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Metabolic engineering of *Saccharomyces cerevisiae* for the production of flavonoids

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Cover: Representation of a double helix of DNA painted with watercolours

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Abstract

The interest in the production of natural products such as flavonoids has been increasing during the last decade. Flavonoids have several attractive bioactivities including antitumoral, antioxidant or antimicrobial properties. To produce these high-value products, we usually recur to chemical synthesis or plant extraction. However, these two options are costly and not environmentally friendly, and microbial production is therefore preferred. *S. cerevisiae* is a thoroughly characterized model organism with a wide range of available tools for engineering, making it an ideal organism for this challenge.

The aim of this thesis was to apply different strategies to engineer *S. cerevisiae* to establish and optimize the production of the flavonoids pinocembrin and naringenin, and their derivatives. Different approaches were used: different heterologous genes were screened, their copy number was increased to achieve the highest production, the competing pathways were eliminated, and the precursors availability was increased. Furthermore, the bottlenecks of the pathways were identified. For pinocembrin production, I established that the accumulation of the toxic intermediate cinnamic acid limits production. Therefore, the transcriptional changes that *S. cerevisiae* undergoes under aromatic acid stress were investigated. My findings indicate that by employing transcription factor engineering it is possible to develop strains that are tolerant to aromatic compounds that can be utilized for the production of valuable natural products. When analysing the naringenin biosynthetic pathway it was found that the distribution of the pathway intermediates in the cell is a major issue. The spatiotemporal distribution of *p*-coumaric acid (a key pathway intermediate) and naringenin was assessed and it was determined that *p*-coumaric acid accumulates extracellularly and cannot be fully utilized. Therefore, a dual dynamic control system that combines a malonyl-CoA biosensor regulator and an RNAi strategy was established, to autonomously control the synthesis of *p*-coumaric acid and downregulate the fatty acid pathways that compete directly for the precursor malonyl-CoA. Finally, the production of naringenin and pinocembrin derivatives was established including kaempferol, quercetin and baicalein which present valuable bioactivities.

Overall, this thesis employs diverse strategies for constructing and optimizing yeast factories for flavonoid production.

Keywords

Genetic engineering, plant natural products, high-value products, cinnamic acid, yeast, microbial cell factories, sustainability

Table of contents

List of publications

Papers included in this thesis:

[I] Tous-Mohedano, M*., Mao, J*., and Chen, Y. (2022) Optimization of pinocembrin biosynthesis in *Saccharomyces cerevisiae*. ACS Synthetic Biology 12, 154-152.

[II] Tous-Mohedano, M*., Konzock, O*., Yu, R., and Chen, Y. Improving the aromatic acid tolerance of *S. cerevisiae* by transcriptomics analysis and transcription factor engineering. (Manuscript)

[III] Mao, J., Tous-Mohedano, M., Fu, J., Li, X., Liu, Q., Nielsen, J., Siewers, V., and Chen, Y. (2023) Fine-tuning of *p*-coumaric acid synthesis to increase (2S)-naringenin production in yeast. Metabolic Engineering 79, 192–202.

[IV] Tartik, M., Liu, J., Tous-Mohedano, M., Mao, J., and Chen, Y. (2023) Optimizing yeast for high-level production of kaempferol and quercetin. Microbial Cell Factories 22, 74

Additional publications not included in this thesis:

[I] Tous-Mohedano, M*., Konzock, O*., and Chen, Y. (2022) Strategies to increase tolerance and robustness of industrial microorganisms. Synthetic and Systems Biotechnology 7, 533–540. Review article

[II] Gast, V., Sandegren, A., Dunås, F., Ekblad, S., Güler, R., Thorén, S., Tous-Mohedano, M., Molin, M., Engqvist, M. K. M., and Siewers, V. (2022) Engineering *Saccharomyces cerevisiae* for the production and secretion of Affibody molecules. Microbial Cell Factories 21, 36.

[III] Konzock, O., Tous-Mohedano, M., Cibin, I., Chen, Y., and Norbeck, J. (2023) Cinnamic acid and *p*-coumaric acid are metabolized to 4-hydroxybenzoic acid by *Yarrowia lipolytica*. AMB express 13, 84.

*Both authors contributed equally to the work

Contribution Summary

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

Paper II
Designed and performed the experiments, analysed, and interpret the results, and wrote and reviewed the manuscript.

Paper III
Assisted in some strain engineering and sample analysis, performed bioreactor cultivations, and edited the manuscript.

Paper IV
Assisted in some strain engineering and sample analysis, performed bioreactor cultivations, and edited the manuscript.

Preface

This dissertation serves as partial fulfilment of the requirements to obtain the degree of Doctor of Philosophy at the Department of Life Sciences at Chalmers University of Technology. The PhD studies were carried out between September 2019 and September 2023 at the Division of Systems and Synthetic Biology (Sysbio) under the supervision of Yun Chen and the co-supervision of Verena Siewers. The thesis was examined by Jens Nielsen. The thesis was mainly funded by Novo Nordisk Foundation grant number NNF18OC0034844.

Marta Tous Mohedano
September 2023

Abbreviations

Term	Abbreviation
Adaptive laboratory evolution	ALE
Artificial Inteligence	AI
By-Product	BY-PR
Chalcone Isomerase	CHI
Chalcone synthase	CHS
Chrysin	CHRY
Cinnamate-CoA ligase	CNL
Cinnamic acid	CA
Cinnamic acid hydroxylase	4CH
Clustered Regularly Interspaced Short Palindromic Repeats	CRISPR
CRISPR-associated systems	Cas
Deoxyribonucleic acid DNA	DNA
Differentially expressed	DE
Double-strand breaks	DSB
Endoplasmic reticulum	ER
Generally recognised as safe	GRAS
Genetically modified organisms GMOs	GMOs
Homologous recombination	HR
Knock Out	KO
Log fold change	logFC
messenger RNA	mRNA
Modular cloning	MoClo
Multidimensional scaling plot of distances	MDS
Naringenin	NAG
Native cytochrome P450 enzymes	P450s
Overexpression	OE
Phenylalanine ammonia lyase	PAL
Pinocembrin	PIN
Polymerase Chain Reaction	PCR
Ribonucleic acid	RNA
RNA interference	RNAi
Transcription factor	TF
Transcription factor activator-like effectors	TALEs
Trimmed mean of M values	TMM

What is essential is invisible to the eye
The Little Prince

L'essencial és invisible al ulls
El Petit Príncep

Introduction

Humans have been using microorganisms for millennia. We have learned to ferment a huge variety of ingredients creating a wide range of delicious food: fermented soybeans (miso, natto and tempeh), fermented cabbage (sauerkraut), fermented spicy cabbage (kimchi), fermented herring (surströmming) or fermented milk (kefir). The credit for those fermented foods goes to mostly filamentous fungi (like *Aspergillus oryzae*) and bacteria (for example *Bacillus subtilis* and *Lactobacillus acidophilus*).

Traces of the first fermented beverage of rice, honey and fruit were found in a bronze vase in the Henan province of China as early as 8000 years B.C[1]. It is speculated that one of the oldest fermented food aside from alcoholic beverages was fermented fish[2]. An example is the *Katsuobushi*, which is a bonito fish that the Japanese would ferment until it is dry and would use as a food flavouring ingredient.

It is estimated that currently between 5% to 40% of the foods that are in our diets are fermented[3]. Food fermentation is not only a way to enhance food flavour but also a method to prevent spoilage. Many early ancestors would, by chance, discover that when food was fermented with the right conditions, the products would remain fresh for a longer period. They would also learn the hard way that the addition of salt prevents food from become toxic[i]. It is apparent that microorganisms have been present in our society for a long time. But how did they end up in our laboratory's Petri Dishes?

How did the first microbes appear on Earth?

This is a very difficult question, and the answer is still uncertain. Nevertheless, many scientists have tried to elucidate how the first prokaryote organisms were formed. It was first proposed by Stanley Miller that the reducing composition of the atmosphere when in contact with sunlight and or electrical discharge would trigger the spontaneous formation of organic molecules[4]. These organic molecules represent the basic materials needed for the first living organisms to arise.

The next step to generate new forms of life was the combination of organic molecules in the form of macromolecules that can reproduce. In the early 1980s, Sid Altman and Thomas Cech discovered that ribonucleic acid (RNA) is a macromolecule able to catalyse its replication using itself as a template[5,6]. Altman and Cech were awarded the Nobel Prize in Chemistry in 1989 for the discovery of this molecular biology milestone. Fossilized microorganisms have been found in different parts of the planet, from Canada to Australia, dating at least four billion years[7]. These first organisms would probably have been simple cells consisting of a phospholipid membrane and a self-replicating RNA chain[8]. RNA is considered to be the origin of the genetic code that deoxyribonucleic acid (DNA) eventually replaced as genetic material. 3.5 billion years ago, the first photosynthetic prokaryotes that produced oxygen as a waste product emerged. Nevertheless, it was not until the Cambrian period (540 million years ago)

[i] Many people that were not adding salt to their food died from botulism. Now we know that this is because of the growth of toxin-producer organisms such as *Staphylococcus aureus* during fermentations.

that microorganisms started to use aerobic metabolism to produce energy and new forms of life as we know them today could eventually evolve[9].

Nevertheless, it will be not until about 360 years ago that scientists reported the existence of microbes. In 1665 Hoke and Van Leeuwenhoek described the first microorganisms: the micro fungus *Mucor* and microscopic protozoa and bacteria[10]. It took another 200 years until Louis Pasteur[ii] confirmed what the German Theodor Schwann had already reported[11] by linking food spoilage to microorganisms[12] and revealed that microorganisms have the ability to transform molecules.

Advances in molecular biology led to the first microbial cell factories

Humans have always tended to use nature at their convenience. And since microbes were discovered, they were not an exception. One of the first attempts to exploit microbes' potential came with the isolation of *Clostridium acetobutylicum* by Chaim Weizmann[13]. This bacterium was used to produce acetone (a compound used during the First World War to produce the smokeless powder cordite), butanol (used as a precursor of synthetic rubber) and ethanol.

However, it was not until the discovery of the deoxyribonucleic acid (DNA) as the "transforming principle" in 1944 by Avery, MacLeod and McCarty that molecular biology flourished[14]. Avery *et al.,* demonstrated that DNA isolated from a bacteria strain could be transformed into another strain conferring new characteristics into the second strain. Later thanks to Watson, Crick, and the so-called *forgotten heroine* Rosalind E. Franklin the double helix structure of DNA was elucidated[15,16]. Shortly after in 1958, Francis Crick defined the central dogma of molecular biology as an explanation of how genetic information flows from DNA to RNA to proteins[17]. Thereafter, protein sequencing techniques were developed. Frederick Sanger was a pioneer that sequenced all the amino acids of insulin in 1955[18,19] and got awarded the Nobel Prize in chemistry twice during his career. Afterwards, it was just a matter of time before the first genetically modified organisms (GMOs) would be developed. Indeed it was in 1973 when Herbert Boyer and Stanley Cohen introduced a plasmid carrying an antibiotic-resistant gene into a bacteria that was sensitive to that antibiotic[20,iii].

The first scientists introduced eukaryotic DNA into bacteria species in 1974[22], and the first stable yeast genomic integration followed in 1979[23]. Few years later, two discoveries that made the amplification of any known DNA sequence possible were instrumental in the development of genetic engineering. Firstly, the discovery of DNA polymerase enzymes[24] that have the function of catalysing the synthesis of DNA from deoxyribonucleotides (dNTPs). Secondly, the invention of the well-known method of Polymerase Chain Reaction (PCR)[25,26] to replicate DNA fragments. During the same period, Sanger accomplished one of the first DNA sequencing using the chain termination method[27]. The sequencing field developed further until 2001 when the human genome was fully sequenced[28].

[ii] Pasteur developed a method to counteract microbial contamination that we still use nowadays termed *pasteurization.*

[iii] The story of how these two scientist that set the foundations of biotechnology is reviewed here [21].

The success of DNA sequencing led to the production of the first recombinant protein in *E. coli* by David Goeddel, insulin[29]. Thanks to the advances in shotgun genome sequencing and genomics, scientists could find production pathways of complex molecules that were interesting for our health, e.g., drugs[30] such as vinblastine[31]. Currently, yeast is one of the most preferred organisms to produce pharmaceuticals.

Using *Saccharomyces cerevisiae* as a host organism

Saccharomyces cerevisiae also known as brewer's or baker's yeast is one of the most commonly used microorganisms to produce different range of products[32]. *S. cerevisiae* is extensively characterized, it has over 6000 genes organised in 16 chromosomes and its genome was the first eukaryote organism fully sequenced[33]. An average of forty thousand publications were published over the last 3 years[iv] making it the most studied eukaryote unicellular organism. In addition, it is a biosafety level 1 organism, and its manipulation is generally recognised as safe (GRAS), which makes it a good candidate to be used in industrial biotechnology. Despite the popularity of *S. cerevisiae*, natural products and pharmaceuticals are also produced in other systems, such as *Escherichia coli*, *Bacillus subtilis* or other non-saccharomyces yeast such as *Yarrowia lipolytica*. However, a perfect host to produce natural products or pharmaceuticals does not exist. *E. coli* can be preferred for its rapid growth and low medium requirements. However, *E. coli* may not be ideal for protein production because it can capture the proteins in inclusion bodies and makes the downstream process very complex[34]. In contrast, *S. cerevisiae* secretes proteins and can do post-translational modifications. *S. cerevisiae* presents other advantages when compared to other yeast. Since it has been deeply characterized, there is a big number of available tools to engineer it. For example, a lot of knowledge regarding gene promoters, terminators, and stable integration sites is already available[35–38]. Thanks to this and depending on what carbon and nitrogen composition is used in the cultivation, or gene expression is preferred for the experiment, many known bio-bricks and tools are available.

Finally, *S. cerevisiae* presents native cytochrome P450 enzymes (P450s). P450s are versatile enzymes that catalyse more than 20 different types of chemical oxidation reactions including nitration, decarboxylation or oxidation[39]. They are part of the biosynthetic pathways for producing natural products, for example, they are needed to catalyse hydroxylation of aromatic molecules[40,41]. The ability to express P450s is especially relevant when we aim to synthesize products of plant origin that require this enzyme to perform certain modifications.

Techniques for engineering *S. cerevisiae*

Building a yeast cell factory requires the expression of heterologous enzymes and often tweaking the native metabolism to favour the flux of carbon towards the desired end-products[42,43]. Initially, new pathways were expressed entirely in plasmids. Even though this is a good option because it makes strain construction easier, it has disadvantages in terms of

[iv] Statistics can be generated in this webtool: https://app.dimensions.ai/discover/publication

stability and gene expression levels inside a population[44]. Therefore, finding well-characterized integration sites that ensure stable gene expression was a crucial step for the development of yeast cell factories[45].

Scientists soon realized that the generation of DNA-double strand breaks (DSB) was necessary to stimulate genome editing through homologous recombination (HR) events. Essentially, when there is a DSB in the genome, *S. cerevisiae* has the machinery to accurately repair the nick using a homologous DNA template[46]. This is a powerful tool when scientists aim to introduce genes into the genome of yeast. If the gene presents homologous regions with the genomic DNA where the DSB was created, *S. cerevisiae* can easily repair the DSB and introduce the gene. To achieve the DSB different customizable DNA binding proteins exist such as zinc finger nucleases[47], transcription factor activator-like effectors (TALEs)[48] and most recently the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated systems (Cas)[49]. Both zinc fingers and TALEs present specificity challenges that require extensive screening and intense labour[50]. Given the drawbacks of the other techniques, CRISPR-Cas9 is the system that provides more efficient results with minimal labour cost. CRISPR history has been shaped by many scientists. CRISPR was first described by Ishino *et al.*, in 1987 in *E. coli*[51] but it was not until 20 years later that Philippe Horvath discovered its biological function[52]. In short, they describe that if a healthy prokaryote is infected by a virus and survives the infection, the prokaryote stores part of the virus DNA on its genome. This DNA would be an immune memory defence mechanism that would guide the native prokaryote CRISPR system to the DNA of a new virus that would infect the cell and the CRISPR nucleases would create a DSB and kill the virus[53]. In 2020, Emmanuelle Charpentier and Jennifer A. Doudna received the Nobel Prize in Chemistry for their contributions to the so-called genetic scissors that enable molecular biologists to easily engineer microbes all over the world.

Currently, a huge number of toolkits are available to engineer *S. cerevisiae*[54] that include the use of CRISPRCas9 technologies and Modular Clonning[55,56]. Thanks to this development, it is possible to integrate more than 25 heterologous genes into a yeast strain in a short period[57]. Therefore, scientists can express whole pathways from plants into *S. cerevisiae*, making microbial cell factories a viable system to produce natural products.

Metabolic engineering microorganisms

The term metabolic engineering was first described by Bailey., as:

> "Improvement of cellular activities by manipulation of enzymatic transport and regulatory functions of the cell with the use of recombinant DNA technology.''[58]

At that moment as Bailey described himself, metabolic engineering was just a few examples of what could be achieved rather than an established science. The field has experienced rapid development, leading to an influx of numerous publications, products, and applications.

Firstly, metabolic engineering has primarily focused on substituting fuel derivatives with sustainable chemicals, also known as commodity chemicals. An example of this is the bioproduction of succinic acid, a four-carbon dicarboxylic acid that is the precursor of industrially relevant chemicals such as 1,4-butanediol or tetrahydrofuran among others. Succinic acid has been produced in many microorganisms[59] including *Actinobacillus succinogenes, Corynebacterium glutamicum, Escherichia coli, Mannheimia succiniciproducens*[60] but despite all the effort and scientific investment it never managed to substitute oil-based production. There are different reasons why the substitution of fuel-derivative products with bio-produced chemicals is facing challenges[61]. Firstly, these chemicals have to be produced in large quantities and developing efficient microbial cell factories capable of meeting such production demands is not currently feasible. Moreover, the production of these chemicals from fuel sources is typically very cost-effective, making it difficult for bio-production to compete with this low cost. For all these reasons the metabolic engineering field is slowly shifting from commodity chemicals production to high-value product production that includes, food ingredients, feed, healthcare, and agriculture products. Some examples of successful products that have been brought to the market are summarized in Table 1.

Table 1. Companies and start-ups that have successfully developed microbial cell factories of high-value products and brought them to the market.

Classification	Product	Description	Company	Ref
Fuel-substitutes	trans-β-Farnesene	Used as a blend in biofuel for diesel and jet fuels	Amyris and Total	[62]
Fine chemicals	Lactic acid	Production of lactic acid using yeast as biocatalyst to reduce costs and environmental footprint	Cargill	
Drug	Artemisinic acid	Antimalarial drug	Amyris[v]	[63]
High-value product	Synthetic Cannabinoids	High-value cannabinoid production for the cosmetic industry	Creo	-
Food ingredients	Animal-free milk substitutes	Production of animal-free milk that has the same nutritional protein content as cow's milk.	Perfect Day	-
Food ingredients	Solein[vi]	Use of chemoautotrophic bacteria to produce protein	Solar foods	-

In the research included in this thesis, a combination of metabolic engineering approaches has been used to establish and optimize the production of high-value plant derivatives in yeast.

[v] Despite being a great example of successful biotech company, Amyris declared bankruptcy to improve its liquidity position the 9th of August 2023.

[vi] The first product containing solein, a chocolate gelato, has been launch to the market in a restaurant, in Singapore. Solein has been granted regulatory approved to be sold in Singapore since September 2022.

Flavonoids, a group of high-value products

Flavonoids are phenolic compounds and are naturally secondary metabolites of plants. There are more than 9000 different flavonoid-like compounds making them one of the largest families of natural products. The flavonoid structure consists of a fifteen-carbon skeleton based on 2 benzene rings linked by a heterocyclic pyran ring (Figure 1). Flavonoids appear as aglycones (basic structure), glycosides (when they have e.g., a D-glucose, glucorhamnose, galactose, arabinose or L-rhamnose) and methylated derivatives. They can be divided into different subclasses depending on the number of substitution patterns of the position indicated in the backbone of Figure 1.

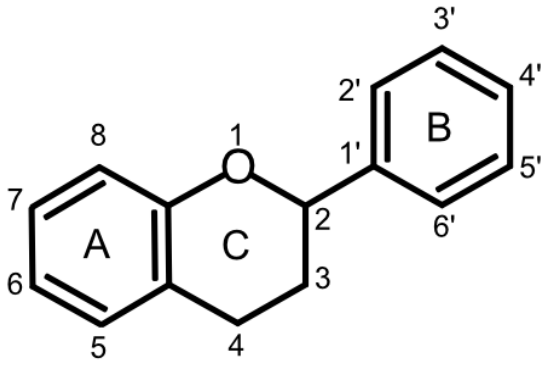


Figure 1. Basic flavonoid structure. Benzene rings are labelled as A and B. Pyran ring is labelled as C.

Table 2. Substitution patterns of a small selection of flavonoids of interest. Adapted from[64]

	Position in the basic flavonoid structure								
	3	5	6	7	8	3’	4’	5’	C_2=C_3
Flavones									
Apigenin	H	OH	H	OH	H	H	OH	H	+
Flavonol									
Quercetin	OH	OH	H	OH	H	OH	OH	H	+
Kaempferol	OH	OH	H	OH	H	H	OH	H	+
Flavanone									
Naringenin	H	OH	H	OH	H	H	OH	H	-
Pinocembrin	H	OH	H	OH	H	H	H	H	-
Flavanonol									
Taxifolin	H	OH	H	OH	H	OH	OH	H	
Isoflavone									
Genistein	-	OH	H	OH	H	H	OH	H	+
Flavan-3-ols									
+ Catechin	βOH	OH	H	OH	H	OH	OH	H	-
Flavylium Salts									
Cyanidin	OH	OH	H	OH	H	OH	OH	H	-

The different subclasses include flavones, flavonols, flavanones, flavanonols, isoflavones, flavan-3-ols and flavylium salts. In Table 2 there is a flavonoid example for each subclass indicating what functional groups are included in each position. Pinocembrin, naringenin, kaempferol and quercetin are four flavonoids that are included in the table and are going to have a major role in the thesis.

Furthermore, flavonoids hold significance in our daily nutrition, being naturally present in fruits, roots, and vegetables that constitute a fundamental part of our diets. Over the past decade, their popularity increased, prompting scientists worldwide to focus not only on their production but also on investigating their applications. Flavonoids exhibit numerous bioactivities, making them appealing for human applications. Many *in vitro* studies have demonstrated their bioactivities. Their properties have been reviewed extensively[65,66] and a small summary is represented in Table 3.

Table 3. Table describing flavonoids bioactivities with examples and references.

Bioactivity	What is known	Ref
Anticancer	Flavonoids can be an alternative to aggressive chemotherapy treatments. Flavopiridol is used for leukaemia treatments. Quercetin is proposed against prostate and breast cancer.	[67,68]
Antibacterial	The fern *Asplenium nidus* that contains the flavonoids gliricidin-7-O-hexoside and quercetin-7-O-rutinoside present inhibitory effects comparable with the drug amoxicillin. Flavonoids could be an alternative to antibiotic use.	[69]
Antioxidant	Flavonoids extracted from the leaves of *Bauhinia variegata* (Pakistan medicinal plant) presented antioxidant properties against ROS.	[70]
Cardioprotective	The methylated flavonol isorhamnetin has a cardioprotective effect against the cardiotoxicity of the anticancer drug doxorubicin.	[71,72]
Anti-inflammatory	Some flavonoids are reported to be inhibitors of proinflammatory signalling molecules such as prostaglandins.	[73]
Protective against UV	Apigenin found in editable plants such as chamomile has skin protective properties. Quercetin has also been demonstrated to inhibit skin damage when applied to hairless mice	[74,75]

This thesis is focused on the production of the pinocembrin and naringenin branches. Pinocembrin has been studied (*in vivo* and *in vitro*) for its application to treat very common diseases such as ischemic stroke, Alzheimer's disease, or cardiovascular diseases[76]. It has very appealing properties that include the ability to cross blood brain barrier and the ability to target neuroinflammation. Pinocembrin derivatives: chrysin, baicalein and norwogonin, also have relevant properties[77–81]. For example baicalein its known to decrease inflammatory markers such as IL-1β, IL-6 and TNF-α[82] and norwogonin and its derivates have excellent

antioxidant properties because of the hydroxyl group presence in C_8[83]. Naringenin, has been studied for its potential antidiabetic properties[84]. Naringenin derivatives, kaempferol and quercetin, have shown antitumoral properties by inhibiting cyclins and increasing tumour suppressor protein concentration[85].

Although flavonoids can be found in many fruits and vegetables they are only found in very small quantities. Therefore, to obtain large amounts, big quantities of plant biomass are required. The extraction of flavonoids from plant material requires a complex purification process. Plant biomass contains a mixture of compounds structurally similar to flavonoids that complicates the purification and separation limiting the large-scale production[86]. An alternative to plant extraction could be chemical synthesis but its complex structure requires toxic reagents and harsh operating conditions. Therefore, microbial production is an attractive alternative to sustainably producing these natural compounds.

Aim of the thesis

The overall aim of this thesis was to develop *S. cerevisiae* platform strains with the capacity to produce flavonoids. We established the flavonoid production in the yeast *S. cerevisiae*. The focus was on the production of pinocembrin and naringenin, two main branches of flavonoids that have very appealing bioactivities and applications. The microbial production of these flavonoids establishes a foundation for the synthesis of many pharmaceutically interesting derivatives. Therefore, in the first chapter (Paper I) the production of the flavonoid pinocembrin *de novo* from glucose in *S. cerevisiae* is presented. In Paper I we identified that the accumulation of the precursor cinnamic acid limits pinocembrin production because of its toxicity. Hence, in the second chapter (Paper II) we aimed to build a strain tolerant to aromatic acids. For that purpose, the transcriptional response of *S. cerevisiae* to three aromatic acids that are precursors of three main branches of flavonoids was studied. In the third and fourth chapters the focus switches to the *de novo* production of naringenin (Paper III) and its hydroxylated derivatives (Paper IV). Firstly, in Paper III we looked into how the cellular distribution of the pathway intermediate *p*-coumaric acid can hamper the production of naringenin. We applied different strategies to control systematically the pathway intermediates and build an efficient naringenin platform strain. Finally in Paper IV the naringenin platform strain was used as a chassis to produce the pathway derivatives kaempferol and quercetin.

Chapter 1 – Establishing a pinocembrin platform in *S. cerevisiae*

Building a proof-of-concept strain

The first reports of *S. cerevisiae* engineered to produce flavonoid-like compounds were published in the early 2000s[87–89]. During the last two decades, several flavonoid production pathways have been described and introduced in yeast. However, the biosynthesis of pinocembrin and its derivatives had not been optimized. This flavonoid was originally found in *Eriodictyon californicum,* belongs to the flavanone subcategory and has been studied for its antibacterial[90], cardioprotective[91] and neuroprotective[92] properties. In addition, its derivatives chrysin, baicalein and wogonin possess very attractive bioactivities[77,78,80,93–95]. Chrysin, for example, has been shown to inhibit thyroid cancer tumour formation (both *in vivo* and *in vitro*)[79]. Baicalein has been studied for its role in mitigating mitochondrial damage[80,VII] and wogonin has been shown to present anxiolytic effects[81]. Therefore, a robust platform strain that can host the production of this flavanone is necessary to produce many of these interesting derivatives.

Having a strain with an increased metabolic flux towards the production of the aromatic amino acids L-phenylalanine and L-tyrosine is preferred to build a cell factory to produce flavonoids. Therefore, we chose a background strain derivative from CEN.PK that was previously developed in our research group. This strain has (i) the allosteric regulation of 3-deoxy-D-arabinoheptulosonate 7-phosphate (DAHP) synthase and chorismite mutase alleviated, (ii) *PHA2*, *ARO1*, *ARO2*, and *ARO3* overexpressed, and (iii) the heterologous shikimate kinase AroL from *E.coli* expressed[43,96].

Pinocembrin is a derivative of the aromatic amino acid L-phenylalanine. The biosynthetic pathway of pinocembrin starts with the conversion of L-phenylalanine to cinnamic acid (CA) by the enzyme phenylalanine ammonia lyase (PAL). CA is then converted to cinnamoyl-CoA by a cinnamate-CoA ligase (CNL). Subsequently, three molecules of malonyl-CoA are added to cinnamoyl-CoA by a chalcone synthase (CHS) to produce trihydroxychalcone. Finally, a chalcone Isomerase (CHI) will produce the final compound to pinocembrin (Figure 2)[87,97,98].

[VII] Mitochondrial dysfunction plays an important role in neurological disease such as Parkinson and Alzheimer's.

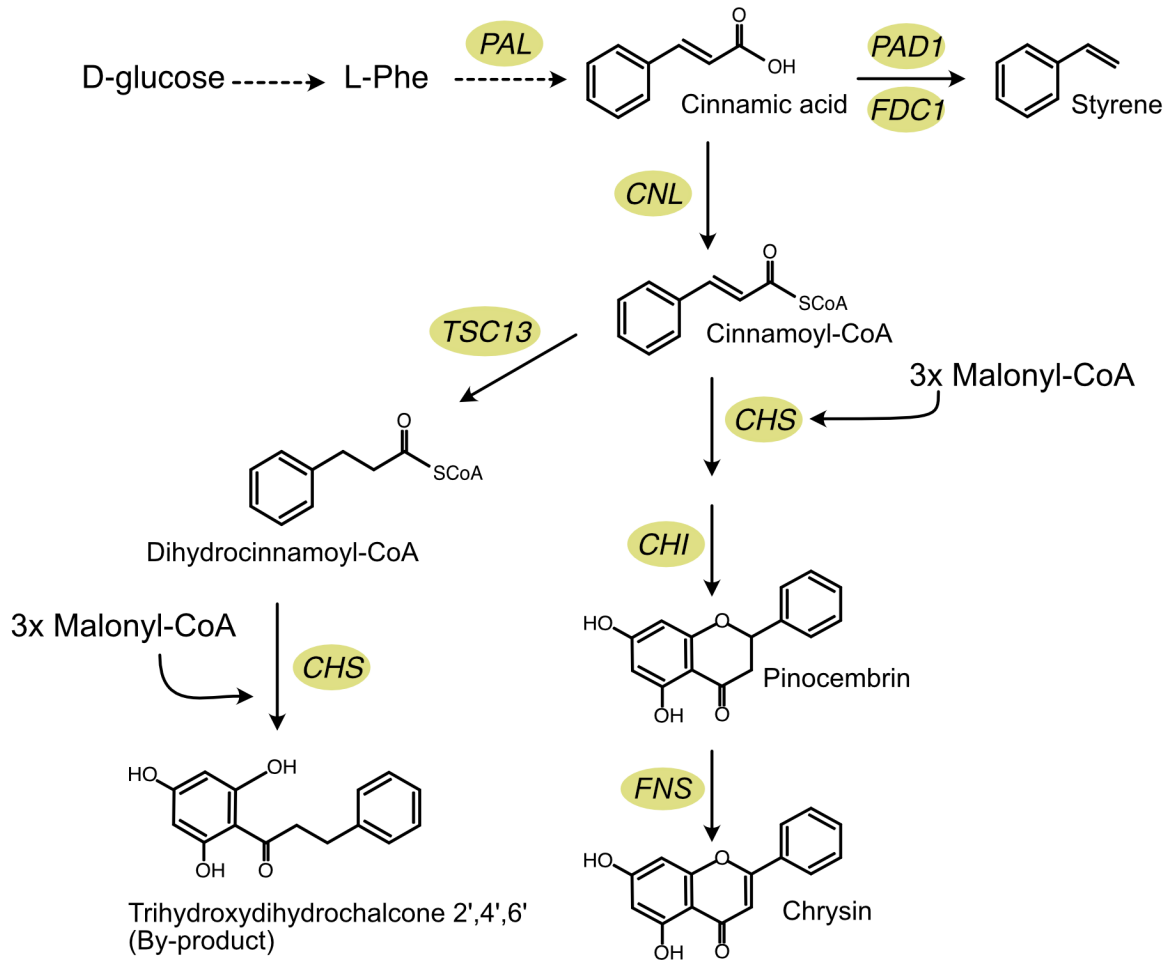
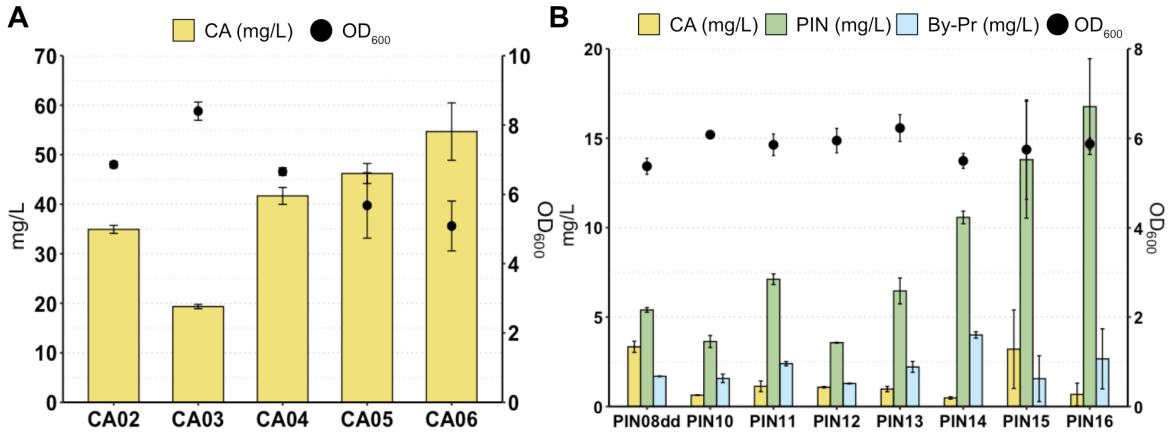


Figure 2. Schematic diagram of the pathway established in S. cerevisiae *to produce pinocembrin and its derivatives. Solid lines represent a single step; dashed lines indicate multiple steps; L-phe, L-phenylalanine; PAL, phenylalanine ammonia lyase; CNL, cinnamate-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; TSC13, enoyl reductase; FNS, flavone synthase.*

A large variety of enzymes capable of catalysing the described reactions are available, from plants, bacteria, and yeast. Firstly, a screening to determine the best enzyme combination suitable for the reactions was needed. The best enzymes must be able to express properly in yeast and have high affinity for the substrate of the reaction. Also, their expression should not compromise cell growth and robustness. Candidates were screened from databases such as UniProt. A collection of enzymes was selected, the encoding genes codon-optimized and tested. To evaluate the efficiency, we checked the growth and the production of the end product pinocembrin and the precursor cinnamic acid on the HPLC. When analysing the results to evaluate the efficiency of the PAL enzymes tested, we found the first bottleneck of the pathway. When cinnamic acid production increased and accumulated in the cell, the growth of the yeast strain was reduced, potentially compromising the production of other compounds downstream (Figure 3A). In Paper I, to mitigate cinnamic acid toxicity we reduced the accumulation of cinnamic acid by improving the pathway flux downstream of this intermediate. Firstly, we selected the best three enzyme versions (CNL, CHS and CHI) that could convert cinnamic acid to a higher pinocembrin concentration (Figure 3A, Paper I). To reduce cinnamic acid accumulation we increased the gene copy number of the three selected

enzymes (CNL, CHS and CHI) until we could not detect any accumulation of CA (Figure 3B). As a result, we obtained the strain PIN16 that had 4 copies of the gene CNL, 3 copies of the gene CHS and 3 copies of the gene CHI (Figure 3B). This strain produced 16 mg/L of pinocembrin, and the accumulation of the toxic precursor CA is neglectable.



AAA^{E}	+	+	+	+	+
*At*PAL	1	-	1	2	3
*Rt*PAL	-	1	1	-	-

*Ph*CNL	1	2	1	1	1	2	3	3
*Rs*CHS	1	1	2	1	2	2	3	4
*Sb*CHI	1	1	1	2	2	2	3	4

Figure 3. Toxicity of cinnamic acid in S. cerevisiae*. (A) Production of cinnamic acid by engineered strains expressing different genes encoding phenylalanine ammonia lyase (PAL) with increased copy number. (B) Pinocembrin production when gene copy numbers were gradually increased. All data represent the mean of n=3 biological independent samples and error bars show the standard deviation. CA, cinnamic acid; PIN, pinocembrin; By-Pr, by-product (trihydroxy dihydrochalcone 2',4',6'); OD_{600}, optical density; CNL, cinnamate-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; Ph,* Petunia hybrida*; Rs,* Rhododendron simsii*; Sb,* Scutellaria baicalensis*; RtPAL phenylalanine ammonia lyase from* Rhodotorula toruloides*; AtPAL, phenylalanine ammonia lyase from Arabidopsis thaliana; AAA^{E}, overexpression of Aro7^{G141S}, Aro4^{K229L}, ARO1/2/3, PHA2, EcAroL from* E. coli.

Eliminating competing pathways

To optimize the production of the desired product, it is important to assess if any side reactions are degrading any pathway intermediates. When examining the biosynthetic pathway to produce pinocembrin, two potential competing pathways were detected. Firstly, cinnamic acid can be degraded to styrene by the action of two native enzymes of *S. cerevisiae*: Fdc1 and Pad1 (Figure 2). These enzymes are known to be ferulic acid decarboxylase and phenylacrylic acid decarboxylase, respectively[99]. To eliminate the risk of precursor degradation we deleted both genes. However, in contrast to what Mukai *et al* described, we could not detect a difference in cinnamic acid production in the tested strains (Supplementary Figure 2, Paper I). After sequencing these two genes in our background strain CEN.PK.113-7D we found that both contain a stop codon in the middle of the ORF making them non-functional.

Secondly, the pathway intermediate cinnamoyl-CoA is potentially a substrate of the native essential enzyme Tsc13, an enoyl reductase involved in very long chain fatty acid elongation[100]. Due to high structure similarity to coumaroyl-CoA, cinnamoyl-CoA can be converted by a 2-step reaction to trihydroxydihydrochalcone 2',4',6', referred to as *by-product* in this thesis for simplification. This reaction requires 3 molecules of malonyl-CoA, also needed to produce pinocembrin. Thus, if the competing reaction is eliminated not only the *by-product* formation

will be eradicated, but the flux of malonyl-CoA will increase and could be utilized to generate pinocembrin. Since *TSC13* is an essential gene and cannot be deleted, different strategies could be applied to reduce its expression. One could be the downregulation of the gene by exchanging the native promoter with a previously characterized weaker promoter. Another strategy could be to substitute the gene with an analogue from another species that would have less affinity for the substrate cinnamoyl-CoA but still fulfil the main function of the Tsc13p enzyme. We chose to follow the last strategy since an analogue of *TSC13* from *Malus domestica* has been successfully used to prevent the formation of phloretic acid and phloretin from coumaroyl-CoA in a naringenin-production strain[101]. By substituting this gene, we could completely eradicate the formation of the undesired by-product and redirect the carbon to the formation of pinocembrin. This resulted in a strain that produced 30 mg/L of pinocembrin, 2-fold more product than the original engineered strain[98].

Increasing the availability of precursors

Pathway precursor availability is a fundamental factor when building any microbial cell factory. When examining the pathway of interest in Paper I, we detected two main precursors that are both part of yeast native metabolism: L- phenylalanine and malonyl-CoA.

In yeast, the aromatic amino acid phenylalanine is produced via the shikimate and chorismate pathway[102]. Previous research developed in our research group by Liu *et al.*, demonstrated that when the allosteric regulation of DAHP synthase and chorismite mutase was alleviated, *PHA2, ARO1, ARO2*, and *ARO3* were overexpressed, and the heterologous shikimate kinase AroL from *E. coli* was introduced the production of aromatic derivates in yeast increased[43]. Therefore, we used a background strain with these modifications incorporated which led to an increased flux of L-phenylalanine.

For producing 1 mol of pinocembrin, 3 moles of malonyl-CoA are needed. Malonyl-CoA is an essential building block for *S. cerevisiae*. It is synthesized from the metabolite acetyl-CoA by the acetyl-CoA carboxylase (Acc1)[103]. Malonyl-CoA is used by yeast as a building block for fatty acid synthesis. During the last decade and with the increased popularity of microbial cell factories, malonyl-CoA is considered an essential precursor for producing many high-value plant natural products in microbes. This is why researchers have focused on increasing the yeast native production of malonyl-CoA. In Paper I, we assessed two different strategies. Firstly, we integrated into the genome a malonate assimilation pathway from *Rhizobium trifolii.* The pathway consists of RtmatC, a malonate carrier protein that transports malonate from the media into the cytosol and RtmatB that is a malonate synthase protein that converts malonate to malonyl-CoA[104]. Secondly, we overexpressed a mutated version of the *ACC1* gene, mAcc1**(Acc1^{ser659ala,ser1157ala}), that presented enhanced activity[105]. The mAcc1** contains two mutations in two phosphorylation sites: ser659 and ser1157. The mutations change both amino acid from serine to alanine. Shi *et al.,* showed that the overexpression of mAcc1** in a wild type *S. cerevisiae* strain increased the ACCase activity 3-fold.

The first strategy has the main disadvantage that it requires malonate feeding, which would be a downside when upscaling the flavonoid production into industry production processes.

Nevertheless, both approaches were tested and the intracellular levels of malonyl-CoA of the resulting strains were measured by using a malonyl-CoA biosensor plasmid[106]. GFP fluorescence that correlates to malonyl-CoA concentration was detected using a flow cytometer. We detected a 21% increase of fluorescence when RtmatC and RtmatB were expressed (strain PIN21) and an increase of 27.5% when mAcc1** was overexpressed (PIN33) (Figure 4). These findings also prove that malonyl-CoA was limiting pinocembrin production and that expressing the enhanced version of *ACC1* improves the pool of this metabolite.

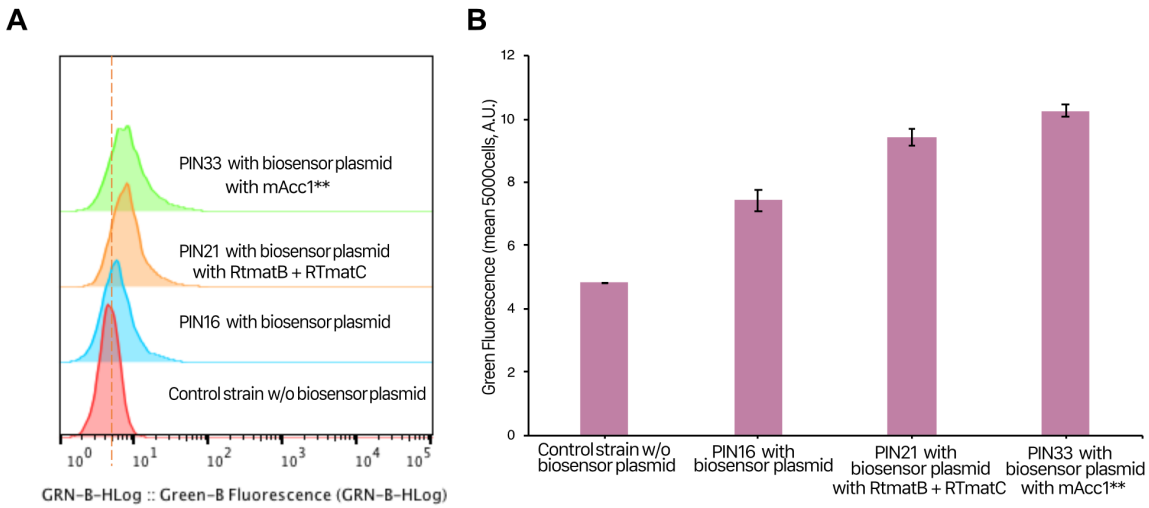


Figure 4. Evaluating malonyl-CoA supply of two different engineering strategies by using a malonyl-CoA sensor. (A) Flow cytometry measurement of different strains carrying the malonyl-CoA biosensor plasmid. (B) Mean green fluorescence intensity of different strains containing the malonyl-CoA sensor. All strains were cultured in a defined minimal medium with 30 g/L glucose as the sole carbon source, and with 5 g/L sodium malonate dibasic supplementation when required. Fluorescence was measured at 12 h after inoculation. All data represent the mean of n= 3 biologically independent samples and error bars show the standard deviation.

When analysing the production profile of the resultant strains, PIN21 and PIN33 (Figure 4, Paper I), we can see that there is an accumulation of the toxic precursor cinnamic acid in strain PIN21. This may be related to the cultivation method difference between the two strains. PIN21 cultivation is supplemented with 5 g/L of malonate dibasic, needed for the expressed bacterial malonate assimilation pathway. We found that malonate acts as a buffer and keeps the pH of the cultivation above 5. In contrast, when malonate is not added, the pH of the cultivation drops below 4.5. At lower pH weak acids, such as cinnamic acid, can diffuse into the cell where they dissociate causing the acidification of the cytosol and affecting cell growth. Since both strategies worked successfully to increase the supply of malonyl-CoA but the addition of mACC1* did not require external malonate feeding and led to less accumulation of cinnamic acid we used PIN33 for further pathway optimization.

As a last step, we combined the strain that has an increased pool of malonyl-CoA (PIN33) with the elimination of the competing pathways, and *by-product* formation, and we achieved a concentration of 40 mg/L of pinocembrin (strain PIN36). The pinocembrin production was further improved (by 2-fold) by cultivating the strain PIN36 in fed-batch like conditions (Figure 5B, Paper I). To mimic fed-batch conditions in shake flasks we used tablets that release glucose slowly overtime. In these cultivation conditions, the carbon supply is limited preventing the overflow metabolism of *S. cerevisiae*.

Using the pinocembrin platform strain to produce downstream flavonoids

Pinocembrin is a molecule of interest for its bioactivities. However, as described in the introduction, derivatives of pinocembrin are being studied to have more strong and appealing properties. Thus, we aimed to test if the platform strain that we developed to produce pinocembrin could be used as a chassis to produce other derivatives of interest.

Chrysin production

The first derivative that can be produced directly from pinocembrin is chrysin which presents a double bond between C_2 and C_3 (Figure 1). It is naturally abundant in propolis, blue passion flower and honey[78]. Chrysin has been studied for its beneficial effects against several cancers including breast, gastric, colorectal, prostate, and cervical cancer among others, delaying tumour formation[77]. To establish chrysin biosynthesis, we tested two enzymes: a flavone synthase I (FNSI) and a flavone synthase II (FNSII). FNSI is a soluble enzyme found in the cytosol whereas FNSII is a cytochrome P450-dependent monooxygenase usually found bound to membranes [107,108]. After genomic integration, we measured the concentration of flavonoids in the cell culture with the strains expressing FNSI and FNSII. We found that strains expressing FNSI could completely convert pinocembrin to chrysin whereas strains expressing FNSII were not as efficient and some accumulation of cinnamic acid and pinocembrin was detected. When expressing FNSI in PIN36, the resulting strain (PIN38) produced 25 mg/L of chrysin in minimal media batch fermentation (Figure 6A, Paper I).

Chrysin derivatives production: the baicalein and norwogonin pathways

Baicalin, wogonin and their glycosides are major bioactive compounds derived from chrysin via the baicalein branch and the norwogonin branch (Figure 5). The bioconversion of chrysin to baicalein is catalyzed by a flavonoid-6-hydroxylase enzyme (F6H) that introduces a hydroxyl group at the C_6 position of chrysin. F6H from *Scutellaria baicalensis* (GenBank ID = MF363006.1)[109] and *Glycine max* (GenBank ID= Y10490.1)[110] have been reported to be functionally expressed in yeast. Subsequently, baicalein can be converted to baicalin by the attachment of D-glucoronate to baicalein (a baicalein 7-O-glucuronosyltransferase is needed, for example, the native enzyme of *Scutellaria baicalensis*, EC:2.4.1.253[111]). Moreover, the expression of a UDP-glucose 6-dehydrogenase is needed to ensure the substrate (D-glucoronate) availability[112].

The second branch starts with the conversion of chrysin to norwogonin. This step can be catalysed by a flavone-8-hydroxylase (F8H) that incorporates a hydroxyl group into the C_8 position of chrysin. The F8H from *Ocimum basilicum* and *Scutellaria barbata* have been reported as candidates to perform this conversion[113,114]. Further, an enzyme with methyltransferase activity such as the flavonol 8-O-methyltransferase from *Mentha piperita* would be needed to transform norwogonin to wogonin[115].

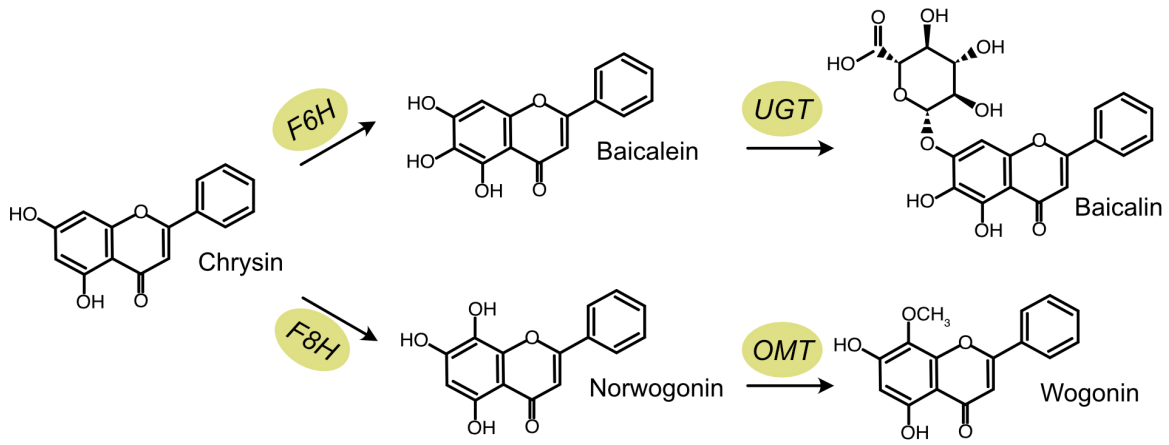


Figure 5. Derivatives of the chrysin pathway. Two branches are described: the formation of baicalein and baicalin (top) and the formation of norwogonin and wogonin (bottom). F6H; flavonoid-6-hydroxylase, F8H; flavone-8-hydroxylase, UGT; baicalein 7-O-glucuronosyltransferase; OMT, flavonol 8-O-methyltransferase.

We attempted to produce baicalein using the developed chrysin production strain *(unpublished data)*. To do so we tested the activity of the two F6H enzymes described previously in the literature. Our data show that the F6H from *Scutellaria baicalensis* (SbF6H) could convert chrysin to baicalein whereas F6H from *Glycine max* was not functional. Nevertheless, the strain that expressed SbF6H presented reduced growth, low production of baicalein and precursors accumulation. P450 enzymes expression such as F6H can cause stress to the cell and lead to growth reduction. Therefore, to uncouple flavonoid production from the growth we tested an inducible promoter to delay the expression of the enzyme SbF6H.

S. cerevisiae prefers to utilize glucose as carbon source; however, it can also use other sugars like maltose, sucrose, or galactose. The galactose metabolism genes are regulated at the transcriptional level in a carbon source dependent manner, and they can be found in three states: (i) glucose repressed state: Mig1 repressor binds and represses GAL promoters. The transcription activator protein Gal4p is also bound to the GAL promoter, but it is inhibited by the repressor Gal80 (ii) derepressed state (glucose is absent but there is no galactose): Mig1 repressor does not bind but Gal4 is still inactivated by Gal80 (iii) Induced state (glucose is absent and galactose is present): Mig1 does not bind to the promoter and Gal3 inactivates the repressor Gal80. Subsequently Gal4 is active and there is transcription of the *GAL* genes[116,117]. We used the divergent promoter from *GAL1* to express the F6H encoding gene[118]. This is the promoter that regulates the expression of the genes encoding the two first enzymes of the galactose metabolic pathway, which in the presence of galactose they are activated. We integrated this cassette into two different backgrounds: the pinocembrin producer strain that expresses FNSI and can produce chrysin at a low level (PIN37, Figure 6A) and the high producer strain that has the mAcc1** integrated and the competing pathways eliminated (PIN38, Figure 6B). The resulting strains PIN41 (Figure 6A) and PIN42 (Figure 6B) respectively, were cultivated with different ratios of glucose/galactose and the flavonoid profile was studied.

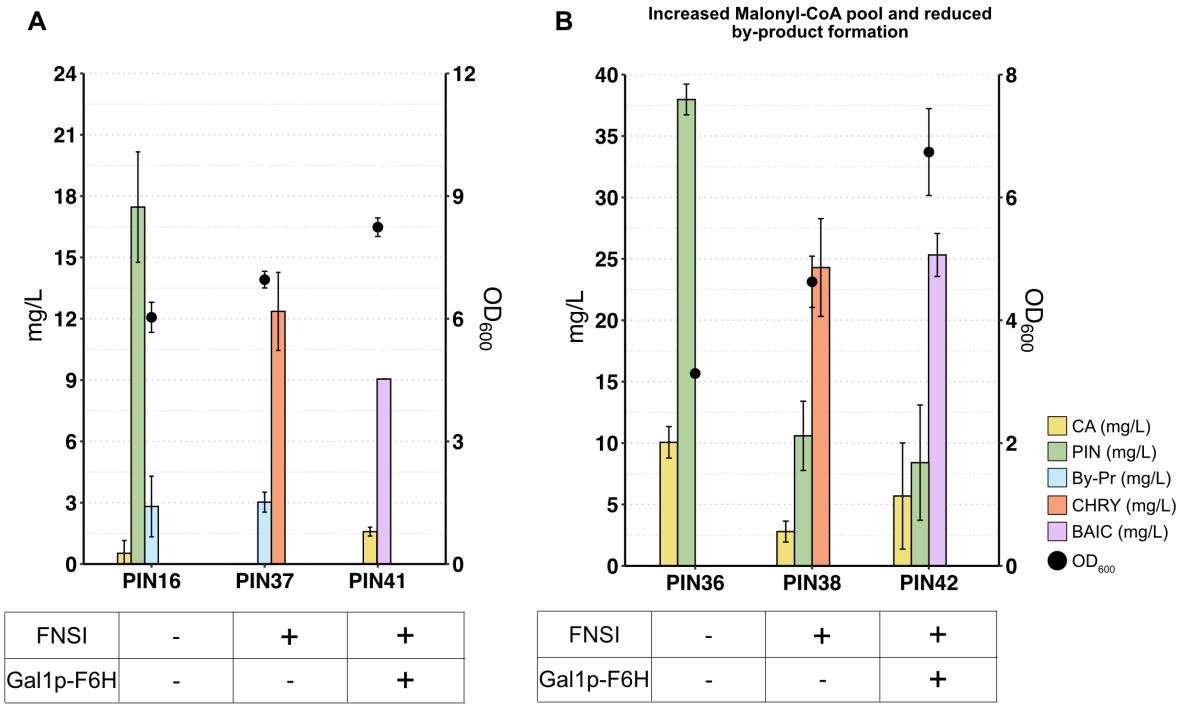


*Figure 6. Pinocembrin, chrysin and baicalein production by engineered strains expressing the genes needed to produce these flavones. (A) Strains expressing the biosynthetic pathway to produce pinocembrin, chrysin and baicalein. (B) strains expressing the optimized (expression of mAcc1** and by-product competing pathway eliminated by substitution of* TSC13 *by MdECR) biosynthetic pathway to produce pinocembrin, chrysin and baicalein. Strains PIN16, PIN37, PIN36 and PIN38 were cultivated in defined minimal media for 72 h at 30 °C with 30 g/L glucose. The strains PIN41 and PIN42 were cultivated in minimal media for 96 h at 30 °C with 10% glucose + 30% galactose as carbon sources. All data represent the mean of at least n=2 biological independent samples and error bars show the standard deviation. CA, cinnamic acid; PIN, pinocembrin; OD_{600}, optical density; By-Pr, by-product (trihydroxy dihydrochalcone 2',4',6'); Chry, chrysin; Baic, baicalein; FNSI, flavone synthase I; F6H, flavonoid-6-hydroxylase.*

The strains PIN41 and PIN42 were tested with 4 different ratios of carbon sources: 1% glucose + 3% galactose, 2% glucose + 2% galactose spiked after 48 h of cultivation, 2% glucose + 2% galactose and 1% glucose + 3% galactose. When analysing the growth and the flavonoid profile of the strains after 96 h of cultivation we found that when cultivating the strain with 3% galactose + 1% glucose the strains presented a larger optical density (OD_{600} always above 6) and the lowest precursor (cinnamic acid and pinocembrin) accumulation. Using these cultivation conditions, the optimized pinocembrin platform strain could produce 25 mg/L of baicalein. However, PIN42 showed some issues despite the high titre of baicalein and the good growth. There was an accumulation of precursors that suggests that the overexpression of the baicalein pathway may be needed to push the production further. Furthermore, to optimize F6H enzyme expression under the control of the *GAL* promoter, the knock-out of the Gal4p repressor, Gal80, can be considered. This approach would induce expression of the *GAL* machinery even in the absence of galactose.

Chapter 1: Take-home message

In this chapter, we established the production of pinocembrin and its derivates in *S. cerevisiae*. Cinnamic acid toxicity is a limiting factor for the production of pinocembrin and its derivatives[114,119]. In Paper I, we found that high pH leads to cinnamic acid accumulation. However, in this study, we did not address the mechanism behind the toxicity of this weak acid. Our strategies focused on directing the flux towards less toxic intermediates. To improve the production titres of pinocembrin and its derivatives it is essential to address cinnamic acid toxicity.

Furthermore, in this chapter we explored the possibility of extending the biosynthetic pathway towards other flavonoids that have more complex structures. We demonstrated that using an inducible promoter to express the P450 enzyme F6H is a good approach to produce baicalein. The platform strain could be used in the future to extend the pathway towards the production of other flavonoids of interest such as norwogonin or baicalin.

Chapter 2 – Alleviating aromatic toxicity by transcriptome profiling

In this Chapter, I address a prevalent challenge encountered when building yeast cell factories: the accumulation of toxic pathway intermediates. Many plant natural products with biotechnological interest such as flavonoids are derivatives of aromatic acids. For example, the flavonoids pinocembrin, naringenin and curcumin are derivatives of cinnamic acid, *p*-coumaric acid and curcumin respectively[98,120]. As shown in Chapter 1 for pinocembrin synthesis, the accumulation of these aromatic acids in *S. cerevisiae* is crucial to ensure successful flavonoid production.

In addition to being precursors of plant natural products, aromatic acids have been studied for their abundance in lignocellulosic hydrolysates. Lignocellulosic hydrolysates are primarily composed of carbohydrates, cellulose, hemicellulose, and lignin, and they are one of the most abundant materials on our planet[121]. Considering the abundance of this material, scientists are investigating the possibility to use it as an alternative to glucose as a carbon source to feed microbial cell factories[122]. The aromatic acid inhibitors, such as cinnamic acid, *p*-coumaric acid and ferulic acid, get released during the pre-treatment of lignocellulosic material required to make the sugars accessible[123]. Thus, to use the lignocellulosic hydrolysates as a carbon source the aromatic compounds should either be removed[124], or the cell factories should be optimized to increase their tolerance.

In this chapter, we investigate the transcriptional changes that *S. cerevisiae* undergoes under aromatic acid stress. We aim to find key regulations that can be used to reverse-engineer yeast to improve aromatic acid tolerance. The study focuses on three aromatic acids that are precursors of three main branches flavonoids and exert different growth inhibition: cinnamic acid (being the most toxic), ferulic acid, and *p*-coumaric acid (being the least toxic).

Determining *S. cerevisiae*'s sensitivity to cinnamic acid

Cinnamic acid (Figure 7A) is an aromatic acid found in the cinnamon spice that is derived from the bark of trees from the *Cinnamomum* genus. Cinnamic acid can be used in flavouring food and drinks, perfumes and detergents, however, the popular cinnamon flavour is given by cinnamyl aldehyde[125,126]. In Paper I we showed that the toxicity of cinnamic acid limited the production of pinocembrin and its derivatives. To comprehend how sensitive *S. cerevisiae* is to cinnamic acid, we monitored cell growth when subjected to an increasing concentration of inhibitor using a Growth Profiler. We observed that a concentration as low as 17.5 mg/L of cinnamic acid would inhibit cell growth in a wild-type laboratory strain (CEN.PK) (Figure 7B).

In Paper II, we aimed to investigate which molecular mechanism is responsible for the aromatic acid toxicity by studying the transcriptome of *S. cerevisiae* when it is exposed to three different aromatic acids: cinnamic acid, ferulic acid and *p*-coumaric acid.

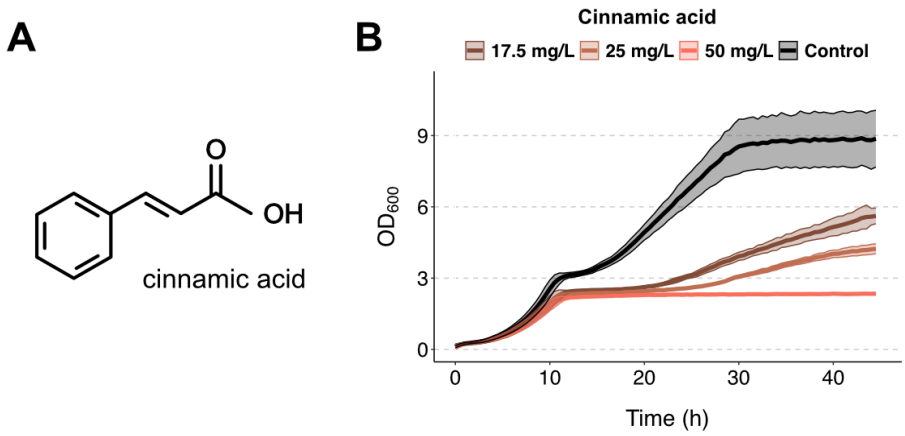


Figure 7. Growth of S. cerevisiae *with supplementation of cinnamic acid. (A) Cinnamic acid structure. (B) Strain IMX851 was grown in Delft minimal media with 17.5 mg/L, 25 mg/L and 50 mg/L of cinnamic acid. The curves and ribbon represent the mean and standard deviation of 3 biological replicates, respectively.*

How can we study aromatic acid toxicity?

Several approaches have been applied in the metabolic engineering field to create robust microbes[127]. In this section, I am going to discuss possible approaches that can be used and explain the method applied in Paper II (Figure 8).

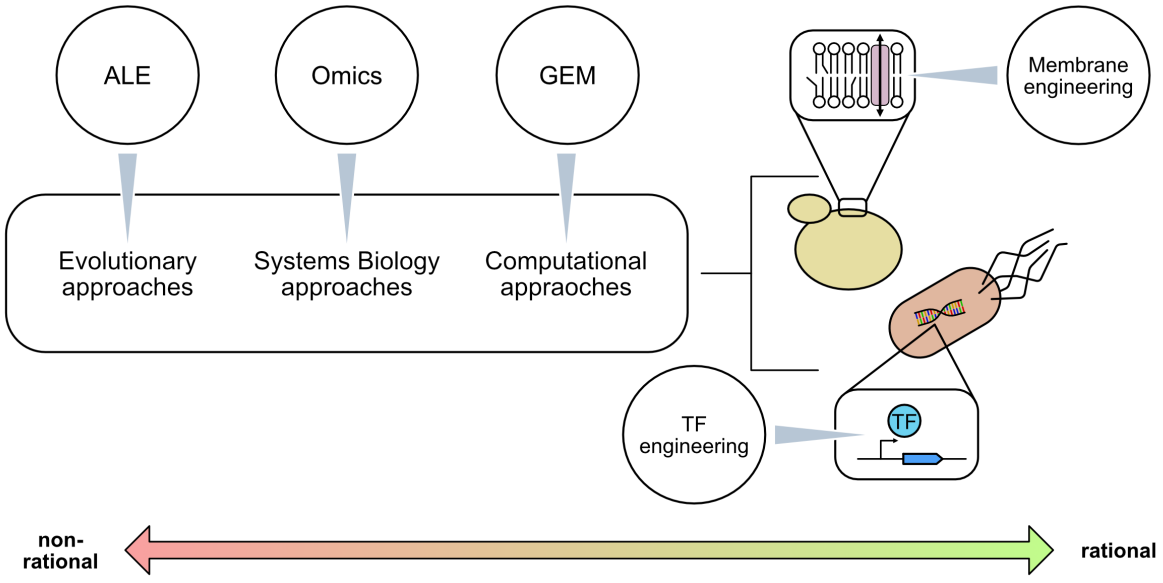


Figure 8. Overview of methods that can be used to increase tolerance and robustness in microorganisms. Adapted from[127]

Non-rational approaches

Non-rational approaches, described also as top-down methods, do not require previous knowledge of the tolerance and toxicity mechanisms. They are usually systems biology, evolutionary or computational methods that aim to understand cell physiology on a theoretical level but usually require strain engineering to improve strain robustness. A popular non-rational approach method that has been used to find strains tolerant to different inhibitors is adaptive laboratory evolution (ALE). ALEs consist of continuous cultivations over several generations under selective pressure, e.g., inhibitor presence[128–130]. ALE has been used to increase the tolerance towards phenolic compounds in *S. cerevisiae,* and it was found

that the upregulation of the aromatic acids transporters relieves the toxicity[131]. Besides ALE, omics-based approaches have also been an essential component of strain engineering. Omics technologies enable researchers to deepen the knowledge of cell physiology at different levels that include mRNA level, protein level and metabolites[132]. Some studies focus on only one level of analysis, e.g., transcriptomics to find gene targets to make *S. cerevisiae* tolerant to acetic acid[133]; while others look into more than one level at the same time, e.g, genomic-proteomic analysis to study ethanol adaptation in *S. cerevisiae*[134].

(Semi-)Rational approaches

Rational approaches, also known as bottom-up methods, are used when there exists previous knowledge of the tolerance mechanisms that are aimed to be improved[127]. An example of a semi-rational approach is membrane engineering. The microbial cell membrane integrity is susceptible to being disrupted by cell stressors as growth inhibitors[135]. Therefore, engineering the membrane to restore membrane homeostasis[136] is a common approach to overcome cell stress. It has been shown that the overexpression of the desaturase Ole1 improved *S. cerevisiae's* tolerance to weak acids by increasing the fluidity and permeability of the membrane[137]. A semi-rational approach employed for improving yeast acid tolerance is transcription factor engineering hence it enables the modification of several gene expressions simultaneously. This approach has been used to increase the tolerance of acetic acid in *S. cerevisiae* by engineering the transcription factor Haa1[138].

Combination of non-rational and rational approaches

Depending on the applications and resources the combination of several methods can also be used. In Paper II, we combine omics technologies to gain knowledge about the transcriptomic response of *S. cerevisiae* to aromatic acids and use transcription factor engineering to obtain a tolerant yeast strain.

RNA sequencing

Why is RNA sequencing valuable? The central dogma of biology establishes that genes (DNA) are transcribed to messenger RNA (mRNA) that can be further translated into proteins that will catalyse many reactions[139]. Sequencing RNA gives an estimation of how much a gene is expressed and to some extent correlates to the protein abundance. Additionally, RNA sequencing allows the identification of genes and pathways that respond to an environmental stress, such as aromatic acid inhibitors[140]. Furthermore, the decreasing cost on transcriptomics makes the method more attractive. Nevertheless, some drawbacks should be considered when using RNAseq. It has been reported that in general the amount of mRNA does not correlate to the amount of proteins in the cell[141]. However, when looking into differentially expressed genes, the correlation between mRNA and protein has been proven to be higher[142].

Sampling and cultivation conditions

For our study, we chose three aromatic acids (cinnamic acid, ferulic acid and *p*-coumaric acid) that exert different toxicity on a wild-type laboratory strain of *S. cerevisiae* (CEN.PK) and determined a concentration of each that inhibited the growth rate to the same level in bioreactors. We did this to minimize the effect that different growth rates would have on the transcriptomic profile. We chose concentrations of 40 mg/L for cinnamic acid, 370 mg/L for ferulic acid and 1000 mg/L of *p*-coumaric acid that inhibit the growth rate around 30% (Paper II, Table 1).

The WT strain was grown in quadruplicates in Dasgip parallel bioreactors (1L) with adequate aromatic acid supplementation for batch and chemostat cultivation. RNA samples were collected in the early exponential phase (OD_{600}=1) and after at least 4 volume changes during the chemostat (Figure 9).

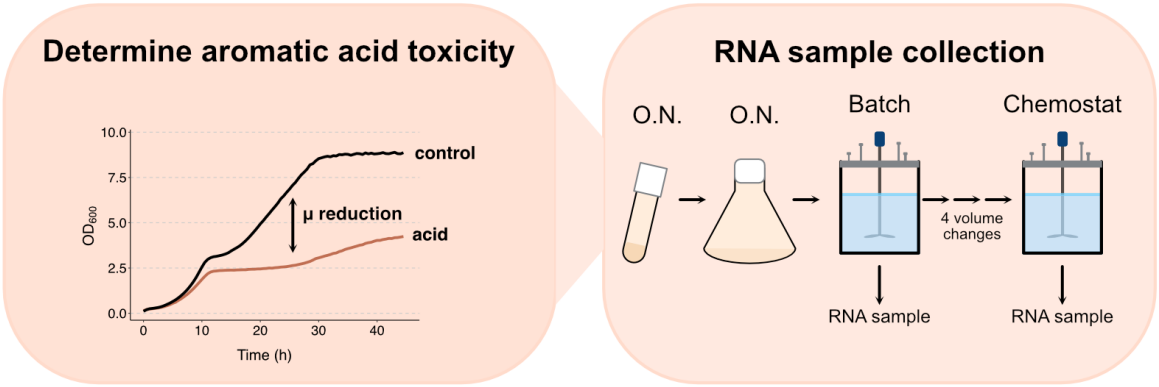


Figure 9. Overview of the pre-screening and sample collection of Paper II. Assessment of the toxicity levels when S. cerevisiae *is grown with cinnamic acid, ferulic acid and* p-coumaric acid *in 1-L bioreactors. Batch and chemostat cultivations were performed and RNA samples were taken as indicated in the second panel. Figure adapted from Paper II.*

How to find targets to reverse engineer *S. cerevisiae*?

To ensure data integrity we removed low-count genes and normalized gene counts between samples with trimmed mean of M values (TMM) method. Thereafter we performed a multidimensional scaling plot of distances (MDS) analysis where we could see how the different samples separate across conditions (Figure 2, Paper II). With this analysis, we saw that the replicates from the same conditions cluster together in all the evaluated samples (from both batch and chemostat). The replicates clustering served as a quality check.

Then we screened for the genes that were differentially expressed (DE)[VIII]. We hypothesised that for improving the tolerance towards cinnamic acid (the more toxic aromatic acid of this study) we could take two approaches:

(1) Genes that were up- or downregulated on exclusively ferulic acid and *p*-coumaric acid conditions could be interesting candidates to test for increasing cinnamic acid tolerance.

(2) Genes that were up- or downregulated only in cinnamic acid conditions could be overexpressed or deleted to increase the natural response of *S. cerevisiae* towards this acid.

[VIII] We filtered for genes with non-directional log fold change (logFC) bigger than 1 and an adjusted *p*-value smaller than 0.01.

Therefore, we focused on the genes that were significantly DE in both *p*-coumaric acid and ferulic acid and the genes that were only differentially expressed in cinnamic acid. To see the distribution, we used Euler diagrams that showed that there was a large percentage of shared DE genes between *p*-coumaric acid and ferulic acid whereas cinnamic acid shared a smaller percentage of DE genes with the other acids (Figure 3, Paper II). The DE gene analysis provided a very long list of targets (336 genes were found to be DE in batch and 893 were found to be DE in chemostat). Even if we would only look into the pool of genes shared between ferulic acid and *p*-coumaric acid, we would still have 247 genes to reverse engineer. This big number was too ambitious for our project, and therefore we sought another strategy to find targets to reverse engineer *S. cerevisiae.*

To narrow down the target list we analysed the gene sets that transcription factors regulate. We selected 16 transcription factors that belong to one of the following four categories:

(1) TFs whose gene sets were significant upregulated for ferulic acid and *p*-coumaric acid compared to cinnamic acid.

(2) TFs whose gene sets were significant downregulated for ferulic acid and *p*-coumaric acid compared to cinnamic acid.

(3) TFs whose gene sets were significant upregulated for cinnamic acid compared to ferulic acid and *p*-coumaric acid.

(4) TFs whose gene sets were significant downregulated for cinnamic acid compared to ferulic acid and *p*-coumaric acid.

Transcription factor engineering

S. cerevisiae has 169 known transcription factors (TFs) making them the most abundant protein cluster[143,IX]. TFs recognize unique sequence-specific DNA elements and can act as activators or repressors. They are likely required to activate the transcription of all protein-coding genes transcribed by RNA polymerase II[143].

Therefore, by choosing to engineer TFs we aimed to alter the expression of gene sets that they control. TF engineering has been applied before when increasing yeast tolerance towards acetic acid[138]. We believe that this can be a fruitful approach because aromatic acid toxicity is likely a polygenic trait. Consequently, the overexpression or deletion of a single gene won't confer resistance to aromatic acids but rather the change in a gene set expression.

In Paper II, we constructed knock-out (KO) and overexpression (OE) strains of the 16 TF that were selected from the transcriptomics analysis. We constructed both KO and OE because it could be that (1) the upregulation of the transcription factor and its gene set would push the cell to deal with the toxicity or (2) the downregulation of the TF and its gene set would realise the stress response and the cell would increase the survival rate. Moreover, it would act as a control because if a positive phenotype would be observed with one of the two strategies, we would expect to see an opposite phenotype using the antagonistic strategy.

[IX] The transcription factors can be found at the Yeastract database[144].

Characterization of engineered mutants

Many parameters can be examined when assessing the fitness of reverse-engineered strains. An easy parameter is to compare the final biomass of the engineered strains when cultivated with the acids with the final biomass of the wild type when cultivated with the acids. If an instrument to monitor the growth is available, growth-curve related parameters could be examined such as the reduction of the lag phase and/or the maximum growth rate. The maximum growth rate is a parameter that can be assessed at different stages. It is commonly calculated during the exponential growth of the microorganism. However, *S. cerevisiae* presents a diauxic growth. During batch cultivations (when there is excess of glucose and oxygen) *S. cerevisiae* prefers to do aerobic fermentation over respiration (a phenomena called Crabtree effect), this phase is named glucose phase. The second growth phase starts when glucose is depleted, and yeast aerobically consumes ethanol (phase commonly called ethanol phase). Altogether, the growth curve of a wild-type *S. cerevisiae* is divided into different stages: the lag phase, the exponential phase (split into glucose phase and ethanol phase) and the stationary phase (Figure 10).

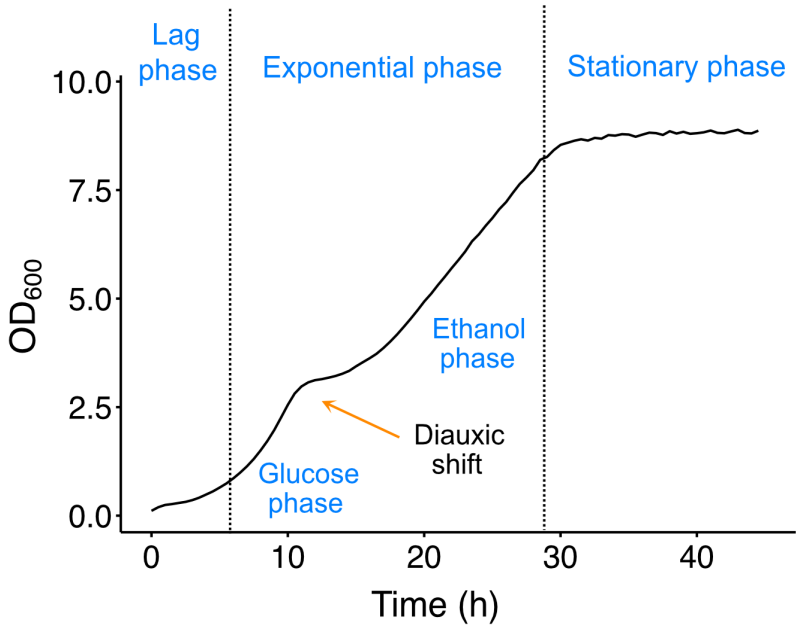


Figure 10. Growth curve of the wild-type S. cerevisiae *laboratory strain CEN.PK indicating the different growth phases.*

Thus, the growth rate can be calculated in different ways: (i) the average growth rate of the whole exponential phase including both the glucose and the ethanol phase. (ii) The maximum growth rate of the glucose phase or the average growth rate of the glucose phase. (III) The maximum growth rate of the ethanol phase or the average growth rate of the ethanol phase. To assess if the reverse-engineered strains build in Paper II showed an improved phenotype when they were cultivated with aromatic acids, we compared the maximum growth rate in the glucose phase and the ethanol phase of the mutants cultivated in the presence of cinnamic acid, *p*-coumaric acid and ferulic acid against the wildtype cultivated in the presence of the acids.

Yeast cells show different behaviour when the cultivation conditions are changed[X]. Previously, we chose acid concentrations that inhibited the growth of *S. cerevisiae* when it was cultivated in bioreactors. To test the mutant's growth, we changed the cultivation method to one that enabled the high-throughput screening of 32 strains in four different media, 96-well plates. To determine the concentration of cinnamic acid, ferulic acid and *p*-coumaric acid adequate for these cultivations chose an acid concentration that had an influence on growth but that did not inhibit completely the ethanol phase of the wild-type. We cultivated the wild-type strain with different acid concentrations. We chose 17.5 mg/L of cinnamic acid, that represents a reduction of the growth rate of 40%. For ferulic acid, we chose 100 mg/L which inhibits growth by 44%. For *p*-coumaric acid stress, we observed that the ethanol phase was shorter, but the growth rate was almost not affected. Consequently, we increased the acid concentration to 600 mg/L which corresponds to 22% growth rate inhibition (Figure 4, Paper II).

The engineered strains were first cultivated in 96-well plates using a Growth Profiler at 30 °C at 200 rpms. The Growth Profiler takes pictures that can be converted to OD_{600} readings every 30 min. We could calculate the growth rate of all the strains during the glucose phase and the ethanol phase. When examining the maximum growth rates during the glucose phase we found that the wildtype's growth rate was the same when the medium was supplemented with the acids than when there was no acid supplementation. Similarly, the mutants' glucose phase growth rate was very close to the wild-type growth rate in the four tested conditions.

In contrast, when examining the growth rates during the ethanol phase, we could see clear differences. Four mutants (Δ*ixr1*, OE *YRR1*, OE *SKN7*, OE *GCN4*) improved the growth rate when the medium was supplemented with ferulic acid compared to the wild type. Two additional mutants (OE *GCR1* and OE *INO2*) presented a shorter lag phase after the diauxic shift when the medium was supplemented with cinnamic acid (Figure 5, Paper II). The six engineered strains that presented an improved phenotype when compared to the wild type were cultivated in shake flasks. Similarly, as done in 96-well plates, the growth was monitored, and the growth rates were calculated. The growth rates were very similar to the ones calculated using the Growth Profiler, confirming that these six modifications can improve the tolerance towards cinnamic and ferulic acid (Table 4).

[X] Surprisingly, even a different material of the cultivation vessel can cause fitness variations[145]

Table 4. The growth rates in the ethanol phase of all the mutant strains cultivated in shack flasks with 25 mg/L of cinnamic acid or 100 mg/L of ferulic acid. The displayed data is the mean of at least 3 biological replicates ± the standard deviation. The improvement percentage is calculated by dividing the WT (IMX585) μ by the mutant μ.

Strain	**CA μ (h^{-1})**	**FA μ (h^{-1})**	**% improvement**	***p*-value**
WT	0.023 ± 0.001	0.013 ± 0.001		
Δ *ixr1*		0.019 ± 0.001	32%	0.0068
OE *YRR1*		0.019 ± 0.002	32%	0.0076
OE *SKN7*		0.030 ± 0.000	57%	0.0006
OE *GCN4*		0.015 ± 0.001	13%	0.2970
OE *GCR1*	0.021 ± 0.002		0%	0.1635
OE *INO2*	0.023 ± 0.003		0%	0.8244

Transcription factor engineering improves aromatic acid tolerance

Increasing amino acid synthesis might mitigate aromatic acid toxicity

In *S. cerevisiae,* the amino acid regulatory network is called GAAC and stands for "general amino acid control". GAAC stimulates the transcriptions of more than 70 amino acid biosynthetic genes. Different factors regulate GAAC in yeast but the activation of GAAC is associated with the synthesis of Gcn4p[146]. GCN4p's main function is to activate the transcription of an array of genes involved in the biosynthesis of amino acids. Gcn4 machinery is triggered when there are amino acids starvation or stress conditions that may cause amino acid imbalance[147]. The overexpression of Gcn4p improved the growth of *S. cerevisiae* under ferulic acid stress. This could indicate that under aromatic acid stress yeast may have amino acid deficiency that effect cell growth. Therefore, the overexpression of *GCN4* can be beneficial to restore amino acid levels.

To test the hypothesis, growth tests with supplementation of single amino acids could be done to determine which one is affected, and then overexpress the production of the deficient amino acids or supplement them.

Overexpression of aromatic acid transporters generates tolerant strains

Aromatic acids in a form of acid (HA) can diffuse through the plasma membrane and enter the cytosol where they dissociate (A^- + H^+) due to a higher intracellular pH[148]. The weak acid dissociation causes a decrease in intracellular pH (due to the realise of protons, H^+) that stresses the cell and can comprise cell viability. In our study, we found that the overexpression of Yrr1p, a transcription factor that regulates the *PDR* genes[149] that encode ABC transporters improved the maximum growth rate of *S. cerevisiae* when it was cultivated with ferulic acid. This suggests that the overexpression of Yrr1p would increase the transport of aromatic acids from the cytosol to the media, alleviating the toxicity. This was also observed in another study where the toxicity of *p*-coumaric acid and ferulic acid was investigated by performing an evolution experiment (ALE)[131]. When the authors analysed the mutations of the evolved strains after the ALE, they found that the up regulation of the transporter Esbp6 relieved the toxicity of the strains grown with these compounds.

Despite improving the growth of the strains this approach may not be optimal for the purpose of our study. To produce downstream flavonoids, keeping the aromatic acids inside the cell is preferred so they can be utilized as precursors.

Aromatic acids may trigger oxidative stress in yeast

The overexpression of Skn7p, a TF that activates the expression of antioxidant genes in *S. cerevisiae*[150] was found to mitigate ferulic acid toxicity in yeast. It was previously described that oxidative stress could be caused by the presence of weak acids[151], such as the ones investigated in our study. Therefore, the overexpression of Skn7p is likely to help reduce ROS triggered by aromatic acids. To further investigate this hypothesis, an experiment to measure the intracellular oxidative stress could be performed. Then we could check if the mutant that has *SKN7* overexpressed presents reduced oxidative stress compared to the wild type when cultivated with aromatic acids.

Endoplasmic reticulum (ER) membrane expansion can mitigate aromatic acid toxicity

The approach of expanding the ER membrane has become very common when designing yeast cell factories that require the expression of pathways that include P450 proteins, e.g., for terpene synthesis[152]. One of the most efficient methods to expand the ER membrane and reduce ER stress in *S. cerevisiae* is the overexpression of *INO2*[153].

We found that the overexpression of this transcription factor could improve the growth under cinnamic acid stress. This result suggests that aromatic acids may cause ER stress that could be regulated by expanding the membrane of this organelle. To test our hypothesis further experiments measuring ER stress could be done.

Chapter 2: Take-home message

In Chapter 2 we explored the transcriptome of *S. cerevisiae* when exposed to different aromatic acids. We found that omics analysis combined with transcription factor engineering is a good combination to find targets to make a yeast strain tolerant towards a growth inhibitor. Our results show that the OE or KO of six promising TF targets could improve the growth of the wild-type CEN.PK when cultivated with certain aromatic acids.

Finally, the TFs that were found to improve growth in Paper II could be incorporated into the platform strains of Paper I to see if they increase the robustness of the pinocembrin platform strains.

Chapter 3 – Optimizing naringenin platform in *S. cerevisiae*

Naringenin, along with pinocembrin, serves as one of the most important scaffolds for synthesizing a diverse range of flavonoids. Naringenin has been studied for its antimicrobial properties and its ability to cross the blood-brain barrier[154]. Some of its derivatives, which belong to the flavonols subclass, will be further discussed later in the thesis.

How can the naringenin production in yeast be optimized?

The naringenin formation pathway is similar to pinocembrin formation (Figure 11)[98]. It is also a derivative of L-phenylalanine. The main precursor of the pathway is *p*-coumaric acid, which is formed from L-phenylalanine by a phenylalanine ammonia lyase (PAL) combined with a cinnamic acid hydroxylase (4CH). Alternatively, it can be produced from L-tyrosine by a tyrosine ammonia lyase (TAL). From here, *p*-coumaric acid is transformed to naringenin by a three-step reaction that needs a coumaroyl-CoA ligase (4CL), a chalcone synthase (CHS) and a chalcone isomerase (CHI). As in the pinocembrin formation pathway, the CHS enzyme requires three molecules of malonyl-CoA.

The naringenin pathway has been previously reconstituted in different hosts. This includes bacterial hosts such as *E. coli*[155,156] or *Streptomyces venezuelae*[157] and eukaryotic hosts like *Yarrowia lipolytica*[158,159] and *S. cerevisiae*[160–163]. Some reports have focused on optimizing the pathway from *p*-coumaric acid to naringenin applying modular engineering[164] and tweaking gene expression, others on regulating the precursor malonyl-CoA supply using a dynamic control and biosensor system[165].

When producing flavonoids in microbial hosts, there is a challenge that is often overlooked. Plant enzymes involved in flavonoid biosynthesis are sometimes bound in specialized plant organelles, such as chloroplasts, which do not exist in yeast cells. Additionally, in plants, pathway intermediates may accumulate in specific subcellular locations, necessitating the presence of active transporters to facilitate their movement to the next enzyme in the pathway[166]. Furthermore, the cellular environment of yeast differs significantly from that of plants since yeast is a unicellular compartmentalized organism, whereas plants have specific organelles for specified functions. These differences in cellular organization and enzyme localization between plants and yeast must be taken into consideration to effectively produce flavonoids in microbial hosts.

In this study, we looked into the distribution of *p*-coumaric acid and naringenin throughout the fermentation process and found that 90% of *p*-coumaric acid is secreted outside of the cell. The naringenin distribution did not follow the same pattern since it was secreted during the first 24h and then accumulated intracellularly (Figure 11). Paper III aimed to optimize the production of naringenin by controlling the pathway intermediates secretion and accumulation.

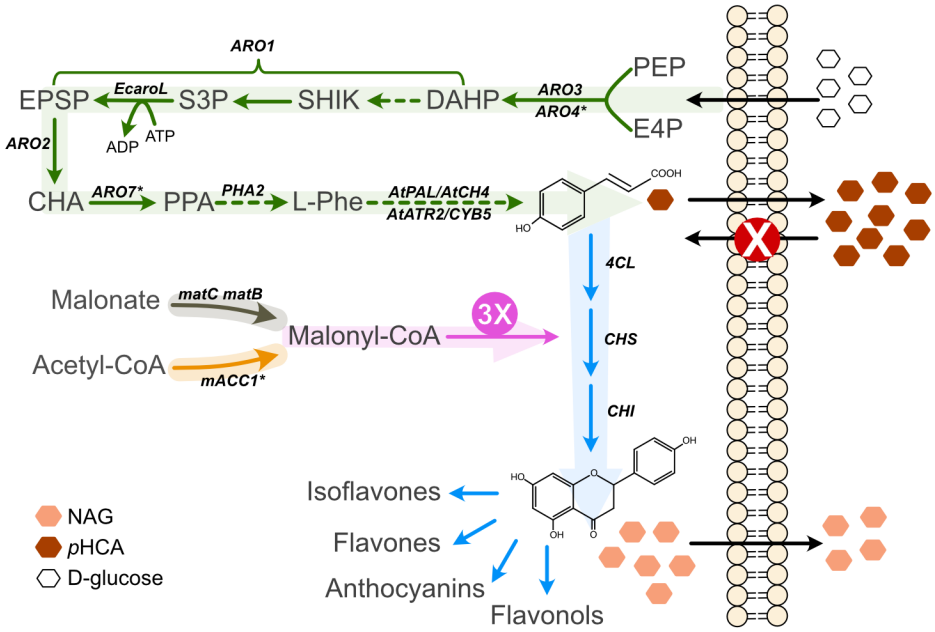


Figure 11. Schematic diagram of the biosynthetic pathway of (2S)-naringenin in S. cerevisiae*. Solid lines represent a single step; dotted lines indicate multiple steps; the blue arrows represent the core (2S)-naringenin biosynthetic pathway and a variety of plant-specific flavonoids derived from (2S) naringenin. ARO3, DAHP synthase; ARO4*, L-tyrosine-feedback-insensitive DAHP synthase (ARO4K229L); ARO1, pentafunctional aromatic protein; EcAroL, shikimate kinase from* E. coli*; ARO2, chorismate synthase; ARO7*, L-tyrosine-feedback insensitive chorismate mutase (ARO7G141S); AtPAL2, phenylalanine ammonia lyase, AtC4H, cinnamic acid hydroxylase and AtATR2, cytochrome P450 reductase, from* Arabidopsis thaliana*; CYB5, yeast native cytochrome b5; 4CL, p-coumaroyl-CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase. E4P, erythrose-4-phosphate; PEP, phosphoenolpyruvate; DAHP, 3-deoxy-D-arabino-2-heptulosonic acid 7-phosphate; SHIK, shikimate; S3P, shikimate-3-phosphate; EPSP, 5-enolpyruvyl-shikimate-3-phosphate; CHA, chorismic acid; PPA, prephenate; L-Phe, L-phenylalanine; pHCA,* p*-coumaric acid; NAG, (2S)-naringenin. Figure adapted from Paper III.*

Approaches for pathway optimization

Naringenin has been produced by many hosts, however, the existing platform strains can be further optimized. Here I will focus on the approaches applied in Paper III to improve the production of this flavonoid in *S. cerevisiae*.

Alleviating the bottleneck downstream of *p*-coumaric acid improves naringenin production

As we did in Chapter I when the production of pinocembrin was established, we used a background strain that had the aromatic acid pathway flux increased. Likewise, the background strain used in this chapter has feedback-resistant variants of Aro4 and Aro7, additional copies of *ARO1/2/3* and expression of EcaroL from *E. coli*[43]. When we expressed in this background the best combination of 4CL, CHS and CHI enzymes screened (Figure 1C, Paper III) we obtained 68 mg/L of naringenin and an accumulation of over 200 mg/L of *p*-coumaric acid (strain NAG10).

We monitored the growth and the location of *p*-coumaric acid and naringenin of the strain NAG10 over 4 cultivation days. The results showed that over 90% of *p*-coumaric acid was found in the supernatant. However, naringenin was located in the supernatant during the first 24 h of the cultivation but afterwards, it accumulated inside the cell (Figure 12).

As a first approach, we checked if the level of expression of the enzymes downstream of *p*-coumaric acid limited the production of naringenin. Variant copies of the genes encoding the enzymes 4CL-CHS and CHI were expressed. We determined that when the gene copy number

was increased to 3:4:4 respectively, there was a 3-fold improvement in naringenin production when compared to the parental strain NAG10. That meant that the resulting strain, NAG3-4, produced over 209 mg/L of naringenin accumulating over 110 mg/L *p*-coumaric acid.

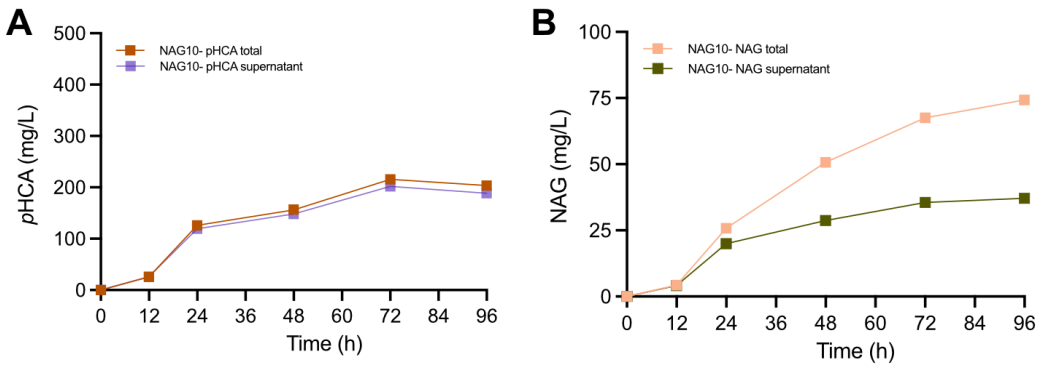


Figure 12. Total and extracellular content of (A) p-coumaric acid *(pHCA) and (B) naringenin (NAG) in NAG10. The cells were cultivated in a minimal medium with 30 g/L of glucose as the sole carbon source. 6 samples were taken during the cultivation and the products of interest were quantified. All data represent the mean of n=3 biological independent samples. Figure adapted from Paper III.*

Increased malonyl-CoA availability improves naringenin production

Comparably to the approach used in Chapter 1, we increased the pool of malonyl-CoA using two methods: (1) by overexpressing the de-regulated acetyl-CoA carboxylase mACC1*[105] and (2) by expressing the malonate assimilation genes from *Rhizobium trifolii*, *matB* and *matC*[104]. Additionally, we build a strain combining both approaches. These three new strains were built using the NAG10 and NAG3-4 background, respectively. For simplification in this Chapter, I am going to only focus on the NAG3-4 derived strains that were named NAG37, NAG38 and NAG39. When examining the production profile under batch fermentation of the three resultant strains, we saw that the production of naringenin did not increase using this strategy (Figure 3B, Paper III). However, the accumulation of *p*-coumaric acid increased indicating that there may be an imbalance of both precursors *p*-coumaric acid and malonyl-CoA. To be certain that the strategies were increasing the pool of malonyl-CoA we used a malonyl-CoA biosensor to quantify its supply in the strains NAG37, NAG38 and NAG39[106]. The results show that the strain NAG39 which has a combination of mAcc1* and matBC showed a significant increase of malonyl-CoA when compared to NAG3-4, NAG37 and NAG38 (Supplementary Figure 4, Paper III).

Furthermore, we tested other cultivation strategies to assess if the production would improve. We cultivated the strains NAG3-4, NAG37, NAG38 and NAG39 in fed-batch-like conditions using slow-release glucose tablets. After 96h of cultivation, we sampled and quantified the concentration of *p*-coumaric acid and naringenin in the cultures. The results showed that in all strains that had an increased pool of malonyl-CoA, the naringenin titre increased (Figure 3D, Paper III). Moreover, strain NAG39 showed the highest increase in production reaching 450 mg/L. Under fed-batch conditions, the yeast cells don't present an overflow metabolism since the carbon availability is limited. Therefore, the cells' metabolism is different than in

normal batch fermentation when there is an excess of glucose and there is a production of by-products such as glycerol and ethanol.
We demonstrated that malonyl-CoA is a limiting precursor for the pathway and that carbon-limited fermentation was favourable to reach high naringenin titres. Nevertheless, the applied strategies did not address the accumulation of extracellular *p*-coumaric acid.

Controlling *p*-coumaric acid synthesis alleviates its accumulation

We employed the FapR-*fapO* system from *Bacillus subtilis*[167] to control the production of *p*-coumaric acid depending on the abundance of malonyl-CoA. In parallel, we tuned the expression of the fatty acid synthase Fas1 using an RNA interference (RNAi) strategy to regulate the malonyl-CoA used for fatty acid synthesis.
The system is activated when there are high concentrations of malonyl-CoA, two parallel mechanisms trigger:
(1) Malonyl-CoA binds to the FapR repressor that releases from the AtPAL promoter activating *p*-coumaric acid production.
(2) Malonyl-CoA binds to the FapR repressor that releases from the promoter controlling the expression of an antisense hairpin RNA mRNA. The small hairpin RNA binds to the mRNA of *FAS1*. *FAS1* translation is inhibited and consequently the synthesis of fatty acids. This approach switches off a major competing pathway for naringenin formation.
In contrast, when the levels of malonyl-CoA are low, FapR inhibits the transcription of AtPAL and the production of the small hairpin RNA, prioritizing fatty acid production.
To implement the system, we first looked into the replacement of the promoter of AtPAL in the background NAG39 with modified promoters that contained the *fapO* site, a sequence of 34 bp. From the four modified promoters tested in Paper III (Figure 4, Paper III), the modified *TDH3* promoter showed the best result since the strain NAG71 could decrease the accumulation of *p*-coumaric acid to 16 mg/L.
Once we demonstrate that the system worked for reducing the accumulation of *p*-coumaric acid, we establish the second part, the tuning of the fatty acid synthase. For that purpose, we used the NAG71 strain as background. The fatty acid synthase complex has two main units Fas1 and Fas2. *FAS2* is regulated by the product of *FAS1*[168]. Therefore, we designed an RNAi system to control only the *FAS1* subunit. To reconstitute the system in NAG71, we constructed and expressed a cassette that consisted of the genes necessary to establish RNAi in *S. cerevisiae* (we tried two *AGO1* and *DCR1*)[169] and a sense-antisense DNA strand of *FAS1* separated by a hairpin loop. We used the *TDH3* promoter with the FapR repressor used before to control AtPAL expression as a promoter to regulate the synthesis of the small hairpin RNA based on malonyl-CoA levels. The resulting strains NAG75 and NAG76, that present 200 bp and 250 bp hairpin length respectively, produced 500 mg/L and 514 mg/L of naringenin (Figure 4D, Paper III). These results suggest that the RNAi system was functional and can be used to improve naringenin production in yeast.

Transporter engineering improves the distribution of precursors and products in the naringenin pathway

To address the challenge of the *p*-coumaric acid and naringenin distribution in *S. cerevisiae* we investigated the effect of some transporters on the distribution of these compounds. The aim was to reduce the export of *p*-coumaric acid from the cytosol to the medium and to increase the export of naringenin from the cytosol to the medium. Several targets were tested in Paper III, however, here, I am going to focus on two transporters that were found to be responsible for increasing the tolerance of aromatic acids in *S. cerevisiae*: Esbp6 and Pdr12[131]. Both transporters were overexpressed in a wild-type strain that had expressed only At4CL, RsCHS and PsCHI (IMX581N4). This strain required external feeding of *p*-coumaric acid to produce naringenin. The flavonoid profile of IMX581N4 overexpressing *ESBP6* and *PDR12* independently was analysed after 96 h cultivation with 300 mg/L *p*-coumaric acid supplementation. We found that IMX581N4 + ESBP6 reduced the accumulation of *p*-coumaric acid but the production of naringenin did not increase compared to IMX581N4. When looking into IMX581N4 + PDR12 was found that there was no accumulation of *p*-coumaric acid and the naringenin titre increased 1.6-fold compared to IMX581N4 (Figure 13).

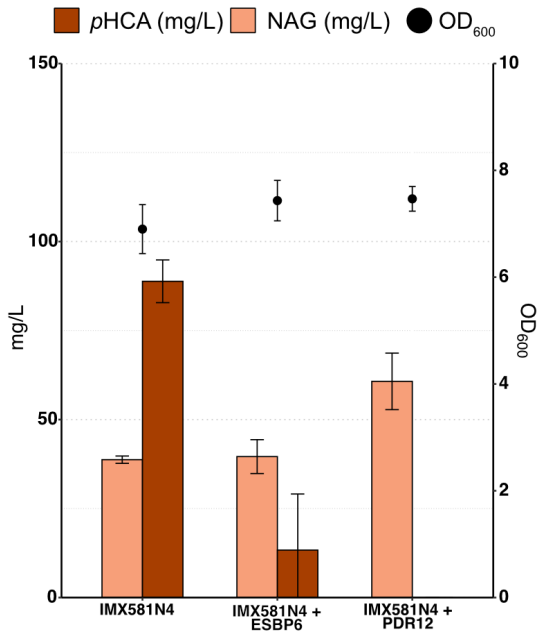


Figure 13. Evaluation of potential transporters to regulate the accumulation of p-coumaric acid and naringenin. Uptake of p-*coumaric acid by overexpressing target transporter genes in IM581N4 background strains. Cells were grown in a defined minimal medium with 30 g/L glucose as the sole carbon source and supplemented with* p-*coumaric acid (300 mg/L) as a precursor. Cultures were sampled after 96 h of cultivation for total p-coumaric acid and (2S)-naringenin detection. All data represent the mean of n = 3 biologically independent samples and error bars show standard deviation. Figure adapted from Paper III.*

The data in Figure 13 suggest that the Pdr12 transporter can transport the extracellular *p*-coumaric acid that is externally fed. From this data, it is unclear if the transporter can also move the produced naringenin outside of the cell. We overexpressed *PDR12* in the NAG39 background strain that has a high flux of the downstream pathway of *p*-coumaric acid. The resulting strain, PIN97, was cultivated under fed batch-like conditions and the naringenin and

p-coumaric acid concentrations were measured (Figure 14). We observed that the *p*-coumaric acid concentration was not significantly decreased whereas the naringenin production was increased from 435 to 460 mg/L. To understand the role of the Pdr12 transporter concerning naringenin we checked the distribution of this compound in the strain NAG39 and NAG97 under batch fermentation (Supplementary Figure 14, Paper III). We found that the naringenin concentration detected extracellularly was 5% higher in the strain that has the transporter overexpressed. This suggests that the Pdr12 transporter may be involved in the transport of the newly produced naringenin outside the cell. A more efficient naringenin transport could pull the flux towards the *p*-coumaric acid downstream pathway reducing the accumulation of *p*-coumaric acid.

Finally, we overexpressed the Pdr12 transporter in the dual dynamic control strain NAG76. We found that the resulting strain, NAG99, improves 9.5% the titre of naringenin under fed batch-like cultivation (Figure 14). Even though the titres of naringenin increase with the overexpression, the role of Pdr12 in the transport of naringenin and *p*-coumaric acid is not clear. Therefore, more investigation should be done to understand the flavonoid transport in *S. cerevisiae*.

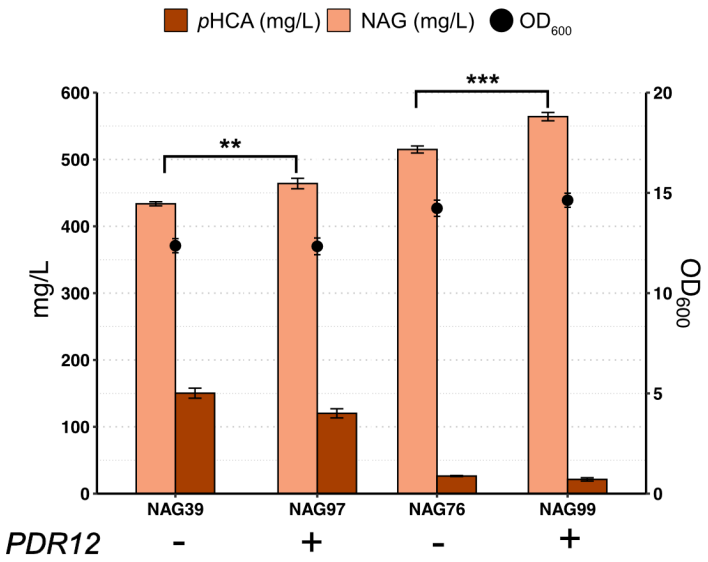


Figure 14. Effect of Pdr12 transporter overexpression on p*-coumaric acid accumulation and (2S)-naringenin production in fed-batch-like conditions. Cells were grown in a defined minimal medium with six tablets of FeedBeads as the sole carbon source and with 2 g/L sodium malonate dibasic supplementation. Cultures were sampled after 96 h of cultivation for total p-coumaric acid and (2S)-naringenin detection. Statistical analysis was carried out by using Student's t-test (two-tailed; two-sample unequal variance; *P < 0.05, **P < 0.01, ***P < 0.001). All data represent the mean of n = 3 biologically independent samples and error bars show standard deviation. Figure adapted from Paper III.*

Testing the scalability of naringenin platform strains

To assess if the production of strains that have incorporated the dynamic control system and the transporter Pdr12p (NAG76 and NAG99) can be scaled up, we cultivated them in 1-L bioreactors under glucose-limited fed-batch conditions. The strain NAG39 that does not have the last modification was also included as a control. In the three tested strains the biomass

increased until around 60 h of cultivation where it plateaued. In strain NAG39, there was an accumulation of *p*-coumaric acid for most of the cultivation time. In contrast, both NAG76 and NAG99 do not present any *p*-coumaric acid accumulation over the cultivation. This confirms again that the dynamic control established in those strains can balance well the precursors supply. The total naringenin was gradually increased along the whole fermentation for strains NAG76 and NAG99 until reaching the highest titre of 1.77 g/L and 2.05 g/L, respectively (Figure 6, Paper III).

Chapter 3: Take-home message

In Chapter 3, we optimized the production of the flavonoid naringenin in *S. cerevisiae*. The extracellular accumulation of the precursor *p*-coumaric acid is a main pathway limitation that hampers production. We established a dual dynamic control that (1) reduces the accumulation of *p*-coumaric acid by controlling its synthesis and (2) improves the naringenin production by downregulating pathway competing for malonyl-CoA. Furthermore, some transporters were tested to assess their capability to export naringenin outside the cell and bring *p*-coumaric acid inside. The optimized strain developed in Paper III (NAG99), could produce over 563 mg/L of naringenin in a fed batch-like shake flask fermentation. However, some other approaches could be considered to further improve the production of naringenin. In Paper III we only considered the utilization of malonyl-CoA as fatty acid building blocks as a competing pathway. Similarly to the pinocembrin biosynthetic pathway, coumaroyl-CoA can be converted to dihydrocoumaroyl-CoA by the *S. cerevisiae* essential native enzyme Tsc13. Dihydrocoumaroyl-CoA is converted to phloretin by the heterologous enzyme CHS and three molecules of malonyl-CoA. Therefore, the substitution of *TSC13* for a homologous gene could help to channel the carbon towards the formation of naringenin.

Chapter 4 – Producing hydroxylated downstream products

In this chapter, the naringenin platform strain is used as a chassis to synthesize other hydroxylated products. In Chapter 1, the pinocembrin platform strain was used as a chassis to produce chrysin or baicalein, but the precursor flux was not as high as for naringenin. Different moieties can be added to flavonoid backbones. For example, hydroxylases from different plant origins have been characterized[109] and tested to successfully produce different products in *S. cerevisiae*. Some of these enzymes have been used in my research and are described in this thesis such as flavone synthase I and II (FNSI and FNSII) from *Petroselinum crispum* and *S. baicalensis* and flavanone 3-hydroxylase (F3H) from *A. thaliana*. The addition of a sugar molecule to a flavonoid backbone, glycosylation, is a common decoration. Flavonoid glycosides are attractive for their water solubility that allows them to spend more time in the human intestine and have better absorption[170]. O-methylations are widely distributed in flavonoids and can happen at different positions of the flavonoid backbone. Methylated flavonoids are suggested to show strong anticancer properties[171]. On the other hand, other modifications have been proven to be more challenging. An example is the expression of plant prenyltransferases that add prenyl groups to (iso)flavonoids[172]. Since these enzymes are usually expressed in plastids that are not present in yeast, they often show very low product conversion when expressed in yeast[173].

In Chapter 4, I focus on the production of hydroxylated derivatives using the naringenin platform strain build in Paper III as a chassis that presents a high flux of precursor and robust growth.

Kaempferol and quercetin

Kaempferol, a flavonoid derivative of naringenin, has been studied for its many bioactivities that are beneficial for human health[174]. Kaempferol belongs to the subgroup of flavonols and is abundant in broccoli, apples, tea, strawberries, and beans. It is studied for its potential as an anticancerogenic molecule. Specifically, it inhibits cancer cell proliferation and promotes cancer cell apoptosis. Additionally, it is less toxic than most standard chemotherapy drugs that are currently in use.

Quercetin is a derivative of kaempferol and has been found to be abundant in the same aliments as kaempferol and especially abundant in berries and red wine. Quercetin has been studied for many bioactivities as many molecules of the same family[175]. Here, I want to highlight quercetin's role in preventing bacterial and viral growth[176]. It inhibits virus replication, e.g., quercetin has been demonstrated to be effective against adenovirus, herpes simplex virus or Japanese encephalitis virus.

To convert naringenin to kaempferol, two consecutive enzymes are needed. The first is a flavanone 3-hydroxylase (F3H) that converts naringenin to the intermediate dihydrokaempferol and secondly a flavonol synthase (FLS) that transforms the intermediate to kaempferol. Finally, to obtain quercetin two enzymes are required, a flavonoid monooxygenase (FMO) together with a cytochrome P450 reductase (CPR) (Figure 15).

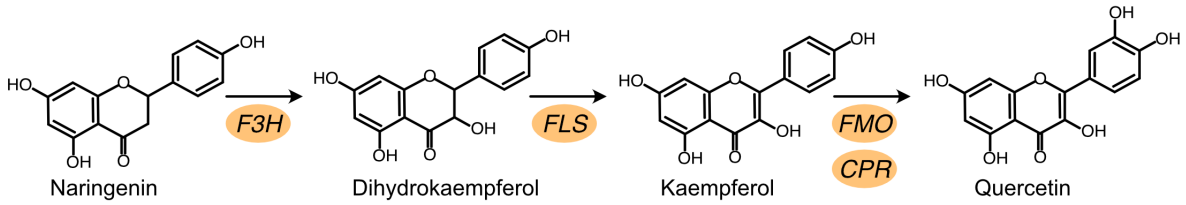


Figure 15. Biosynthetic pathway to produce kaempferol and quercetin from naringenin in S. cerevisiae*. Naringenin is converted to kaempferol by flavanone 3-hydroxylase (F3H) and flavonol synthase (FLS). Kaempferol is converted to quercetin by a flavonoid monooxygenase (FMO) together with a cytochrome P450 reductase (CPR).*

Enzyme screening

To establish the production of kaempferol, enzymes from different origins were screened in two different naringenin-producing strains: NAG10, a low-level naringenin producer, and NAG3-4, a high-level producer. For simplification in this chapter, I am going to talk about derivatives of the high producer strain, NAG3-4 (this strain has the genes downstream of *p*-coumaric acid (4CL:CHS:CHI) overexpressed in the ratio 3:4:4). An F3H enzyme from *Arabidopsis thaliana* could convert all the available naringenin to dihydrokaempferol. However, the three tested FLS enzymes were not as efficient and some accumulation of dihydrokaempferol was always found. The strain MTK4 that expresses F3H and FLS from *A. thaliana* showed the best production profile among all the enzymes tested (Figure 2B, Paper IV).

To reduce the accumulation of the pathway intermediate we increased the copy number of the gene encoding FLS intending to increase the flux. As a result, we obtained the strains MTK42 and MTK43 with 2 and 3 copies of the FLS encoding gene that reduced the accumulation of dihydrokaempferol to 26 mg/L and 19 mg/L respectively (Figure 3A, Paper IV). To reduce dihydrokaempferol accumulation other strategies could be used including looking for other versions of FLS that may be more efficient than the ones that were screened in this study. Moreover, random mutagenesis techniques to mutate the coding region of the gene to find enzyme versions with increased affinity for the subtract or that presented better expression in yeast could be employed.

Finally, to extend the pathway towards our final target, we screened three FMO versions from three different origins. When a copy of FMO from *Glycine max* was integrated into MTK43, the resultant strain (MTQ13) produced around 200 mg/L quercetin and a small accumulation of 10 mg/L of kaempferol (Figure 4B, Paper IV). It is worth mentioning that two CPR were already expressed in our naringenin platform strain, one from *A. thaliana* and another one (*CYB5*) overexpressed from the same *S. cerevisiae*.

Testing fed-batch-like cultivation

Since *S. cerevisiae* presents an overflow metabolism when there is excess of glucose in the media, we wanted to test the production profile be in glucose-limited cultivation conditions. Therefore, the strains MTK43 and MTQ13 were tested under fed-batch-like conditions by

using FeedBeads. Changing the cultivation conditions was a success since the kaempferol producer strain (MTK43) could produce 266 mg/L of kaempferol and the quercetin producer strain (MTQ13) could synthesize 319 mg/L of quercetin (Figure 16).

Increased flux of malonyl-CoA improves kaempferol and quercetin production

When establishing the platform strain to produce naringenin, we have seen that increasing the flux of malonyl-CoA increases the titres of flavonoids further, especially in fed-batch-like cultivations[120]. We followed the same strategy, and we also increased the availability of malonyl-CoA by overexpressing the enhanced version of the acetyl-CoA carboxylase enzyme[105] (mACC1*). This is the same enzyme that was used to increase the malonyl-CoA flux when building the pinocembrin and naringenin platform strains in Chapters 1 and 3.

When introducing mACC1* into the best kaempferol and quercetin producer strains, MTK43 and MTQ13 respectively, the titres of the final products increased significantly. The strain MTK43A produced 347 mg/L of kaempferol and the strain MTQ13A produced 355 mg/L of quercetin under fed-batch-like conditions (Figure 16).

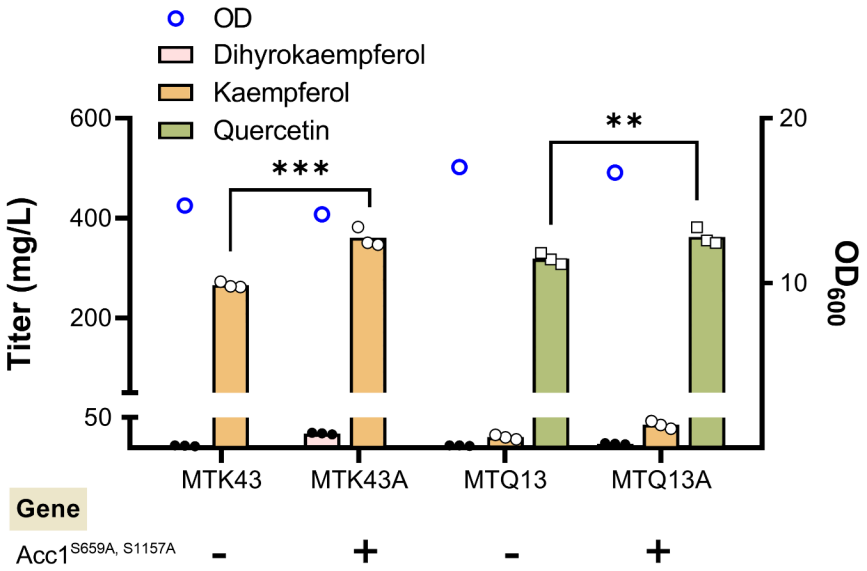


Figure 16. Production of kaempferol and quercetin strains with an increased supply of malonyl-CoA. Strains were cultivated for 96 h in minimal medium with 6 tablets of FeedBeads that release glucose at a slow rate as sole carbon source. Statistical analysis was performed by using one-way ANOVA, Tukey's multiple comparison tests (< 0.05, ** < 0.01, *** < 0.001). All data represent the mean ± standard deviation of 3 biologically independent samples.*

In Paper IV, we decided to increase the malonyl-CoA supply only by overexpressing mACC1* instead of also using the matBC system. The reason why the latter strategy is not preferred is because it requires the external feeding of an extra substrate, malonate. This is a drawback when the process is to be scaled up to industrial levels since it will increase the cost of production. Keeping the cultivation media as simple as possible is always a preferred option.

Testing the scalability of the kaempferol and quercetin production strain

Finally, we wanted to evaluate if the fermentation process could be scaled up keeping a glucose-limited feeding. Limiting the carbon source is important to avoid *S. cerevisiae*

overflow metabolism and maintain purely respiratory metabolism avoiding by-products formation. Therefore, we cultivated the strains MTK43A and MTQ13A in 1-L bioreactors. To maintain respiratory metabolism, we used a slow glucose feeding rate, starting with 0.4 mL/h, and finishing the cultivation at 14.6 mL/h. During the cultivation, the pH was controlled by KOH addition at 5.6 and the airflow was set to 30 L/h. To ensure oxygen availability, when the dissolved oxygen would decrease from 30% the airflow would automatically increase from 30 L/h to a maximum of 48 L/h. Under these cultivation conditions, we achieved a maximum titre of kaempferol after 100 h of cultivation, reaching 956 mg/L and achieving 930 mg/L of quercetin after 75 h of cultivation (Figure 6, Paper IV).

However, the cultivation process was not optimal since there was an accumulation of by-products such as ethanol towards the end of the process which indicates that the cells were not in purely respiratory metabolism.

Chapter 4: Take-home message

In Chapter 4 we engineered a naringenin platform strain to successfully produce two flavonoids of interest: kaempferol and quercetin. By reconstructing and balancing the biosynthetic pathway, increasing the availability of the precursor malonyl-CoA and optimizing fermentation conditions we achieved produced 956 mg/L and 930 mg/L of kaempferol and quercetin respectively.

In order to improve the production of kaempferol and quercetin, the strain NAG76 built in Paper III could be used as a chassis. During the development of these projects, the experiments of Papers III and IV were carried out in parallel and therefore this was not possible at that time. With all the results available I believe that using a strain where the precursors malonyl-CoA and *p*-coumaric acid are balanced will be beneficial to push the titres further.

In addition, the activity of the FLS enzyme could be improved to reduce the accumulation of dihydrokaempferol. To do that approaches of *in vitro* mutagenesis targeting the open reading frame of the gene with error-prone PCR or *in vivo* mutagenesis using a base editor such as EvolvR could be used[177]. However, both approaches would need the previous development of a biosensor to enable high throughput screening.

Summary and Conclusion

In this thesis, the production of pinocembrin and naringenin, two main branches of flavonoids, and its derivates were established and optimized in *S. cerevisiae*. Diverse strategies for constructing and improving yeast cell factories for flavonoid production were presented and discussed.

In the first chapter, we established the production of pinocembrin. Firstly, we successfully implemented and balanced the biosynthetic pathway to produce pinocembrin. Secondly, we eliminated the formation of a by-product that directly competed with the pathway precursors. Finally, we increased the flux of malonyl-CoA, an essential building block that limited the production of pinocembrin. In the process, we determined the main bottleneck that hampers the production of this flavonoid and its derivatives: the accumulation of the toxic precursor cinnamic acid, which must be addressed to improve the titres of the engineered strains.

In the second chapter, we looked into the transcriptome of *S. cerevisiae* exposed to cinnamic acid, ferulic acid and *p*-coumaric acid. These aromatic acids are precursors of flavonoids and exert different levels of toxicity for the cell. Looking into the transcriptome we found that 16 transcription factors presented differences in the expression levels of their gene sets. In Paper II, we build knock-out and overexpression strains for the targeted TFs and found that changing the expression of six of them had a positive impact on the growth of *S. cerevisiae* under at least one of the tested aromatic acids. The characterized transcription factors from Paper II could be used to increase the tolerance to aromatic acids in flavonoid microbial cell factories.

In the third chapter, we optimized the production of naringenin in *S. cerevisiae*. The naringenin biosynthetic pathway was first optimized. We increased the expression of the enzymes downstream of *p*-coumaric acid and increased the pool of precursor malonyl-CoA. However, we detected an accumulation of the precursor *p*-coumaric acid outside of the cell. This limitation hindered the production of naringenin since the precursor could not be utilized. To address the bottleneck, we established a dual dynamic control that (1) reduced the accumulation of *p*-coumaric acid by controlling its synthesis and (2) improved the naringenin production by downregulating a pathway competing for malonyl-CoA. Additionally, we identified that the overexpression of the Pdr12 transporter led to a reduction of *p*-coumaric acid accumulation and an increase in naringenin production. The strategies applied in Chapter 3 highlight the importance of balancing pathway intermediates when establishing the production of heterologous pathways in yeast.

In the fourth chapter, we used a naringenin *S. cerevisiae* platform strain to produce two structurally complex flavonoids: kaempferol and quercetin. Firstly, the production of

kaempferol was established by screening various F3H and FLS enzymes. Afterwards, we reduced pathway intermediate accumulation by overexpressing the limiting enzyme FLS. Secondly, we established the production of quercetin by FMO enzyme screening. To increase the production, we enhanced the availability of precursor malonyl-CoA by overexpressing a deregulated Acc1 version. We finally tested the microbial cell factory scale-up potential by cultivating the strain in 1-L bioreactors under fed-batch cultivation.

Challenges and how they can be addressed

As I have discussed in the thesis, building microbial cell factories to produce high-value compounds that are originally synthesized in plants comes with challenges. In this section, I am going to discuss some limitations that I encountered when establishing the production of flavonoids and I will propose some strategies to overcome the challenges.

Toxicity of pathway precursors/intermediates

Plants generate secondary metabolites like flavonoids because they have different benefits, such as defend them against UV radiation, serving as signalling molecules for pollination, and protecting plants from parasites due to their antimicrobial characteristics. When introducing the production pathways into yeast, the expression levels are not well balanced and sometimes pathways intermediates can accumulate. Since these compounds are not native to yeast, sometimes they are toxic, and their accumulation can limit cell growth.

An example of this is the cinnamic acid accumulation in the pinocembrin biosynthetic pathway (Paper I). To avoid this problem, research has focused on the production of other flavonoids that are not limited by the toxicity of a precursor. For example, the production of naringenin and its derivatives has been explored extensively in different hosts reaching titres of 2 g/L in fed-batch fermentations in *S. cerevisiae*[120]. In Paper I when we attempted the optimization of pinocembrin and its derivatives in yeast, we channelled the pool of cinnamic acids towards less toxic intermediates. However, without the capacity of accumulating the precursor, our achieved titres were still low compared to other flavonoids produced in *S. cerevisiae*. Therefore, in Paper II we found six candidates that could increase the ethanol phase growth-rate of the wild-type cultivated with aromatic acids. The transcriptomic analysis provided a lot of information on aromatic acid response and the reverse engineering performed in Paper II is just a small fraction of what could be found. Our decision to look into TF proved fruitful but could be expanded by combining some of the targets or looking into other growth parameters of the reverse-engineered mutants such as final biomass. In addition, the reverse-engineered strains could be tested with increased concentrations of acids to see if other stages of the growth are affected, e.g., the duration of the lag phase. Finally, some of the identified transcription factors could be incorporated in the pinocembrin production platform strain of Paper I and assess the impact in growth and production.

Nevertheless, aromatic acid tolerance by *S. cerevisiae* is still a challenge and other approaches could be used to produce these flavonoids. Finding an alternative host to produce pinocembrin and its derivatives could be an easier approach. *Yarrowia lipolytica* is an

alternative that has been proven to be an excellent host for producing plant derivates for its tolerance to inhibitors[178] and high flux of malonyl-CoA. However, *Y. lipolytica* can degrade some aromatic acids[179]. There are other non-conventional yeasts that have potential as excellent microbial hosts[180,181] such as *Debaryomyces hansenii*, known for its high salt tolerance[182]. Additionally, natural yeast strains libraries of non-characterized yeast could be screened under cinnamic acid selective pressure to find novel strains tolerant to aromatic acids.

Low efficiency of plant enzymes

Flavonoids are secondary metabolites of plants, and they are produced in small quantities. Plant enzymes involved in flavonoid production pathways are usually inefficient in their host organisms and may not be efficient when they are transferred to microbial hosts. An example is the enzyme chalcone synthase (CHS) involved in the conversion of coumaroyl-CoA to trihydroxychalcone in the naringenin biosynthetic pathway. CHS is promiscuous and inefficient and triggers the accumulation of the reaction substrate coumaroyl-CoA. The accumulation of coumaroyl-CoA has been shown to be toxic for *S. cerevisiae* limiting its growth[163]. To reduce the accumulation of the toxic coumaroyl-CoA *S. cerevisiae* might hydrolyse it back to *p*-coumaric acid, restricting naringenin production. A more efficient CHS version could reduce the coumaroyl-CoA accumulation and increase the flux toward naringenin production. Another example is the expression of prenyltransferases that transfer prenyl groups to flavonoid backbones[172]. Prenyltransferases have been expressed in the past in yeast, but their efficiency has been reported to be very low[173,XI]. The low efficiency of these enzymes can be attributed to different reasons. In plants, prenyltransferases are membrane-bound enzymes, usually bound to plastids. This localization is beneficial because the plastid synthesizes large amounts of isoprenoids in plants. Therefore, localizing the enzyme in that organelle is an advantage for the production of prenylated isoprenoids in plants but yeast does not have plastids. Targeting the enzymes to the cell membrane or to the membrane of an organelle may be a good strategy to improve its efficiency.

Up-scaling flavonoid production

Building microbial cell factories to produce a compound of interest is a long and complex process. It can be divided into two main parts: (1) the upstream process and (2) the downstream process. In my thesis, I mostly covered the upstream process that consists of strain engineering strategies that aim to obtain robust proof-of-concept strains. However, the downstream processing is very important to bring the proof-of-concept strain into industry. The downstream processing consists on the optimization of fermentation conditions and the product purification[183]. In this thesis, we have tested different fermentation conditions to improve the fermentability of the platform strains. We performed cultivations in shake flasks mimicking fed-batch conditions and we have scaled up the fermentations of naringenin,

[XI] In our group we attempted to produce prenylated naringenin but achieved very low titres (data unpublished).

kaempferol and quercetin to 1-L bioreactors. However, other factors should be considered before bringing to industry strains that produce high-value products (such as flavonoids). The media used is one of the factors of great importance. This is because the cost of the growth medium represents around 30-40% of the production costs of the culture[184]. Another factor is maximizing the titre ($g*L^{-1}$), yield ($g*g^{-1}$), and productivity ($g*L^{-1}*h^{-1}$)[185], especially important when producing low-value products[XII]. Unfortunately, both factors are sometimes not compatible. To reach maximum productivity often expensive carbon sources have to be used that increase the cost of the whole process. A possible solution to this problem is to optimize the cell factories to be able to grow in cheap substrates[XIII].

Microbial cell factories- an outlook

The metabolic engineering field started by producing commodity chemicals and started to shift to produce high-value compounds. One of the reasons for the shift was economical. To produce commodity chemicals, the microbial fermentations have to compete with petroleum-based production. During the last decade, there was a rising interest on producing food ingredients, feed, healthcare related products or compounds of agricultural interest. Another reason was the lack of knowledge and tools to express the biosynthetic pathways to produce high-value products such as flavonoids.

I believe that during the next years the fields of metabolic engineering and synthetic biology will develop further, and the production of microbial cell factories will be accelerated. Advances on Artificial Intelligence (AI) will help the development of enzyme engineering. Nowadays AI can already help to predict protein structures, for example alpha-fold. It is likely that in the near future AI could predict how to optimize a protein sequence to be expressed and functional in a microbial host. Furthermore, thanks to the development of robotics and high-throughput screening methods such as biosensors, research laboratories will be streamlined. An example of lab automation is the foundation of the first Biofoundries that essentially are molecular biology and synthetic biology facilities that integrate robotic liquid handling with high-throughput analytics and systems biology approaches. Biofoundries allow the automation of laboratory protocols and facilitate the scale-up of experiments that can be run in parallel.

Altogether, I believe that the new biotechnological advances will push the production of microbial cell factories further.

[XII] When producing low value products yield is very important so the maximum amount of product is produced using the least substrate to make as much profit as possible.

[XIII] Some start-ups are using industrial side-streams to grow their microbes and reduce costs. An example is the Swedish start-up Cirkulär.

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