**) [1][9]. In most cases, the oils are extracted from the oil-rich seeds of different crops by mechanical or solvent extraction [83].

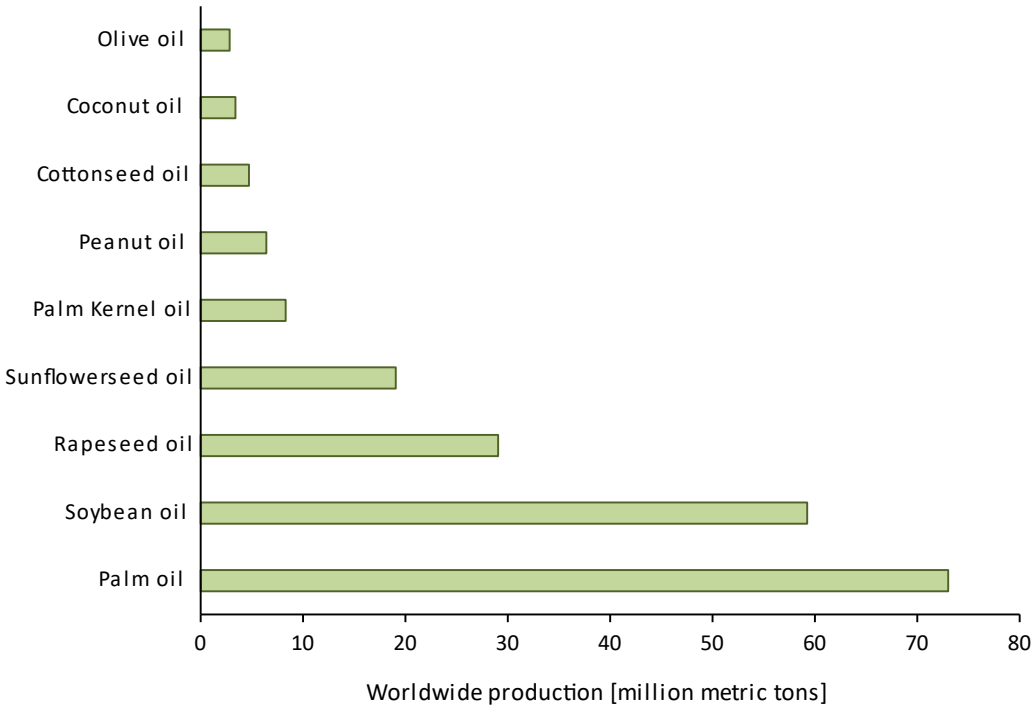


Figure 3: Worldwide vegetable oil production in the crop year 2020/2021. Data obtained from [1].

Vegetable oils mainly consist of triacylglycerides (TAGs), three fatty acids covalently bound via ester bonding to a glycerol backbone. The three fatty acids can vary in length (number of carbon atoms), saturation (number and position of double bonds) and position on the glycerol backbone (sn-1, sn-2, sn-3 position) (**Figure 4**). Fatty acids of vegetable oils commonly have a length of 12 to 20 carbon atoms. They are either unsaturated (no double bonds), mono-unsaturated (one double bond) or polyunsaturated (two and more double bonds). Each

[9] Let me put this into perspective. With an average vegetable oil density of 0.915 g/mL [82] the worldwide vegetable oils production can fill 86.8 Great Pyramids of Giza or a cube with an edge of over 600 m.

vegetable oil is a mix of different TAGs that determine the oils' physical and chemical behaviour, e.g. density, melting point, and viscosity [84].

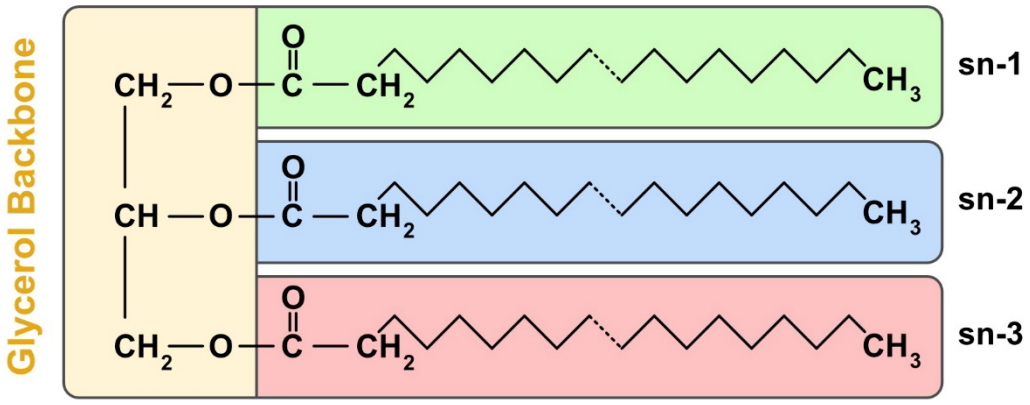


Figure 4: Structure of a triacylglyceride. Three fatty acids of various lengths and saturation are attached to a glycerol backbone at the sn-1 to sn-3 position.

The most FAMEous method of this thesis[10]

For our project, we need to measure lipids in general but also the different fatty acids. The most common way to analyse fatty acids is to convert them to fatty acid methyl esters (FAME), which then can be detected via gas chromatography-mass spectroscopy (GC-MS).

Gas chromatography (GC) separates compounds based on their boiling point and interaction with a stationary phase (column). Afterwards, the compound is ionized, and its mass charge ratio is detected in a mass spectrometer (MS). The resulting spectrum is characteristic for each molecule, and by comparing it to large libraries, the compound can be identified. However, this method requires the molecules to be volatile enough to *fly* in the GC, which lipids are not. Therefore, lipids need to be converted to FAME, which have a higher volatility and separate in the GC. [85–87]

The transesterification reaction to convert lipids into FAME requires alcohol and a catalyst. We used methanol as an alcohol and methyl group donor and sodium hydroxide (NaOH) as a catalyst for our protocol [88]. The NaOH captures a proton from methanol to form a methoxide ion, which reacts with the lipids. For each TAG, three FAME and one glycerol[11] are produced.

It is important to point out that this method converts all lipids to FAME. Therefore, the detected fatty acids come from neutral lipids (e.g. cholesterol, cholesterol ester, TAG, DAG

[10] Göteborg is famous for its very special humor that is characterized by bad puns. I have been living here for more than four years and I simply could not resist.

[11] Transesterification is also used to produce biodiesel. With the increased biodiesel production glycerol has become a waste product that can be used as a cheap carbon source. [89]

and MAG), free fatty acids, and phospholipids. If a more detailed analysis of the fatty acid distribution of different lipid classes is wanted, additional lipid class separation is required prior to the FAME analysis. This lipid class separation can be achieved with *solid-phase extraction* (SPE). The solid phase is a column filled with silica gel on which the sample is loaded. By applying a series of different solvents, different lipid classes are eluted. The fractions are collected and further converted to FAME and analysed via GC-MS [90]. However, since this approach requires significantly more time and material, we only used SPE for some of our strains. Additionally, TAGs are the dominant lipid class in *Y. lipolytica* following nitrogen starvation, with more than 90% of the total lipids (**Paper II Figure 5**). Therefore, a FAME extraction of the total lipid fraction is still a good representation of the fatty acid composition TAGs [91].

Triacylglycerols in *Y. lipolytica*

As previously mentioned, *Y. lipolytica* is an oleaginous yeast, which means it is naturally producing a high amount of lipids. The lipid synthesis can be divided into acetyl-CoA production, fatty acid synthesis, and their incorporation into different lipids.

From sugar to acetyl-CoA

Y. lipolytica can naturally use various carbon sources, but for simplicity, we will look at the most common carbon source: glucose[12]. Cells take up glucose from the media and transport it into the cytosol. Then, glucose undergoes a process called glycolysis, in which it is converted to dihydroxyacetone phosphate (DHAP) and pyruvate. A portion of the DHAP is further converted to glycerol-3-phosphate (G3P), while pyruvate is transported into the mitochondria and enters the tricarboxylic acid (TCA) cycle as acetyl-CoA. Under nitrogen-limiting conditions, the adenosine monophosphate (AMP) deaminase is causing an AMP depletion, which inhibits the TCA cycle. This leads to an excess of citrate in the mitochondria, which is transported into the cytosol by a malate/citrate transferase. In the cytosol, the ATP-citrate lyase cleaves citrate to form acetyl-CoA. Acetyl-CoA is then further converted to malonyl-CoA by the acetyl-CoA carboxylase (**Figure 5**). [92–94]

[12] Other carbon sources are discussed in the next chapter.

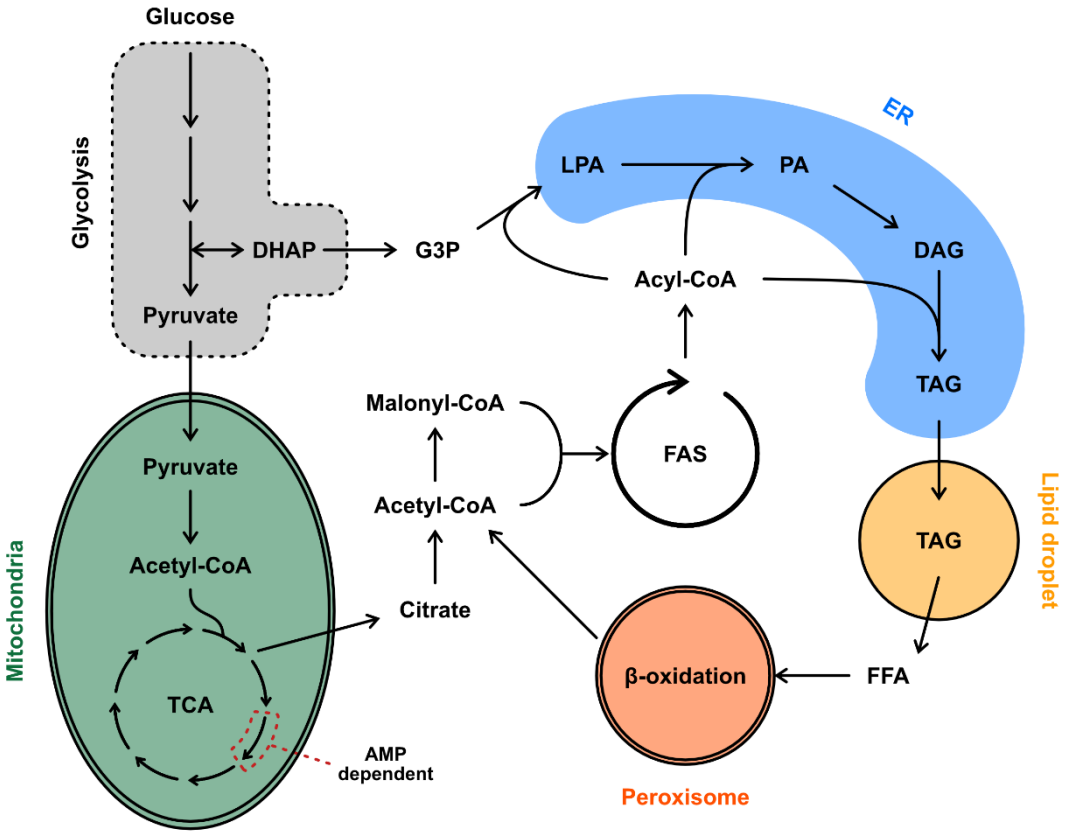


Figure 5: Simplified overview of lipid metabolism. Marked in red dashed lines is the isocitrate dehydrogenase, which is AMP-dependent and inhibited during nitrogen limitation. TCA tricarboxylic acid cycle, ER endoplasmatic reticulum, DHAP dihydroxyacetone phosphate, G3P glycerol-3-phosphate, LPA lysophosphatidic acid, PA phosphatidic acid, DAG diacylglycerol, TAG triacylglycerol, FFA free fatty acid.

Fatty acid biosynthesis

During the fatty acid synthesis, malonyl-CoA and acetyl-CoA are used by the fatty acid synthase complex (FAS) to produce acyl-CoA. The starting point is one acetyl-CoA (C2), which is elongated by the addition of malonyl-CoA (C3). During each elongation, CO_2 is released, resulting in an elongation of two carbons per malonyl-CoA, which is why even-chained fatty acids are common in most microorganisms[13]. Additionally, each elongation consumes two molecules of NADPH. This energy can later be released during β-oxidation in the peroxisomes. Once the fatty acid chain reaches a length of 16 or 18 carbons, it is released from the FAS and can be further elongated in the ER to long and very long-chained fatty acids or desaturated. [96–98]

Alternatively, to the *de novo* synthesis of fatty acids, *Y. lipolytica* can directly take up hydrophobic substances (e.g. alkanes or fatty acids) from the environment [99]. The

[13] With genetic engineering, *Y. lipolytica* can also produce odd-chain fatty acids from propionyl-CoA as elongation block [95].

hydrophobic substances can either be degraded by different pathways (e.g. alkane oxidation or β-oxidation) or be directly integrated into storage lipids [100]. This ability can be used to produce tailored lipids (e.g. cocoa-butter-like-lipids) from fat-rich waste streams [101, 102].

The different lipid classes

Acyl-CoA is used to form different lipids such as sterol esters (SE), TAGs, phospholipids, and sphingolipids. Phospholipids are the main component of biological membranes, and sphingolipids are an essential part of those membranes and are e.g. involved in stress responses and signalling [103]. Similarly, sterols are an essential component of the plasma membrane and are stored as SE to prevent toxicity from their excess [104].

TAGs are formed through the Kennedy pathway. As a first step, G3P, which is a by-product of glycolysis, is acylated to lysophosphatidic acid (LPA). LPA gets further acylated to phosphatidic acid (PA), which is dephosphorylated to diacylglyceride (DAG). The last acyl chain can either be donated from acyl-CoA (catalysed by the diacylglycerol acyltransferase Dga1,2) or from phospholipids (catalysed by the phospholipid diacylglycerol acyltransferase Lro1) (**Figure 6**). [96, 99]

SE and TAGs are stored in lipid droplets. Lipid droplets are cell organelles with a mono-phospholipid layer and are formed and mature from the ER [105]. The cell can use the stored lipids to re-modulate the fatty acid composition of phospholipids or transport them to the peroxisome for β-oxidation.

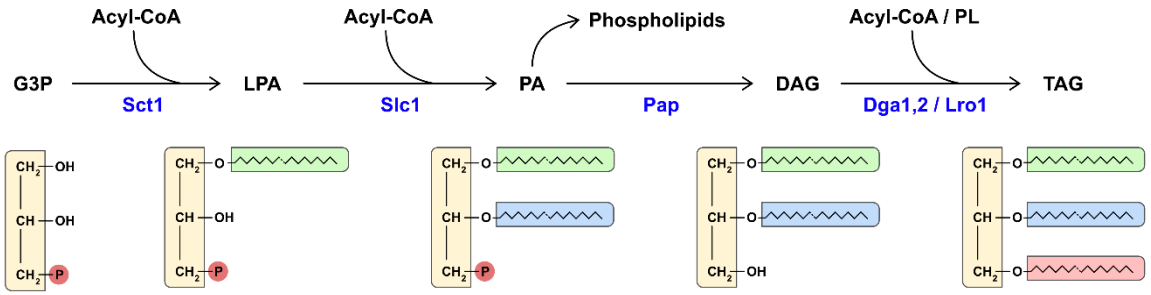


Figure 6: TAG formation pathway. The enzymes catalyzing the reactions are marked in blue. Sct1, Slc1 and Dga1,2 use acyl-CoA as an acyl donor, while Lro1 uses phospholipids (PL). G3P glycerol-3-phosphate, LPA lysophosphatidic acid, PA phosphatidic acid, DAG diacylglycerol, TAG triacylglycerol.

Increasing lipid production by genetic engineering

Now that we have an overview of the lipid production of *Y. lipolytica:* can we use that knowledge to improve the lipid production further? Yes! Although *Y. lipolytica* has a naturally high lipid production capacity, genetic engineering can further improve it to reach lipid contents of up to 90% of its cell dry weight [106]. To improve the lipid production of our strain,

we chose to overexpress the diacylglycerol acyltransferase (Dga1), a critical enzyme for TAG formation and delete acyl-CoA:sterol acyltransferase (Are1) to abolish the formation of sterol esters. These modifications increased the lipid content from around 20% to over 50% after four days of cultivation in lipid production media [91]. We did not further try to improve the production since the project's main goal was to alter the composition and not the amount of lipids[14].

Now that we have a well-performing platform strain, the next step is to adjust the fatty acid composition.

Cocoa butter

We chose cocoa butter as the first food oil to produce in our microbial cell factory. Cocoa butter is a high-value vegetable oil that is one of the main ingredients of chocolate[15] but is also used in cosmetics. Cocoa butter is extracted from the seeds of the cocoa tree (*Theobroma cacao*[16]), which is native to Central America, but its cultivation has spread to almost all tropical countries. It takes five years until the tree bears fruits and another five years until it reaches its maximum yield [109]. However, global warming [110], pests and diseases [111], as well as year to year weather fluctuations can influence the production of cocoa butter.

The idea to produce cocoa butter in yeast is not new. Multiple studies have already shown the production of cocoa butter equivalents in *S. cerevisiae* [112–114]. However, *S. cerevisiae* is not an oleaginous yeast and it requires many genetic modifications to reach the lipid content of an oleaginous organism [115]. Different oleaginous yeasts have been explored to produce different lipids [116]. In a comparison of six different oleaginous yeasts Wei et al. identified *Trichosporon oleaginosus* as the best organism to produce cocoa-butter-like lipids. However, the genetic tools to engineer this yeast species are still scarce and limit the unfolding of this yeast's full potential [117].

The primary fatty acids of cocoa butter are palmitic acid (C16:0, 26.2%), stearic acid (C18:0, 35.8%) and oleic acid (C18:1, 33.6%), and a marginal amount of linoleic acid (C18:2, 2.7%) [118]. These four fatty acids and palmitoleic acid (C16:1) are also found in *Y. lipolytica* (**Figure 7**). Palmitic acid (C16:0) and stearic acid (C18:0) are synthesized de novo from acetyl-CoA. Both fatty acids can be bound to Coenzyme A (CoA) and desaturated by the Δ9 desaturase Ole1 to form palmitoleic (C16:1) and oleic acid (C18:1), respectively. Oleic acid (C18:1) can be converted to phosphatidylcholine, which then is desaturated again by the Δ12 desaturase Fad2 to linoleic acid (C18:2) [119]. Both desaturases are located in the

[14] Additional approaches to increase the lipid content are discuses in end of the thesis.
[15] In the EU, chocolate needs to have at least 18% cocoa butter content [107].
[16] Theobroma: '*Food of the Gods*' from the Greek words *broma* (Food) and *Theo* (God) [108].

endoplasmic reticulum (ER) membrane and use an NADH-dependent cytochrome b5 reductase to obtain electrons [120–122].

Although *Y. lipolytica* naturally produces all fatty acids found in cocoa butter, their relative content is not identical to cocoa butter. To mimic the fatty acid content of cocoa butter, we have to remove palmitoleic acid (C16:1) from *Y. lipolytica*. Additionally, we need to reduce the linoleic acid (C18:2) content and alter the distribution of the remaining three fatty acids (**Figure 7**).

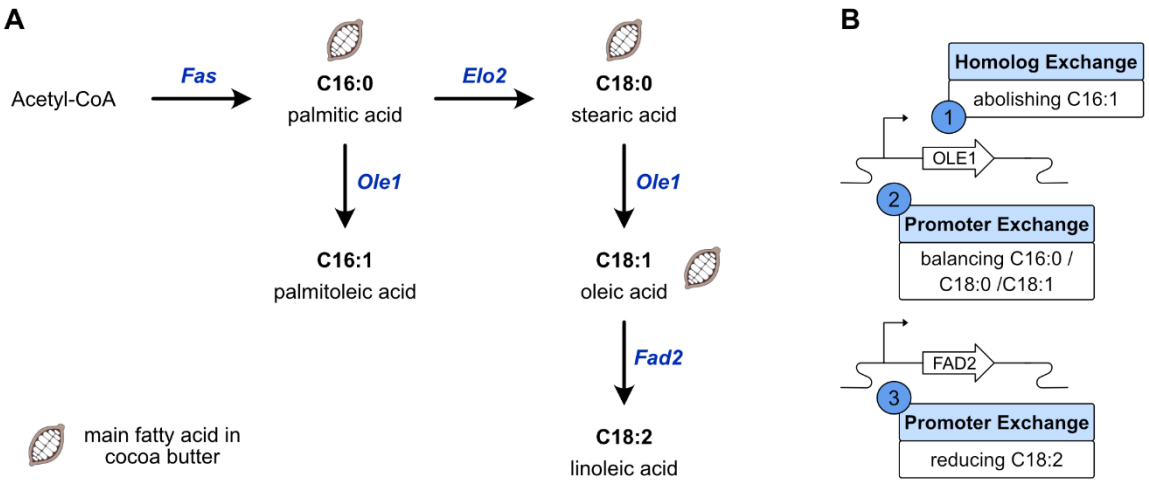


Figure 7: Cocoa butter production in *Y. lipolytica*. A Main fatty acids in *Y. lipolytica* and cocoa butter (marked with cocoa pod. **B** Engineering strategy to mimic cocoa butter in *Y. lipolytica*. From [91] (Paper II).

Genetic engineering of desaturases to mimic cocoa butter

We applied a three-step genetic engineering strategy to mimic cocoa butter in *Y. lipolytica*. First, we abolished palmitoleic acid (C16:1) by exchanging the native *OLE1* with homologs from other species with low affinity for palmitic acid (C16:0). We then exchanged the native *OLE1* promoter (pOLE1) to balance the distribution of C16:0, C18:0 and C:18:1 to mimic cocoa butter. Finally, we exchange the native *FAD2* promoter to reduce the linoleic acid (C18:2) content.

Abolishing palmitoleic acid (C16:1)

To abolish palmitoleic acid, the first thought that might come to mind is the deletion of *OLE1*. Since Ole1 is the enzyme catalysing the desaturation of palmitic acid (C16:0) to palmitoleic acid (C16:1), deletion of that gene would stop this reaction, and we could abolish palmitoleic acid (C16:1) from the fatty acid profile of our cells. However, Ole1 also desaturates stearic acid (C18:0) to oleic acid (C18:1), a fatty acid that we want to keep, albeit at a reduced level. Additionally, unsaturated fatty acids are vital, and deletion of *OLE1* is only viable if the cells are supplied with unsaturated fatty acids from the medium [91, 123]. It appears that we need

to keep Ole1. But could we modify the enzyme to only react with stearic acid (C18:0) and not palmitic acid (C16:0)?

Every enzyme has a particular task in the metabolism of the cell. Over millions of years of evolution, each enzyme was shaped to fulfil this task efficiently. This efficiency usually comes with the cost of a relatively narrow substrate range. Mutating the gene and thereby changing the enzyme can alter the substrate range. With biomolecular tools (e.g. error-prone PCR [124]), we can create libraries that contain thousands of versions of an enzyme, which can be tested for the desired reaction. However, this procedure is very time and labour intensive. Instead, we accessed the library of evolution.

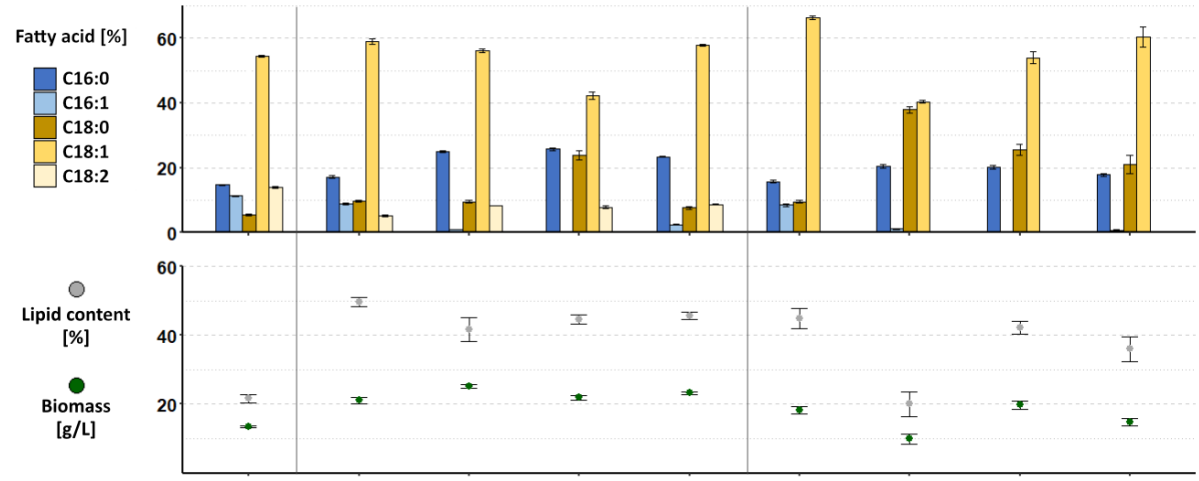
Throughout evolution, species divided and adapted to different environments. Genes mutate during this process, and their corresponding enzymes can change their function or affinity. To abolish palmitoleic acid (C16:1) while keeping oleic acid (C18:1), we can look for different microorganism species with a desired fatty acid profile and express their *OLE1* homologs in *Y. lipolytica*. A previous study screened different *OLE1* homologs to produce high-oleate oils [123]. Among their screened candidates, we found the *OLE1* homologs of *Arxula adeninivorans* (*AaOLE1*), *Gloeophyllum trabeum* (*GtOLE*), and *Rhodotorula toruloides* (*RtOLE1*) to be suitable for our purpose[17].

When we exchanged the ORF of the native *YlOLE1* with those codon-optimized homologs, we found *RtOLE1* to be the best candidate (**Figure 8**). In the strain expressing *RtOLE1* (OKYL056) we could no longer detect palmitoleic acid (C16:1), while the growth and lipid production remained high. Additionally, the stearic acid (C18:0) content was increased compared to the parental strain, which is another desired trait to mimic cocoa butter.

Additionally, we tested all three *OLE1* homologs in a fad2Δ strain. Fad2 is the Δ12 desaturase that converts oleic acid (C18:1) to linoleic acid (C18:2), which only exists in small amounts in cocoa butter. The deletion of *FAD2* removes linoleic acid from the fatty acid profile of *Y. lipolytica* without a significant impact on the growth or lipid production (AOYL02).

In the fad2Δ strain background, *GtOLE1* showed the best fatty acid profile (KSYL14). However, the growth and lipid production of this strain was strongly reduced. Based on these results, we decided to continue with *RtOLE1* and *AaOLE1* in the subsequent experiments.

[17] Recently, the approach to replace *OLE1* was used to produce high oleic oils [125].



FAD2	WT					Δ			
OLE1	Yl	Yl	Gt	Rt	Aa	Yl	Gt	Rt	Aa
Lipid production	WT	engineered for obese phenotype							
Strain	OKYL029	OKYL049	OKYL057	OKYL056	OKYL059	AOYL02	KSYL14	KSYL15	KSYL16

Figure 8: Replacement of OLE1 with OLE1 homologs. Strains were cultivated for 96 h in LPU media, and the fatty acid profile was determined by FAME extraction. Lipid content represents g FAME per g cell dry weight. Displayed is the mean and standard error of n ≥ 4 replicates. Yl *Y. lipolytica*, Gt *G. trabeum*, Rt *R. toruloides*, Aa *A. adeninivorans*, WT wild type, Δ deletion. From [91] (Paper II).

Balancing the fatty acid composition

The exchange of *YlOLE1* with homologs has successfully removed palmitoleic acid (C16:1) from the fatty acid profile of *Y. lipolytica*. However, the distribution of stearic (C18:0) and oleic acid (C18:1) can still be improved to better mimic cocoa butter. We tried to balance the distribution of the fatty acids by exchanging the native *OLE1* promoter (pOLE1) with other promoters (**Figure 9**).

We aimed to have enough expression of the OLE1 gene during the exponential growth phase, to allow a healthy growth but a lower expression in the lipid production phase to reduce the oleic acid (C18:1) content. Based on published transcriptomics analysis, we identified pRP30 and pGAPDH as potentially suitable promoters (**Figure 9B**) [126].

We found that the expression of pRP30 was too low to sustain healthy growth. Strains expressing different *OLE1* homologs under the control of pRP30 showed reduced growth and lipid content. When we supplemented the media with unsaturated fatty acids, the growth of these strains could be recovered, showing that the lack of these fatty acids was responsible for the impaired growth.

The strains expressing the *OLE1* homologs under the control of pGAPDH did grow well. However, the additional deletion of Fad2 reduced the growth and lipid production (KSYL09 and KSYL12). Overall, *RtOLE1* showed the best results in both experiments regarding growth and fatty acid profile and was used for the next experiment.

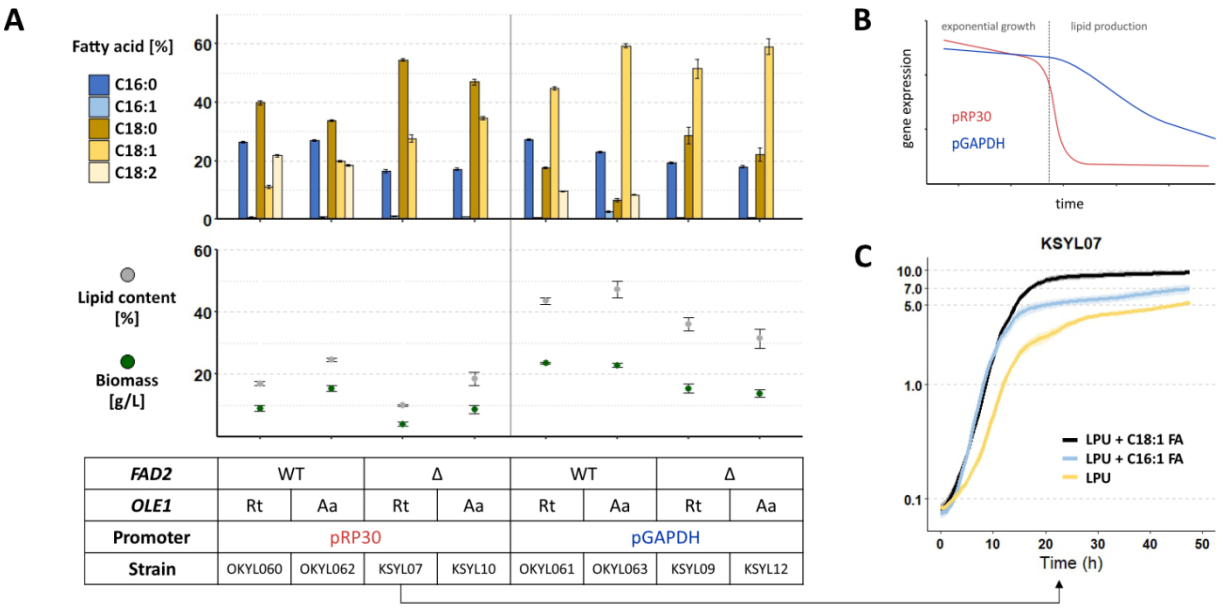
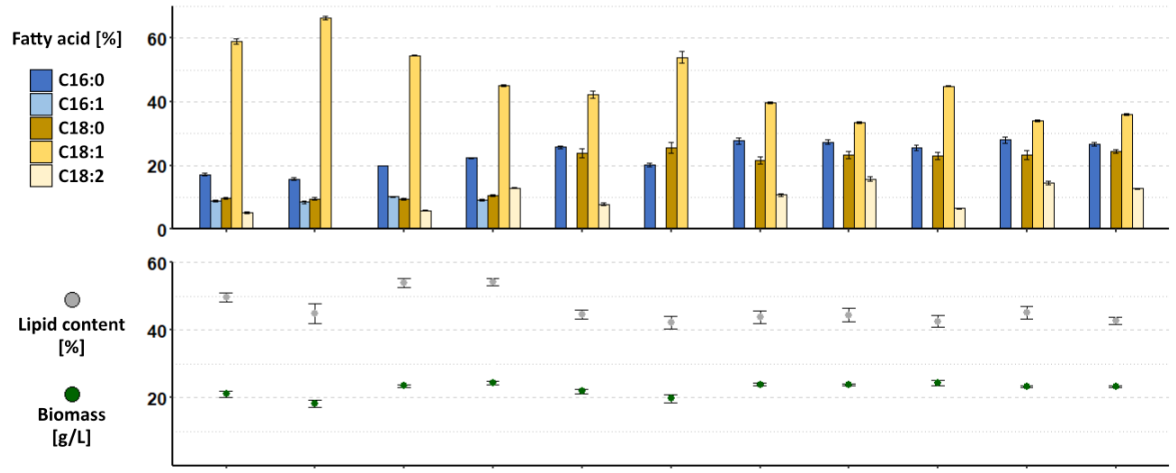


Figure 9: Promoter exchange of OLE1 homologs. The native *OLE1* gene was exchanged for either *RtOLE1* or *AαOLE1*, and the native promoter was exchanged for either pGAPDH or pRP30. **A** Strains were cultivated for 96 h in LPU media, and the fatty acid profile was determined by FAME extraction. Lipid content represents g FAME per g cell dry weight. Displayed is the mean and standard error of n ≥ 4 replicates. **B** Scheme of expected promoter activity of pRP30 and pGAPDH based on data from [126]. Dashed line marks the onset of lipid production due to nitrogen limitation. **C** Growth curves of strain KSYL07. Cells were cultivated in 96-well plates in LPU media with or without the addition of palmitoleic acid (C16:1) or oleic acid (C18:1) (500 mg/L in 1% Tween 20), and OD_{600} was measured every 30 min. The lines and shadows represent the average and standard deviation of quadruplicates, respectively. Figure from [91] (Paper II).

Reducing the linoleic acid (C18:2) content

Our best cocoa butter strain (OKYL056) has a linoleic acid (C18:2) content of around 9%, which is about 3-times higher compared to cocoa butter. Linoleic acid (C18:2) is produced via the desaturation of oleic acid (C18:1) by the Δ12 desaturase Fad2. We exchanged the native promoter of *FAD2* with different strong and weak promoters to explore the range of linoleic acid (C18:2) contents that *Y. lipolytica* can produce.

In contrast to our findings with the *OLE1* promoter exchange, we found that a wide range of *FAD2* expression levels is possible, resulting in various linoleic acid (C18:2) contents without significant effect on the growth or lipid production (**Figure 10**). Additionally, there appears to be an inverse correlation between linoleic and oleic acid (C18:2 and C18:1). For example, when *FAD2* is deleted, linoleic acid (C18:2) disappears from the fatty acid profile and oleic acid (C18:1) increases by approximately the same amount, while the other fatty acids remain primarily unchanged.



FAD2 promoter	WT	Δ	pRP30	pGAPDH	WT	Δ	pRP30	pGAPDH	pICL1	pTEF1	pOLE1
OLE1	pOLE1 - YlOLE1				pOLE1 - RtOLE1						
Strain	OKYL049	AOYL02	OKYL064	OKYL065	OKYL056	KSYL15	YMYL01	YMYL03	YMYL02	YMYL04	YMYL05

Figure 10: Promoter exchange of native FAD2 gene. Strains were expressing the native *FAD2* gene under the control of different promoters. The strains were either expressing the native *YlOLE1* or *RtOLE1* under the control of the native OLE1 promoter, respectively. Strains were cultivated for 96 h in LPU media, and the fatty acid profile was determined by FAME extraction. Lipid content represents g FAME per g cell dry weight. Displayed is the mean and standard error of n ≥ 4 replicates. Figure from [91] (Paper II).

Can we produce chocolate in *Y. lipolytica* now?

We constructed numerous different strains with various fatty acid profiles during this project. But can we make chocolate out of any of the strains? Unfortunately, it is not that easy.

First of all, we only mimicked the fatty acid composition of cocoa butter but ignored the TAG composition. As described at the beginning of this chapter, each TAG has three fatty acids in the sn-1 to sn-3 position. Additionally, food oils do not simply consist of one TAG species but a mix of many. Cocoa butter mainly consists of POP, POS, and SOS (Palmitic acid (C16:0), Oleic acid (C18:1), Stearic acid (C18:0)) [127]. In *Y. lipolytica,* the sn-1 and sn-2 positions are occupied mainly by saturated and unsaturated fatty acids, respectively [117]. Therefore, the sn-3 position would be a suitable future engineering target. This can be achieved by expressing DGAT and PDAT from *T. cacao*, as has been shown in *S. cerevisiae* [113]. An example of successful altering of the fatty acids in TAGs in *Y. lipolytica* is the production of human milk substitute [128].

With these additional modifications, the product would be a true cocoa butter equivalent (CBE), with similar physical and chemical properties that could be mixed with cocoa butter without changing its properties [127]. These properties define the melting and crystallization that make chocolate melt on your tongue. However, they are not giving the taste of chocolate.

The flavour and aroma of chocolate result from the fermentation, drying, and roasting of the cocoa beans. The fermentation mainly removes the pulp from the cocoa beans but does not produce the actual flavour. However, it produces acetic acid and ethanol, which kills the cocoa bean, causing a membrane breakdown between the storage and pigment cells of the bean. The cell contents mix and the reactions of various, mostly endogenous, enzymes yield flavour precursors, which develop into the characteristic chocolate flavours upon roasting. [129, 130]

The mimicking of these complex flavours and aromas is an ongoing challenge.

The world is saved - almost

Now, we have strains that can express different fatty acid profiles that can be used for different applications. Instead of ravaging tropical rain forests to make space for oil crop plantations, we can produce microbial oil at any place and preserve the biodiverse hotspots of our planet. Mission accomplished – right?

Almost. So far, our strains consume glucose as a primary carbon source. Glucose is mainly produced from sugarcane, sugar beet and sweet sorghum [131], which occupy vast farmland areas worldwide. The large-scale production of microbial food oil from glucose would result in increased farmland for sugar crops, ultimately leading to the destruction of ecosystems. It also raises ethical questions when food crops are used for microbial cultivations if almost 10% of the world population is affected by severe food insecurity [132].[18]

We need to find an alternative carbon source to overcome this problem. A cheap and abundant alternative to glucose is lignocellulosic biomass, such as agricultural waste or forestry residues. However, the utilization of lignocellulosic biomass requires additional treatments that bring new challenges, which we will explore in the next chapter.

[18] The biofuel industry faced a similar problem. While the first-generation biofuels used glucose as carbon source, the second-generation biofuels use lignocellulosic material that in general is not used for consumption. The third-generation is based lipid production in algae, which avoids any competition with farmland [133].

Chapter 3 – Hydrolysates as a carbon source

Two of the most important objectives of a modern production process are economic feasibility and environmental sustainability. Unfortunately, those two things rarely go hand in hand. Increasing a process's sustainability often requires substituting toxic and environmental-harming substances for more expensive alternatives or implementing expensive additional downstream procedures to clean up waste streams. However, for microbial cultivation, we have the chance for a win-win situation: the carbon source.

The carbon source can make up to 60% of the total cost of industrial-scale cultivations [134]. A widely used carbon source is glucose, but its production can cause conflicts between land usage and food supply. Cheap and sustainable alternatives are carbon-rich waste streams. Those waste streams are by-products of other industries rich in sugars or lipids and can be used as energy and carbon sources (**Table 1**).

Table 1: List of different waste streams and their origin. These and further examples were also reviewed by [134].

Waste stream	Origin	Ref
Glycerol	biodiesel plants	[89, 135]
Volatile fatty acids	syngas fermentation or anaerobic organic waste digestion	[136, 137]
Flour-rich waste streams	bakery and wheat mills	[138]
Lipid rich waste streams	olive oil mills	[139, 140]
Whey permeates	cheese and butter creameries	[141]
Lignocellulose	agricultural residue, municipal solid waste	[142, 143]

What is lignocellulosic biomass?

A well-studied and widely available carbon alternative is lignocellulose, e.g. from agricultural or forestry residue. Lignocellulose consists of three main components: lignin, cellulose, and hemicellulose (**Figure 11**). Lignin is a complex, branched polymer that gives structure, strength and protection from microbial degradation and is the most abundant non-carbohydrate component [144]. Cellulose is a polysaccharide of glucose that can form microfibrils and is the main component of plant cell walls [145]. Hemicellulose is another structural component and is a polysaccharide of different hexoses (glucose, galactose and mannose) and pentoses (arabinose and xylose) [146]. The exact composition of lignocellulose varies between different species (e.g. softwoods versus hardwoods), the age of the plant (e.g. earlywood vs latewood), and the part of the plant that is used (e.g. leaves vs bark) [146].

Although the main building blocks of lignocellulose are sugars, which microorganisms can metabolize, they are built into long and complex polymers, making them inaccessible for most microorganisms. Therefore, if we want to use lignocellulose as a carbon source, we first need to break it into single sugars.

As a first step, the biomass must undergo pre-treatment. The primary purpose of that treatment is to improve accessibility by removing the lignin and breaking the hemicellulose. The different methods of pre-treatments can be classified as mechanical (e.g. chipping or grinding), chemical (e.g. metal chlorides or acid), physio-chemical (e.g. steam explosion or wet oxidation), and biological (e.g. microorganisms or enzyme treatment). The chosen method depends on the exact composition of the biomass, and often a combination of multiple methods is applied. After the pre-treatment, the surface area of the biomass has significantly increased, and the biomass is accessible for enzymatic hydrolysis, which releases the single sugar monomers. [147, 148]

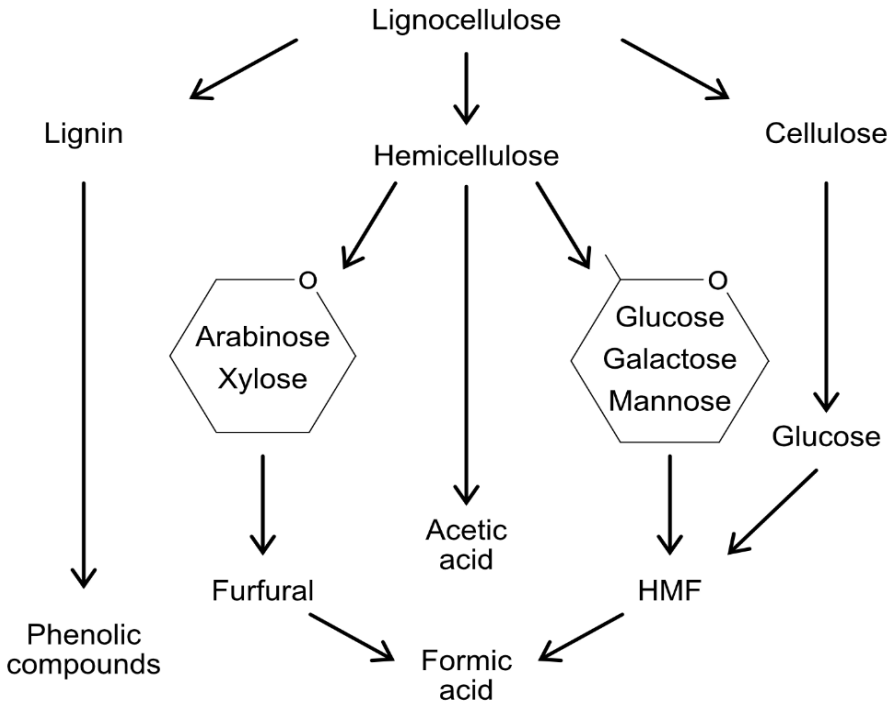


Figure 11: Components of lignocellulose and the inhibitors resulting from its treatment. Inspired by [148].

However, the harsh conditions of pre-treatment also yield other chemical compounds that inhibit microbial growth[19]. The most commonly found inhibitors are furanic aldehydes (e.g.

[19] The *dilute-acid pretreatment* for example, uses acids and temperatures of 160°C. It is not surprising that these conditions allow numerous additional reactions to occur [147].

furfural and 5-hydroxymethylfurfural (HMF)), weak acids (e.g. acetic and formic acid), and aromatic compounds (e.g. cinnamic acid and coniferyl aldehyde) (**Figure 11**).

Furfural and HMF are formed by the dehydration of pentoses and hexoses, respectively [149, 150]. Both compounds have reactive aldehyde groups that cause the formation of reactive oxygen species (ROS), which induce DNA mutation, membrane damage, protein misfolding, and apoptosis [151].

Acetic acid is produced during hemicellulose degradation, while formic acid is a breakdown product of furfural and HMF [149]. Both inhibit microbial growth by *uncoupling* and *anion accumulation*. In its protonated form, weak acids can diffuse through membranes. The pH inside the cell is higher and causes the acid to dissociate, which decreases the cellular pH. To maintain their cellular pH, cells invest energy (in the form of ATP) to pump out protons. This phenomena is referred to as uncoupling, and leads to ATP depletion and to the accumulation of the acid anion in the cell. [152]

Phenolic compounds are derived from lignin. Lignin is a complex polymer that can have different connections to cellulose and hemicellulose, resulting in a wide range of phenolic compounds affecting membranes. Additionally, some phenolic compounds have weak acidic characteristics that result in inhibition by uncoupling. [148, 153]

To efficiently use lignocellulosic biomass as a carbon source for our cultivation, we needed to achieve two things: first, we must engineer our strain to metabolize other sugars than glucose; secondly, we need to understand the effect of the different inhibitors on *Y. lipolytica*.

Xylose as an additional carbon source

To use lignocellulosic biomass in the most efficient way, the cultivated organism should consume multiple sugars. The most abundant sugars in most hydrolysate are glucose and xylose[20]. *Y. lipolytica* consumes glucose naturally, but xylose consumption appears to be strain and condition dependent (e.g. adaptation or starvation periods) [155]. When we tested our lipid producing strain (OKYL049), we did not observe any growth in xylose as a sole carbon source. To enable our strain to metabolize xylose, we again used genetic engineering.

In general, xylose can be metabolized by two different pathways, the XR-XDH and the XI pathway. During the two-step XR-XDH pathway, xylose is first reduced to xylitol by a NAD(P)H-dependent xylose reductase (XR), which is further oxidized to xylulose by the NAD^+-dependent xylitol dehydrogenase (XDH). In contrast, the xylose isomerase (XI) pathway isomerized xylose directly to xylulose without any co-factor. In both pathways, xylulose is phosphorylated by the

[20] As mentioned before, this highly depends on the exact kind of biomass that has been used. For example, softwood hydrolysates contain more mannose than xylose [154].

xylulose kinase (XK) to D-xylulose-5-phosphate (X5P), which enters the non-oxidative part of the pentose phosphate pathway (PPP) (**Figure 12**). [155, 156]

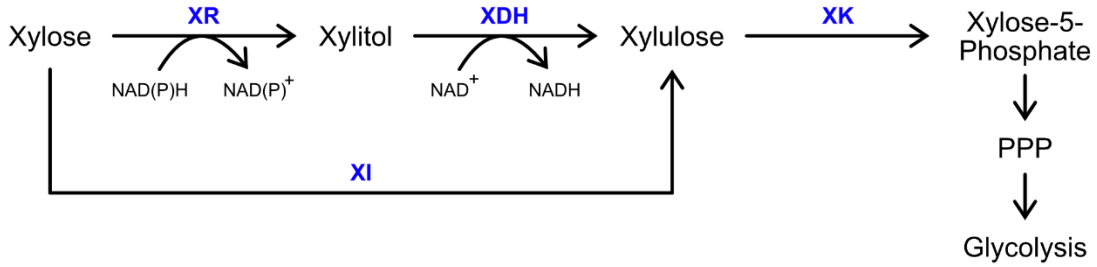


Figure 12: Xylose pathway in microorganisms. XR Xylose reductase, XDH Xylose dehydrogenase, XK Xylulose kinase, XI Xylose isomerase. PPP Pentose Phosphate pathway.

The PPP runs in parallel to glycolysis and can be divided into oxidative and non-oxidative parts. The oxidative part converts glucose-6-phosphate in two steps to ribulose-5-phosphate and generates NADPH. In the non-oxidative part, metabolites are reversibly converted by transaldolase and transketolase reactions. The intermediates fructose-6-phosphate and glyceraldehyde-3-phosphate connect the PPP with the glycolysis. [157]

The genome of *Y. lipolytica* contains *XR*, *XDH* and *XK*, although their expression is strain and condition dependent. To improve the xylose metabolism, the expression of *XR* and *XDH* homologs from *Scheffersomyces stipites* in *Y. lipolytica* has proven efficient [158, 159]. The expression of a codon-optimized XI did not show any activity in *Y. lipolytica* [158].

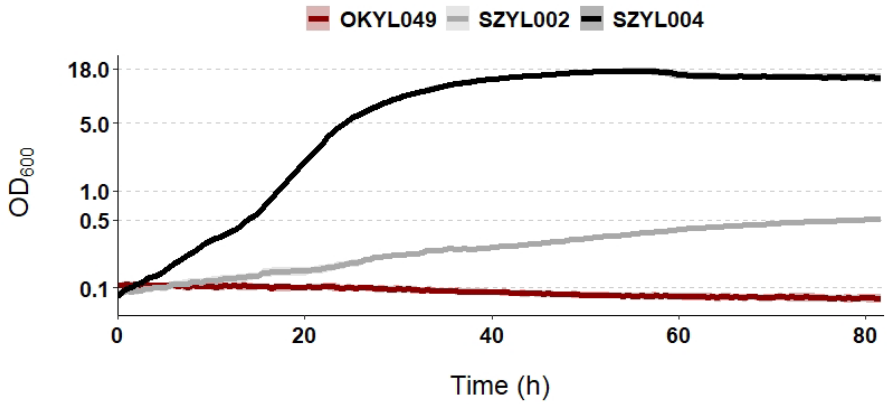


Figure 13: Growth curves of *Y. lipolytica* strains expressing xylose genes. Strains were precultured for 18 hours in LPU media with 10% glucose and then grown in LPU media with 10% xylose as sole-carbon source. Cells were cultured in 96-well plates and the OD_{600} was measured with the growth profiler every 30 minutes. The curves and shadows represent the average and standard deviation of triplicates, respectively. Data from [160] (Paper III).

Based on the results of these previous studies, we decided to overexpress the native *Y. lipolytica* genes for the XR-XDH pathway. Quantitative RT-PCR analysis indicated that *XR* is sufficiently expressed in wild type strains and that XDH and XK are the limiting enzymes [159]. To test if this also applies to our strain, we constructed two strains: one expressing XDH and XK (SZYL002) and one expressing XR, XDH and XK (SZYL004) (**Figure 13**). We found that the expression of XDH and XK does allow some growth on xylose, but that the growth was limited, and the additional expression of XR clearly led to a strongly improved growth rate and final biomass formation [160].

Inhibitors and *Y. lipolytica*

As a next step to efficiently using lignocellulosic biomass, we investigated different inhibitors' growth effects on *Y. lipolytica*. We tested six inhibitors commonly found in hydrolysates: acetic and formic acid (weak acids), cinnamic acid and coniferyl aldehyde (aromatic compounds), and furfural and HMF (furanic aldehydes) [161]. We first tested different concentrations of each inhibitor (**Figure 14**).

Tolerance of individual inhibitors

We tested two weak acids, acetic acid and formic acid, ranging from 7.5 to 75 mM. We observed a similar trend for both acids: with increasing acid concentration, the growth rate decreases, the lag phase increases, but the final biomass is unaffected. In both cases, too high concentrations inhibited the growth entirely. However, we found that formic acid is less tolerated than acetic acid. The higher toxicity of formic acid can be linked to its smaller size, which allows formic acid to diffuse into the cells easier [153]. Additionally, acetate (the dissociated form of acetic acid) can be metabolized after being activated to acetyl-CoA by the cytosolic acetyl-CoA synthetase [162], which provides a likely explanation for the lower toxicity of acetic acid. In comparison, *Rhodosporidium fluviale*, another oleaginous yeast, showed strong growth inhibition at concentration of 16.7 mM and 10.9 mM of acetic and formic acid, respectively [163].

We tested two aromatic compounds, cinnamic acid and coniferyl aldehyde, in concentrations ranging from 0.1 to 1 mM. Both compounds were tolerated in the initial concentration range. In a second experiment, we increased the concentration to 2.5 and 5 mM and found that cinnamic acid was still tolerated at 2.5 mM, but growth was inhibited at 5 mM. For coniferyl aldehyde, we saw that 2.5 mM shifted the lag phase substantially (to about 48 h), and 5 mM inhibited the growth entirely (**Figure 4 Paper III**). The toxicity of coniferyl aldehyde was linked to ROS formation in *S. cerevisiae* [164], while the toxicity of cinnamic acid could be associated with decreased membrane integrity [165].

We tested two furanic aldehydes, furfural and 5-hydroxymethylfurfural (HMF), at concentrations ranging from 3 to 30 mM. We found that the lag phase increases linearly with the concentration of furfural, while the growth rate and final biomass stay unaffected. In

contrast, HMF does not affect the lag phase, nor the final biomass, but the growth rate. Both compounds cannot be metabolized naturally by *Y. lipolytica*. However, non-specific enzymes can reduce or oxidize them to less toxic alcohols or acids [166]. Furfural appears to inhibit the growth of *Y. lipolytica* entirely. Still, it allows the cell to slowly detoxify the media until the furfural concentration is low enough for normal cell growth to continue. We decreased the lag phase induced by furfural by increasing the inoculum size (**Figure 5, Paper III**), similar to

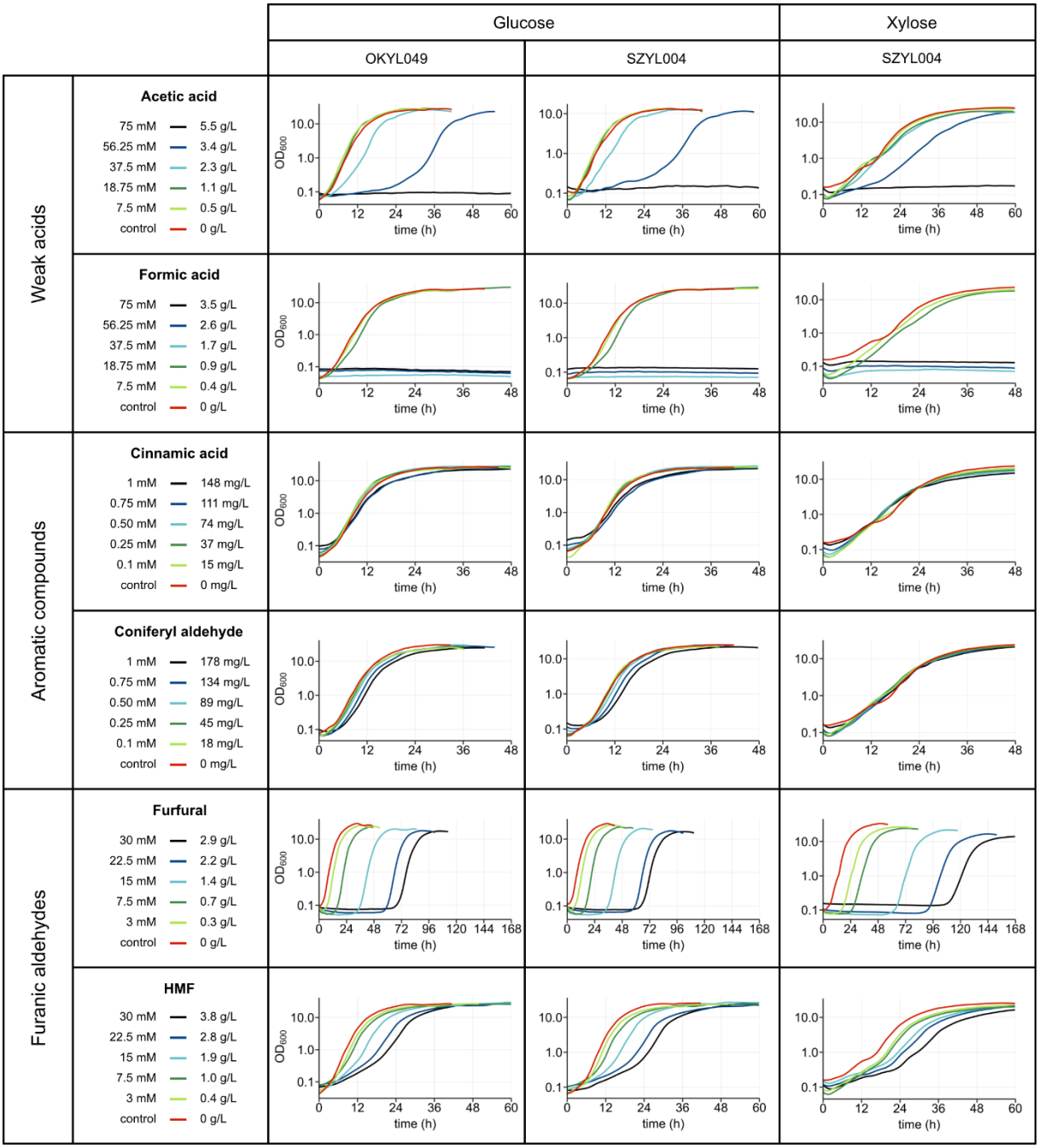


Figure 14: Growth curves of *Y. lipolytica* strains with different inhibitors. Strains were cultured in 96-well plates with media contained 100 g/L glucose or xylose. The OD_{600} was measured every 30 min with the growth profiler. The curves represent the average of triplicates. For clarity of the graphs error bars have been removed. Figure from [160] (Paper III).

results reported in *S. cerevisiae* [167]. Alternatively, the expression of aldehyde dehydrogenases can increase the furfural detoxification [168]. The inhibition of HMF appears to be similar to furfural. However, HMF does not block the growth but only reduces the growth rate until an OD_{600} of approximately 0.3. It is likely that at that time, enough HMF has been detoxified to allow normal cell growth again.

How does the carbon source affect the tolerance?

Since we engineered our strain to consume xylose, we also investigated if the carbon source (glucose or xylose) influences the inhibitor tolerance. We found that the carbon source does not affect formic acid, cinnamic acid, and HMF tolerance. For coniferyl aldehyde and acetic acid, we saw a slight tendency for lower sensitivity when the cells were grown in xylose compared to glucose. For furfural, we observed an extended lag phase for xylose cultivations. A possible explanation for that behaviour could be co-factor imbalance. The detoxification of furfural and the xylose metabolism require NADPH as a co-factor. When cells are grown with xylose as a carbon source, the NADPH pool for furfural degradation might be exhausted, resulting in a prolonged lag phase. However, in a real hydrolysate, both glucose and xylose are present. *Y. lipolytica* prefers glucose over xylose and would most likely detoxify the hydrolysate from furfural with energy coming from glucose.

Is there any synergetic effect between the inhibitors?

In our first experiments, we only investigated the effect of individual inhibitors on *Y. lipolytica*'s growth. However, a real hydrolysate is a mix of different inhibitors, which can have a synergistic effect on the cells [148]. To simulate a real hydrolysate, we mix the six previously tested inhibitors in media with xylose as a carbon source. The concentration of each

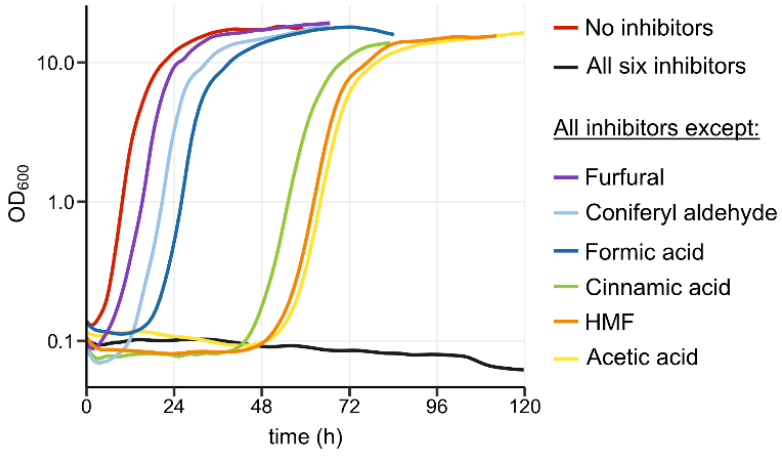


Figure 15: Growth curves in different inhibitor mixtures. SZYL004 was cultured in LPU + 100 g/L xylose in 96-well plates and the OD_{600} was measured with the growth profiler every 30 min. The curves represent the average of quadruplicates. Inhibitor concentrations used: 15 mM acetic acid, 15 mM formic acid, 0.2 mM cinnamic acid, 0.2 mM coniferyl aldehyde, 6 mM furfural, and 6 mM HMF. Figure from [160] (Paper III).

inhibitor was 20% of the maximum tested in the first individual experiment. None of the individual inhibitors caused any considerable growth inhibition at those concentrations. However, we found that in combination, the mixture did not allow any growth, illustrating the strong synergistic effects of the inhibitor mix (**Figure 15**). To understand further which inhibitor causes the most substantial effect in a mix, we repeated the experiment but left out one of the six inhibitors at a time. The closer the growth curve of five inhibitors is to the control without inhibitors, the stronger the effect of the inhibitor left out of the mix.

Interestingly, each mix of five inhibitors allowed growth, showing that it is not one compound inhibiting the growth but simply the combined effect of too many toxins. Additionally, we saw that furfural, coniferyl aldehyde and formic acid appear to have the strongest effect in the inhibitors mix. Furfural and formic acid strongly affected the growth before when tested individually (**Figure 14**). Coniferyl aldehyde, on the other hand, was very well tolerated as a single inhibitor but showed a strong effect in combination with other inhibitors. Likely this is because of the accumulation of ROS. Multiple tested inhibitors have been reported to cause ROS formation. While the cell can manage the ROS formation of one inhibitor, the combined ROS formation was too severe [160].

Our results show a strong tolerance of *Y. lipolytica* for individual inhibitors, especially for aromatic compounds. We have shown that the increased lag phase caused by furanic aldehydes can be overcome by increasing the inoculum. Additionally, we highlighted the strong synergetic effect that a mixture of inhibitors could have.

Future efforts should focus on increasing the tolerance to formic acid because of its strong individual effect and aromatic compounds such as coniferyl aldehyde because of its strong synergetic effect in inhibitor mixtures. Different non-rational (e.g. adapted laboratory evolution) or rational approaches (e.g. membrane or transcription factor engineering) could be used to increase the tolerance [169].

These results show that *Y. lipolytica* is suitable for using lignocellulosic biomass as an alternative carbon source. As mentioned at the beginning of this chapter, the carbon source is the highest cost-contributor of the cultivation media. However, there is still some room for improvement in media composition. Next, we will look at the second-highest cost-contributor of cultivation media, the nitrogen source.

Chapter 4 – Urea as a nitrogen source

The nitrogen source is the second biggest cost factor in microbial cultivation media [134]. Nitrogen is an essential part of amino acids and nucleotides, which are part of proteins and DNA/RNA, respectively.

Nitrogen can be assimilated from inorganic sources (e.g. ammonium salts) or organic sources (e.g. yeast extract, peptone, amino acids). Depending on the nitrogen source, the cellular behaviour can change. For example, it can influence the lipid accumulation of oleaginous yeasts [170] and the product formation during the anaerobic growth of *S. cerevisiae* [171]. In *Y. lipolytica,* the nitrogen source can influence filamentation [55] or impact protein production (e.g. lipases) [172].

Ammonium sulphate and urea as nitrogen sources

Most laboratories use ammonium sulphate (AS) as a nitrogen source, which is produced by treating ammonia with sulfuric acid. However, ammonium production consumes vast amounts of energy and releases substantial greenhouse gases[21] [173]. A more sustainable alternative is urea, which can be produced from municipal wastes in the future[22] [175]. Additionally, per mol of nitrogen urea is cheaper than AS [176].

The pathways for ammonium and urea utilization are well characterized in *S. cerevisiae*. The genome of *Y. lipolytica* contains potential homologs of the involved genes, which indicates that a similar mechanism is present in our host organism.

In *S. cerevisiae*, ammonium is transported into the cell by three ammonium permeases (Mep1,2,3) and deprotonates to ammonia, releasing a proton to the cytosol (**Figure 16**). To maintain the intracellular pH, the plasma membrane H^+-ATPase (Pma1) pumps protons out, acidifying the media throughout the cultivation [177]. Urea enters the cell via a plasma membrane transporter (Dur3) and is converted to CO_2 and two ammonia molecules by a urea amidolyase (Dur1_2). This reaction consumes one ATP per urea, or half an ATP per ammonia [178]. This makes the urea pathway energetically more efficient than the ammonium pathway, which requires one ATP per ammonia for proton transport.

In both pathways, ammonia is converted to glutamate by the NADP-dependent glutamate dehydrogenase (Gdh1) and further to glutamine by the glutamine synthetase (Gln1). These two reactions can be reversed by the NAD(+)-dependent glutamate synthase (Glt1) and

[21] The fossil-fuel based Haber-Bosch process is responsible for almost 1% of global annual greenhouse gas emissions. [173]

[22] Today, urea is almost exclusively produced by the reaction of ammonia with CO_2 at high temperatures and pressures [174].

dehydrogenase (Gdh2), which allows cells to use glutamate and glutamine as nitrogen sources [179]. Glutamate and glutamine are the precursors for other amino acids.

Since urea is cheaper, more energy-efficient, and does not acidify the media, urea seems an attractive alternative to AS. However, many established cultivation processes are based on AS, and switching the nitrogen source could affect cell behaviour and productivity. Therefore, we investigated how great the cellular differences are between the two nitrogen sources.

For the primary goal of producing food oils, we cultivated the lipid producing strain (OKYL049) in nitrogen limitation (C/N 116). To better understand the general differences between the two nitrogen sources, we also tested carbon limiting conditions (C/N 3) and the wild type (OKYL029) and a storage-lipid free strain (JFYL007). Strains with reduced or abolished storage lipid capacity can be used to produce other compounds that derive from citrate or acetyl-CoA, e.g. terpenoids or polyketides [180]. We tested both nitrogen sources in three different strains (lipid production strain, wild type, and lipid-free strain) in either nitrogen or carbon limiting conditions (C/N ratio 116 or 3). We cultivated our strains in chemostats to exclude the influence of different growth rates. Chemostats are a special kind of continuous cultivation in which the biomass concentration is determined by the limiting quantity of one of the nutrients. The growth rate is equal to the dilution rate in this setup and was set to 0.1 h^{-1} for all strains and conditions.

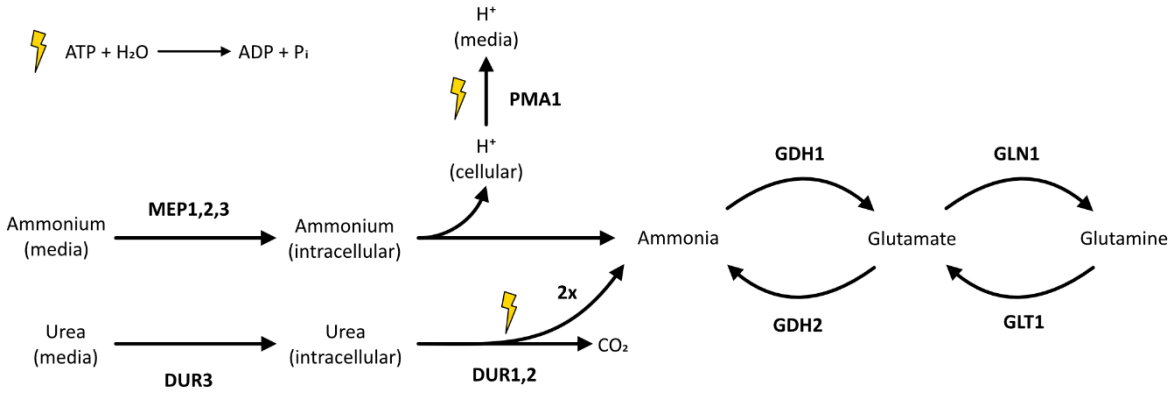


Figure 16: Overview of ammonium and urea utilization in yeast. Arrows represent reaction and gene names are based on *S. cerevisiae* nomenclation.

Cell physiological changes

As a first step, we had a closer look at some cell physiological parameters. We monitored the biomass, lipid content, and glucose consumption to calculate yields (**Table 2**). Overall, the cell physiology was mainly unaffected by the nitrogen source. For the lipid production strain (OKYL049), we found small significant changes in biomass, lipid content, biomass yield and glucose consumption rate under nitrogen limitation. However, none of these parameters were significantly changed in the wild type (OKYL029) under the same conditions. In the lipid-free strain (JFYL007), we found significant changes in biomass, lipid content, and

corresponding yields under carbon limitation. Interestingly, the lipid content was also significantly changed in nitrogen limitation; however, in the opposite direction[23].

Since the differences in the measured parameters are not consistent between strains and conditions, we concluded that the nitrogen source does not significantly impact overall cell physiology.

Table 2: Physiological changes of the strains in different C/N ratios and nitrogen sources. Displayed is the mean ± standard deviation of at least three replicates.

	N-source	OKYL029		OKYL049		JFYL007	
C/N ratio		3	116	3	116	3	116
Biomass (g/L)	AS	3.5 ± 0.2	2.0 ± 0.3	3.6 ± 0.1	2.2 ± 0.1 **	3.5 ± 0.1 *	1.4 ± 0.2
	U	3.7 ± 0.3	1.7 ± 0.1	3.5 ± 0.1	1.8 ± 0.2	3.8 ± 0.0	1.4 ± 0.4
Lipid content (%)	AS	5.8 ± 0.1 *	8.2 ± 0.4	9.0. ± 0.9	22.5 ± 1.5 *	7.0 ± 0.6 **	3.4 ± 0.5 **
	U	5.3 ± 0.3	8.2 ± 0.4	9.8 ± 1.2	18.1 ± 2.4	4.2 ± 0.3	5.7 ± 0.6
Biomass yield (gCDW/gGlucose)	AS	0.46 ± 0.03	0.48 ± 0.10	0.48 ± 0.01	0.36 ± 0.01 *	0.47 ± 0.02 *	0.42 ± 0.05
	U	0.50 ± 0.03	0.42 ± 0.02	0.47 ± 0.01	0.42 ± 0.04	0.51 ± 0.01	0.36 ± 0.1
Lipid yield (gLipid/gGlucose)	AS	2.7 ± 0.1	4.0 ± 0.6	4.3 ± 0.5	8.0 ± 0.6	3.3 ± 0.3 **	1.5 ± 0.4
	U	2.6 ± 0.3	3.5 ± 0.4	4.6 ± 0.6	7.7 ± 1.5	2.1 ± 0.2	2.1 ± 0.7
qGlucose (mmol / gCDW h)	AS	1.26 ± 0.04	1.22 ± 0.14	1.24 ± 0.05	1.63 ± 0.07 *	1.22 ± 0.05	1.4 ± 0.14
	U	1.17 ± 0.07	1.41 ± 0.09	1.26 ± 0.04	1.45 ± 0.08	1.13 ± 0.05	1.7 ± 0.55
qBiomass (mmol / gCDW h)	AS	4.53 ± 0.15	4.59 ± 0.06	4.63 ± 0.08	4.54 ± 0.02	4.53 ± 0.13	4.56 ± 0.11
	U	4.56 ± 0.20	4.68 ± 0.07	4.60 ± 0.15	4.76 ± 0.22	4.53 ± 0.19	4.51 ± 0.16

Significance was calculated between the two nitrogen sources amonium sulphate (AS) and urea (U) with a two-tailed homoscedastic t-test, * and ** indicate a p-value <0.05 and 0.01, respectively.

The primary goal of this thesis is focused on the production of food oils. Therefore, we also performed FAME extraction on all samples to see the effect of the nitrogen source on the fatty acid composition (**Table 3**). For the wild type and lipid production strain, we only found some low significant changes ($0.01<p<0.05$) for some of the fatty acids under either carbon or nitrogen limitation that did not show consistency between the strains. The fatty acid composition of the lipid-free strain[24] was significantly ($p<0.01$) changed for almost all fatty acids in both conditions. This most likely reflects great changes in the phospholipids that could, e.g. be related to the acidification caused by AS. The higher lipid content could conceal this change in the other two strains.

[23] The lipid free strain showed a very different and unexpected behavior during our experiments and is the subject of an additional research project we are working on. However, since this strain is not the main focus of this thesis, I will not discuss every anomaly of it.

[24] It is important to note, that although I'm referring to this strain as *lipid-free*, it is only free of the storage lipids TAGs and sterol esters. It still contains lipids e.g. as phospholipids in its membranes.

Table 3: Changes in the strains' fatty acid composition (% of total fatty acid) in different C/N ratios and nitrogen sources. Displayed is the mean ± standard deviation of at least three replicates.

	N-source	OKYL029		OKYL049		JFYL007	
C/N ratio		3	116	3	116	3	116
C16:0	AS	12.2 ± 2.3	15.8 ± 0.8	11.8 ± 1.9	16.5 ± 0.4	16.1 ± 2.1 **	16.3 ± 0.5 **
(%)	U	10.6 ± 0.6	16.6 ± 2.4	12.1 ± 2.3	15.2 ± 2.2	9.6 ± 0.9	22.5 ± 0.5
C16:1	AS	9.5 ± 0.6 *	8.5 ± 0.1	8.9 ± 0.6	6.0 ± 0.1 *	11.5 ± 0.7 **	11.1 ± 1.4
(%)	U	11.1 ± 0.9	8.3 ± 0.3	9.4 ± 0.8	5.3 ± 0.5	14.4 ± 0.8	10.5 ± 2.5
C18:0	AS	8.5 ± 5.3	6.5 ± 0.4	9.2 ± 4.0	11 ± 0.5	18.8 ± 3.7 **	2.6 ± 0.3 **
(%)	U	4.9 ± 1.2	8.1 ± 5.7	10 ± 5.1	10.7 ± 2.7	3.4 ± 1.6	17.2 ± 3.4
C18:1	AS	36.4 ± 5.1	43.5 ± 0.5	43.7 ± 4.0	53.6 ± 0.7	22.8 ± 2.6 **	15.9 ± 2.6 **
(%)	U	37.3 ± 1.6	39.8 ± 5.7	42.6 ± 5.0	52.8 ± 3.8	31.5 ± 1.0	9.4 ± 0.6
C18:2	AS	33.4 ± 2.9	25.7 ± 1.4	26.4 ± 1.6	12.9 ± 0.5 *	30.8 ± 2.7 **	54.1 ± 4.5 **
(%)	U	36.2 ± 2.4	27.3 ± 2.5	25.9 ± 2.1	15.9 ± 1.7	41.1 ± 1.4	40.4 ± 1.3

Significance was calculated between the two nitrogen sources amonium sulphate (AS) and urea (U) with a two-tailed homoscedastic t-test, * and ** indicate a p-value <0.05 and 0.01, respectively.

The good news for producing food oils is that urea seems not to impact the biomass titre, lipid production or fatty acid composition in lipid producing strains. However, the great changes in the fatty acid composition of the lipid-free strain indicate that there might be changes we did not capture with the measured parameters. Additionally, there might be changes in metabolites that we did not measure at all. Therefore, we performed RNA sequencing to analyse if gene expression is affected by urea compared to AS.

RNA sequencing

The central dogma of molecular biology states that genes are transcribed to messenger RNA (mRNA) and further translated to proteins, which can catalyse different reactions [181]. RNA sequencing is a powerful tool to investigate the expression of genes. By collecting and sequencing the RNA of a cell, we estimate the presence of the correlating proteins and assume that the reactions they catalyse are occurring in the cells. However, this is a simplification. Multiple studies have shown that the correlation between the transcriptome (all of the RNA in a cell) and proteome (all protein of a cell) is insufficient [182]. Additionally, the protein activity can be further regulated by posttranslational modifications (e.g. phosphorylation, acylation, or ubiquitylation) [183]. Nevertheless, RNA sequencing has become an affordable tool to get a glimpse of the overall transcriptional changes of a cell.

Principal component analysis

To get a first overview of the similarities as well as dissimilarities of samples, we performed a principal component analysis (PCA) (**Figure 17**). In this analysis, samples that are similar cluster

together while being separated by their overall differences. Consequently, replicates from the same condition should cluster together – which is the case for our samples.

We found that the clearest separation of conditions is based on the nitrogen versus carbon limiting conditions (C/N ratio) (**Figure 17A**). Among the nitrogen-limited samples (C/N 116) the samples further separated based on the strain (**Figure 17B**). The nitrogen source only contributed minorly to the separation of the samples (**Figure 17C**). These results are an indication that the nitrogen source (AS vs urea) only impacts the cells' behaviour in a minor way.

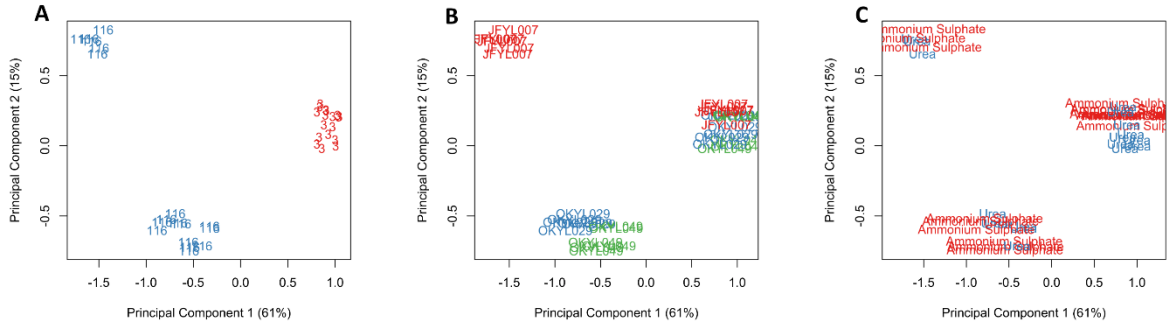


Figure 17: Principal component analysis (PCA) plot of RNA-seq samples. The panels display the same PCA result, but samples are labelled differently based on either (**A**) C/N ratio, (**B**) strain or (**C**) nitrogen source. PCA analysis was performed with log2 count per million, after removing low count genes, and normalizing gene counts between samples with trimmed mean of M values (TMM) method.

Differential gene expression analysis

The PCA gives an overview of the similarity of the samples. To identify genes that change with the nitrogen source, we performed a differential gene expression analysis. Hereby, the expression of each gene is compared between a sample grown with urea versus AS while keeping the other variables (strain and C/N ratio) constant. The result of each comparison was filtered to only contain significant (p.adj<0.05) and differentially expressed (DE) (>2-fold change) genes and then visualized in a network plot (**Figure 18**). In the network plot, each shape represents a gene, and the lines connect the genes to the conditions in which they were identified.

The most interesting genes are DE in all six conditions (cluster A). However, we only identified two genes belonging to this cluster – a protein of unknown function and a protein with similarity to VPH2 of *S. cerevisiae*. VPH2 is essential for the V-ATPase assembly, and its DE could be associated with the acidification caused by AS but not urea [184]. Since the PCA analysis indicated that the C/N ratio has a strong overall effect on the samples, we also looked for genes that are similar in all three strains under either nitrogen or carbon limiting conditions. We found three significantly DE genes in all strains under nitrogen limitation (cluster B) and three in carbon limitation (cluster C). All three genes in cluster B and two in cluster C encode uncharacterized proteins. The remaining gene of cluster C encodes a S-

(hydroxymethyl) glutathione dehydrogenase, for which we could not find a link to the nitrogen source.

Since the lipid-free strain (JFYL007) showed a very different cell physiological profile and was additionally separated in the PCA from the other two strains (**Figure 17B**), we decided to also look at genes that are significantly DE in the wild type strain (OKYL029) and the lipid producing strain (OKYL049) for both C/N ratios (cluster D and E). For those two strains in nitrogen and carbon limitation, we found six and 15 genes, respectively. 16 of these 21 genes are of unknown function, and for the remaining genes, we did not find any direct link to the nitrogen source[25].

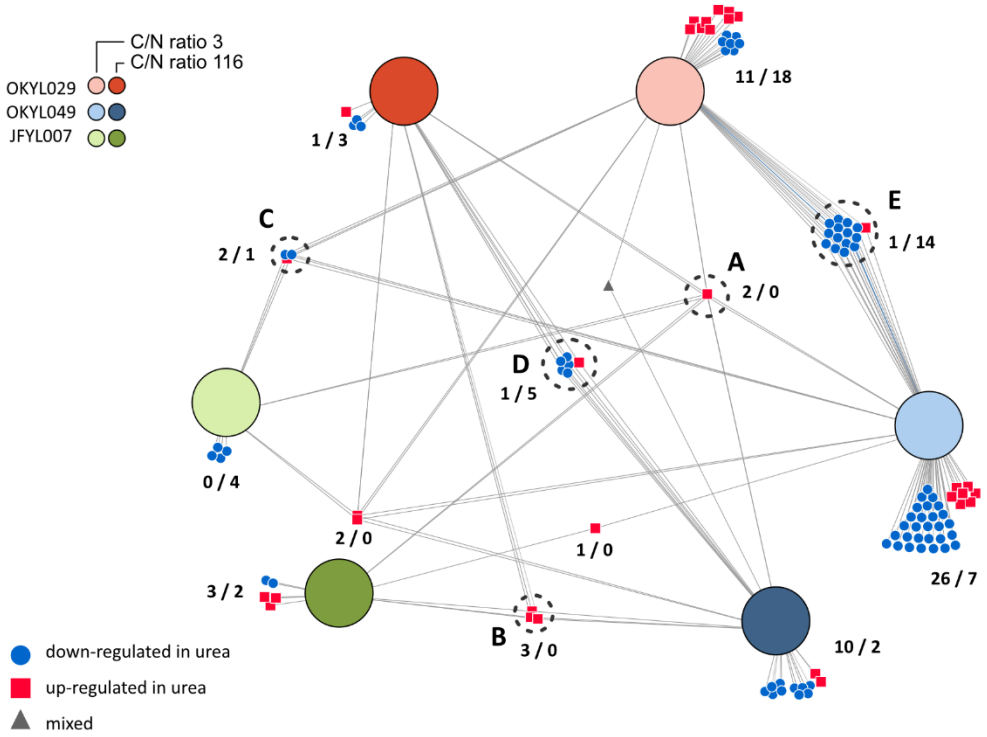


Figure 18: Visualization of overlap of differentially expressed genes in different conditions. Displayed are the significant (p.adj<0.05) differentially (>1-fold change) expressed genes of each strain and C/N ratio in urea compared to ammonium sulphate. Numbers indicate the number of down/up-regulated genes. Letters A to E are gene clusters of interest. Genes of each cluster are listed in table S2 Paper IV. For visualization the DiVenn web-tool was used [185].

In summary, the number of clustered genes is very low and supports the finding of the PCA that the tested nitrogen sources have a very low impact on the overall gene expression. Additionally, the high number of uncharacterized genes highlights the limitation of working with non-conventional organisms today. However, it can be expected that the number of

[25] The list of genes and their function of each cluster can be found in supplementary table 2 of paper IV.

uncharacterized genes will continue to decrease over the coming years. The function of genes can either be directly characterized in *Y. lipolytic*a or in other species, which will allow functional prediction via sequence homology.

Genes of urea and AS pathway

Since the overall transcriptional changes between the two nitrogen sources appear to be marginal, we investigated the expression of selected genes of the urea and ammonium pathway introduced at the beginning of this chapter.

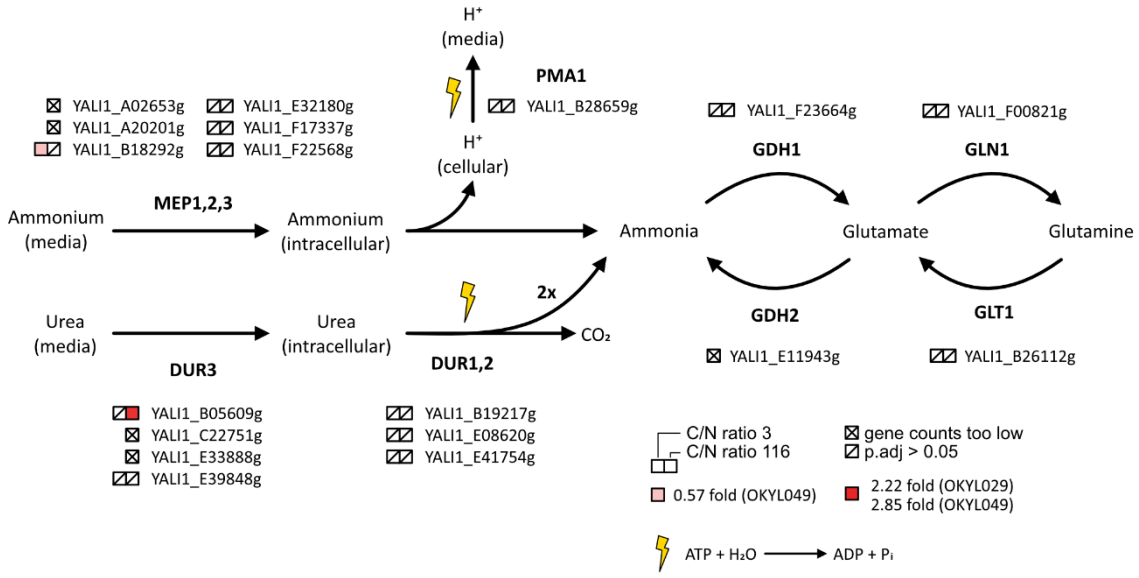


Figure 19: Influence of urea as nitrogen source compared to ammonium sulphate on the expression of the urea metabolic pathway genes. Displayed are *Y. lipolytica* genes with potential homologous functions, and their expression change from ammonium sulphate to urea as a nitrogen source. The genes were identified from different sources, as listed in Table S1 Paper IV. Genes marked with x have been removed during the filtering of the gene counts; genes marked with / did not show any significant (p.adj<0.05) change between the nitrogen sources.

In *Y. lipolytica*, most of the AS and urea pathway enzymes have multiple homologs [186, 187] (**Figure 19**). Some of the genes have been removed during the filtering of the RNA sequencing raw data (marked with X). Others were not significant (p.adj>0.05) (marked with /). Only two genes showed a significant change in expression between the two nitrogen sources in either carbon or nitrogen limitation for one or more strains: an ammonium transporter (YALI1_B18292g) and an urea transporter (YALI1_B05609g).

These results were somewhat surprising for us since the genes of the urea pathway have been reported to be induced by the presence of urea in other species, e.g. *C. albicans* and *S. cerevisiae* [188, 189]. The upregulation of only one of the four DUR3 homologs indicates that YALI1_ B05609g is the true homolog to *ScDUR3* with a similar regulation. However,

further analysis would be needed to confirm that hypothesis, e.g. knockout strains. The genes downstream of ammonia were not expected to be differently expressed since both ammonium and urea end in ammonia.

Should we use urea?

Yes. Although there might be some minor changes in the phospholipid composition, changing the nitrogen source from AS to urea has many advantages. With urea as a nitrogen source, a cultivation process can become cheaper and more sustainable while maintaining productivity.

Conclusion and outlook

In the last section of this thesis, I will first summarize the main findings. Then I will outline the room for improvement that can be applied to this project in the future. Finally, I will give a more general outlook of what might lay ahead of us in the future of synthetic biology and biotechnology.

Things we have learned

As a first step, we looked at *Y. lipolytica's* ability to form filaments. Since they can trigger unfavourable cultivation conditions in large-scale processes, we decided to abolish this ability. We tested three different knockout candidates and found Δmhy1 to be the most reliable to abolish filamentation. After testing its impact on lipid production and stress tolerance, we implemented this knockout as the foundation of all our strains.

In the second chapter, we tinkered around with desaturases to change the fatty acid profile of *Y. lipolytica* to mimic cocoa butter. By testing different homologs of *OLE1* and varying their expression levels and the expression level of *FAD2*, we were able to build strains with different fatty acid profiles. Our collection of strains can be used to mimic cocoa butter but can also mimic other food oils and can also be of interest for other applications (e.g. bio-fuels).

In the third chapter, we searched for an alternative carbon source. To avoid conflicts with food crops, we focused on hydrolysates of lignocellulosic biomass. We engineered our strain to consume the second most abundant sugar in hydrolysates, xylose. Furthermore, we documented the tolerance of *Y. lipolytica* to different inhibitors commonly found in hydrolysates, which can help to set priorities for future tolerance engineering.

In the final chapter, we investigated the effect of urea as a nitrogen source compared to ammonium sulphate. Both cell physiological data (i.e. biomass, lipid content and fatty acid composition) and transcriptomics analysis suggest that urea can be used as a cheap and sustainable alternative nitrogen source.

Things we can improve

Carl Sagan once said: *Science is an ongoing process. It never ends.* Of course, this project is no exception, and although we managed to do the first steps to produce food oils in *Y. lipolytica*, there are still plenty of things that can be improved.

Lipid content

We performed our experiments in a high lipid producing strain, which reached about 50% of lipid content after four days of shake flask cultivation. While this is clearly an oleaginous

phenotype, multiple studies have shown that the lipid content of *Y. lipolytica* can be improved further [92].

Besides overexpressing endogenous genes to increase the TAG production and deleting competing pathways (e.g. lipid degradation pathways), some studies have tuned transcription factors to improve lipid production. For example, the deletion of *SNF1*, a glucose repression regulator, can improve the lipid content 2.6-fold [190]. Additionally, heterologous genes expression can improve lipid production. While the overexpression of the native *ACL* gene did not improve lipid production, the homolog from *Mus musculus* improved the lipid content by over 3-fold [191].

Two other bottlenecks for lipid production that have been investigated are the acetyl-CoA and NADPH supply. Acetyl-CoA is the precursor for malonyl-CoA, which is further converted to acyl-CoA. The overexpression of *CAT2* from *S. cerevisiae* can establish an alternative cytosolic acetyl-CoA pathway that approximately doubled the lipid titre [192]. The acyl-CoA synthesis consumes large amounts of NADPH (14 NADPH / C16:0 FA). Redox engineering can be used to convert glycolytic NADH to NADPH and thereby improve lipid production [193].

In addition to the rational approaches described above, non-rational approaches can be used to improve the lipid content [e.g. adapted laboratory evolution (ALE)]. In ALE cell are randomly mutated (either naturally or e.g. by UV-light or mutagenic substances) and then exposed to a selection pressure (e.g. ferulic acid [194]). Cells that harbour a beneficial mutation, enrich under the restrictive conditions and can be subject to additional rounds of mutations [195]. For the enrichment of high-lipid-content cells, Liu et al. selected *floating cells*. Cells with a high lipid content, have a lower density and float at the top of a culture, while cells with low lipid content settle at the bottom. The evolutionary approach allowed them to identify *UGA2* (succinate semialdehyde dehydrogenase) as an important lipid production enhancer [196].

Because of the vast amount of existing research, we focused on the composition rather than the amount of lipids. Nevertheless, the lipid content of production strains needs to be further improved for future applications to ensure sufficient production titres and, ultimately, process feasibility.

TAG composition

In our project, we aimed to mimic the fatty acid profile of cocoa butter. However, as mentioned before, cocoa butter consists of TAGs. Thus, mimicking the fatty acid profile is only the first step. Future efforts should focus on analysing and modifying the composition of the TAGs to mimic the physio-chemical properties of different food oils (see page 20 for more detailed discussion).

Carbon source

The sustainability of microbial cultivation can be improved by using hydrolysate as a carbon source. However, using hydrolysates still comes with an environmental cost. Even if the lignocellulosic biomass only consists of waste streams (e.g., the wood industry), the pre-treatment often consumes a large amount of energy or uses harmful chemicals [147].

Instead, utilising existing waste streams that do not require additional treatment would be ideal, such as glycerol from biodiesel production [135] or lipid-rich waste streams requiring expensive remediation, e.g. waste cooking oil [197].

An even better approach would be switching to a third-generation production organism, e.g. micro algae [133]. While the first-generation production organisms rely on processed sugars (e.g. glucose), the second generation could use complex carbon sources (e.g. hydrolysates or waste streams). The third generation uses phototrophic organisms (e.g. micro algae), which do not need any sugar as a carbon source. Instead, they use sunlight and CO_2 from the air to grow. Additionally, CO_2 can be fed directly from industrial off-gas streams, increasing growth and capturing CO_2. However, the biomass density of micro algae cultivations is limiting its application [198], with 20 g/L biomass in high-density cultivation in open thin-layer photobioreactors [199].

Down-stream processing

The down-stream processing is an essential aspect of food oil production in microorganisms that this thesis has not touched. In our experiments, we extracted and converted the lipids of the cells in a one-step FAME extraction protocol. However, in an industrial setup, the lipids need to be extracted without conversion to FAME and preferably without the use of toxic solvents. Traditional methods use mechanical extraction, steam distillation, solvent extraction or enzymatic extraction [83].

In addition to those traditional methods, nanotechnology could provide additional solutions for extracting lipids from cells. Magnetic nanoparticles coated with graphene spikes can release lipids from *Y. lipolytica* and could be used in future applications to improve the downstream processing [200].

Things we might see

Climate change is one of the biggest challenges humanity is facing. We will need to find sustainable ways to produce our everyday goods and food and replace fossil fuels with

renewable alternatives[26]. I believe that synthetic biology can play a crucial role to reach these goals.

As outlined in the introduction, our knowledge of biology and the number of available tools to engineer microorganisms exploded over the last century. In all areas of biotechnology, we are making new discoveries and inventions and are approaching the *Golden Age* of biotechnology [201]. The biggest revolution in metabolic engineering in the last decade was the discovery and engineering of CRISPR/Cas9. CRISPR guided transformations have been a game-changer, especially for non-conventional organisms, which often struggle with low-efficiency transformations and problems with genome manipulation methods [202]. We constantly see more tools and new organisms they can be applied in, expanding our stable of microbial workhorses [203]. This extended repertoire allows us to use different organisms for different challenges instead of relying on a few well-established model organisms. Therefore, I expect to see more non-conventional organisms, such as *Yarrowia lipolytica*, both in academic studies and industrial applications.

Nevertheless, we can only harvest the true potential of biotechnology with the support of the public and policymakers. Scientists have faced the consequences of a misinformed public for years when discussing climate change or the risks of vaccinations. Especially in times of misinformation and fake news, it has never been more critical to build trust in the scientific process [204]. It will require the joined effort of all parts of society to achieve this goal.

[26] Additionally, recent times have shown that cutting loose from fossil fuels is also important to secure an economy's independence from foreign oil and gas imports, which otherwise can limit political leeway.

Acknowledgements [27]

”Which is more important” asked Big Panda, “the journey or the destination?”

“The company.” said Tiny Dragon [205]

I couldn’t agree more with James Norbury’s Tiny Dragon. The last four years have been an amazing journey for me. I had the chance to gather knowledge and work on various topics. And although I’m looking forward to the destination of this PhD, the thing I cherish the most are the many amazing people I met along the way.

First of all, I would like to thank my supervisor and co-supervisor for the countless discussions, advice and general guidance. You have supported me at all stages of this thesis and given me the freedom to explore different topics and follow my curiosity, which I’m very thankful for. You have not only taught me the state-of-the-art technologies but also the beauty of simple *old-school* and pilot experiments. I will also never plot a growth curve in a linear scale again.

I’d also like to thank all my co-authors for their contributions to our shared projects. It has been my great pleasure to work with all of you. You have taught me new methods and approaches, and I’m very proud of our achievements.

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[27] The acknowledgments are probably one of the most read parts of any PhD thesis. In accordance with that, the pressure for the writer is very high. To not disappoint or even offend anyone, I will not mention any names at all. I’m sure though, that you will feel addressed in the appropriate section.

Finally, thank you to my family for your love and support throughout my life. You have raised me to question things and sparked my curiosity, which ultimately led to my desire to become a scientist - a career choice I couldn't be happier with. And very finally, from all my heart, thank you Marta[28]. For caring for me, baring me and yet loving me. For all the laughs, adventures, and memories we share. I can't wait to see what the future holds for us. Ets la meva mitja taronja.

[28] … almost no names.

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