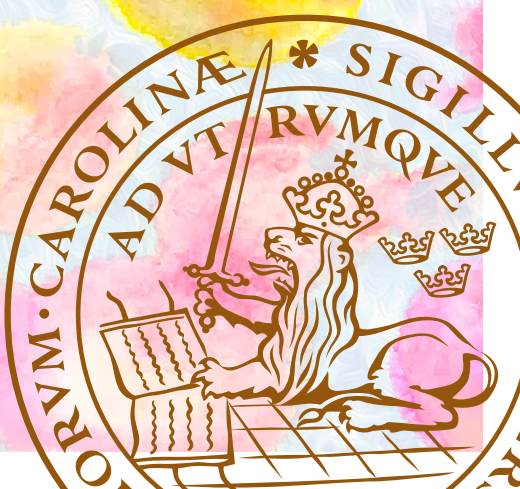
Biosynthesis of capsaicinoids by recombinant Saccharomyces cerevisiae

Muratovska, Nina

2023

Biosynthesis of capsaicinoids by recombinant *Saccharomyces cerevisiae*

NINA MURATOVSKA | APPLIED MICROBIOLOGY | LUND UNIVERSITY



Biosynthesis of capsaicinoids by recombinant *Saccharomyces cerevisiae*

Nina Muratovska

Division of Applied Microbiology
Department of Chemistry
Lund University

LUND
UNIVERSITY

Front cover

"Watercolor representation of baker's yeast" by Nina Muratovska & Matej Stare

Back cover

"*Piperche*" by Natalija Stojanovska

Applied Microbiology
Department of Chemistry
Faculty of Engineering
P.O Box 124
SE-221 00 Lund
Sweden

ISBN: 978-91-7422-928-8 (print)
ISBN: 978-91-7422-929-5 (digital)

Printed in Sweden by Media-Tryck, Lund University
Lund 2023

To my dear family

"Education is the most powerful weapon which you can use to change the world."

Nelson Mandela

Abstract

Throughout history, people have used products from different microorganisms and plants for many medicinal and nutritional applications. Chilli peppers have been used for spicing up food, but also for their pharmacological properties. They contain alkaloid molecules called capsaicinoids, which have been shown to activate and desensitise the heat receptor - Transient Receptor Potential Vanilloid type 1 (TRPV1) cation channel. The TRPV1 receptor is a target for pain relief treatments for a range of health conditions, therefore capsaicinoids are important drug candidates.

Capsaicinoids can be manufactured by extraction directly from the chili pepper, which typically results in relatively small amounts and therefore requires large areas of land. Furthermore, capsaicinoids yield can be affected by environmental and genotypical factors. Another way of production can be through the use of synthetic biology and metabolic engineering strategies to introduce enzymatic reactions in a model microorganism. This way, plant-derived or novel capsaicinoids with potentially improved therapeutic properties can be produced from given precursors, simple sugars or renewable raw materials.

The aim of this work was to engineer baker's yeast *Saccharomyces cerevisiae* for a whole-cell biocatalytic production of capsaicinoids. The structure of these compounds is comprised of a vanilloid moiety and a fatty acyl chain joined by an amide bond. The last step for synthesis of these compounds in the plant is through condensation of the precursors vanillylamine (derived from vanillin) and a fatty acyl-CoA forming the amide bond. Amide forming reactions are very important in chemical synthesis and research has been directed towards finding efficient enzymes to perform them. Furthermore, yeast as a model microorganism has been modified to produce vanillin from glucose; however, reductive amination of vanillin to vanillylamine, or production of capsaicinoids in yeast has not been achieved previously.

This thesis addresses how capsaicinoids can be produced, the challenges for *in vivo* reductive amination of vanillin to vanillylamine and the identification and characterisation of several heterologous *N*-acyltransferase (NAT) and CoA-ligase (CL) amide-forming enzymes. Vanillylamine was produced from vanillin, by overexpressing a vanillin aminotransferase. The transamination reaction was improved by the co-expression of an alanine dehydrogenase, which also removed

the need for amine donor supplementation. Cultivation conditions were shown to have an effect on the transamination, with anaerobic conditions and ethanol as a co-substrate leading to decrease in by-product formation and improved reductive amination of vanillin to vanillylamine. Moreover, combinations of NAT and CL enzymes were evaluated for production of nonivamide (a model capsaicinoid) from vanillylamine and nonanoic acid. Finally, nonivamide, was successfully produced *in vivo* from precursors vanillin and nonanoic acid, after implementing strain engineering strategies and evaluating reaction conditions. Even though the NAT and CL enzyme cascade effectively produced nonivamide, the titres and yields of the process can be further improved, indicating that the amidation step is the current bottleneck in the production and requires further optimisation.

Lastly, the potential of *S. cerevisiae* as a biosensor was evaluated by overexpressing the TRPV1 receptor and modulating its activation. This was done as a first step towards the goal of using yeast as a screening platform for strains producing capsaicinoids with activity on the TRPV1 receptor.

Transfering the capsaicinoid enzyme cascade in a cell host is not straightforward and requires optimal expression of several heterologous enzymes, adjustments of reaction conditions and strategies in reducing inhibitory effects and by-products. However, biosynthesis in whole-cells may offer advantages due to simple cell-derived generation of enzymes, intermediates and co-factors, positively affecting the cost and performing the reaction in aqueous environment as a more environmentally sustainable way of production.

Popular science summary

Have you ever eaten a chili pepper and felt the spicy and pungent feeling in your mouth? If yes, this is because the chilli peppers contain compounds that are responsible for this spiciness by interacting with heat receptors in our body. These compounds are named capsaicinoids and are important molecules that can cause this painful reaction, but more importantly, in a specific dose they can block the pain signal and are therefore used in pain-relief creams and as part of treatment for some diseases like rheumatoid arthritis and neuropathy.

These compounds can be extracted from the plant itself; however, in small quantities and require land resources that can be directed towards food purposes. In the lab, plant-derived, but also new compounds, can be created using chemical synthesis methods. This can be challenging for complex molecules and would require careful handling and disposal procedures of chemicals and materials to protect the environment. There are also biological ways of producing capsaicinoids through the use of enzymes and live microorganisms as small "production factories". Enzymes are proteins which are present in all cells and greatly speed up the rate of chemical reactions serving very important functions in the metabolism.

In this thesis, enzymatic reactions were introduced in the well-known microorganism baker's yeast (*Saccharomyces cerevisiae*) through metabolic engineering methods in order to produce a type of capsaicinoid compound called nonivamide. Performing enzymatic reactions in a microorganism is beneficial due to the use of the cell's metabolism to produce the enzyme and other needed substances for the production, while only supplying feed and/or precursors to the cell for the product in question. Yeast was engineered to produce several enzymes originally found in plants and bacteria and was able to convert the starting compound vanillin to the intermediate vanillylamine and finally to the product nonivamide. Several challenges were encountered such as formation of other unnecessary by-products and inhibitory interactions between the cell and the product and its precursor.

Microbes have great potential as factories for production of a range of compounds identified in or inspired by nature. They offer an environmentally friendly alternative to other ways of production. This kind of whole-cell production system coupled with the use of renewable raw materials as feed can make a huge difference in today's design and production of useful natural compounds.

Populärvetenskaplig sammanfattning

Har du någon gång ätit en chilifrukt och känt den starka, kryddiga och stickande känslan i munnen? Det beror på att chilipeppar innehåller substanser som orsakar denna kryddighet genom att interagera med värmereceptorer i vår kropp. Dessa substanser kallas capsaicinoider och är viktiga molekyler som utöver att de orsakar denna smärtsamma reaktionen, kan vid specifika doser blockera smärtsignalen, och används därför i smärtlindrande krämer och som en del av behandling av vissa sjukdomar som reumatoid artrit och neuropati.

Dessa substanser kan extraheras från själva chiliplantan; dock i små mängder, och kräver odlingsbar marks som annars kan användas till livsmedelsändamål. I labbet kan dessa växtsubstanser eller nya substanser skapas med kemiska syntesmetoder. Detta kan vara utmanande för komplexa molekyler och skulle kräva noggrann hantering och bortskaffande av kemikalier och material för att skydda miljön. Det finns också ett annat sätt att producera capsaicinoider: genom användning av enzymer, eller levande mikroorganismer som små "produktionsfabriker". Enzymer är proteiner som finns i alla celler och avsevärt påskyndar hastigheten för kemiska reaktioner som tjänar mycket viktiga funktioner i ämnesomsättningen.

I den här avhandlingen introducerades enzymatiska reaktioner i den välkända mikroorganismen bakjäst (*Saccharomyces cerevisiae*) genom gentekniska metoder för att producera en typ av capsaicinoidförening som kallas nonivamid. Att utföra enzymatiska reaktioner i en mikroorganism är fördelaktigt på grund att man kan använda cellens egen ämnesomsättning för att producera enzymet och de andra nödvändiga ämnen som behövs för att producera sin önskade molekyl, och det enda man behöver tillföra cellen är olika typer av näringsämnen och eventuellt några få kemiska byggstenar. En modifierad jästsvamp konstruerades för att producera flera enzymer som ursprungligen fanns i växter och bakterier och kunde omvandla ämnet vanillin till mellanprodukten vanillylamin och slutligen till produkten nonivamid. Flera utmaningar påträffades dock, såsom bildning av oönskade biprodukter och hämmande interaktioner mellan cellen och produkten och dess prekursor.

Mikrober har stor potential som fabriker för produktion av en rad substanser identifierade i eller inspirerade av naturen. De erbjuder ett miljövänligt alternativ till andra produktionssätt. Den här typen av helcellsproduktionssystem i kombination med användning av förnybara råvaror som matning kan göra en enorm skillnad i dagens design och produktion av användbara naturliga substanser.

Популарно научно резиме

Дали некогаш сте јаделе лута пиперка и сте почувствувале печење во устата? Ако одговорот е да, тоа е затоа што чили пиперките содржат соединенија кои се одговорни за оваа пикантност и кои ги активираат таканаречените топлински рецептори во нашето тело. Овие соединенија се именувани капсаициноиди и се важни молекули кои можат да ја предизвикаат оваа болна реакција, но уште поважно, во одредена доза тие можат да го блокираат сигналот за болка. Затоа се користат во креми за ублажување на болка и како дел од третманот за некои болести како ревматоиден артритис и невропатија.

Овие соединенија може да се изолираат од самото растение; но, во растението се присутни во мали количини. За доволна количина би биле потребни големи насади наменети само за производство на овие соединенија, кои инаку би можеле да се насочат кон прехранбени цели. Во лабораторија, може да се создадат соединенија исти како оние од растително потекло, но и нов тип на соединенија користејќи хемиска синтеза. Но, хемиската синтеза не секогаш може да биде едноставна за создавање вакви сложени молекули и е потребно внимателно ракување и употреба на процедури за отстранување на несаканите хемикалии и материјали со цел да се заштити животната средина. Исто така, постојат биолошки начини за производство на капсаициноиди преку употреба на живи микроорганизми, во функција на мали „фабрики за производство“, и ензими. Ензимите се протеини кои се присутни во сите клетки и во голема мера ја забрзуваат брзината на хемиските реакции кои служат многу важни функции во метаболизмот.

Во оваа докторска теза, беа употребени ензимски реакции во добро познатиот микроорганизам - пекарски квасец (*Saccharomyces cerevisiae*), воведени преку методи на метаболно инженерство, со цел да се произведе еден вид капсаициноидно соединение наречено нонивамид. Вршењето ензимски реакции во микроорганизми е корисно поради тоа што се употребува самиот клеточен метаболизам за производство на ензимот и другите потребни супстанции, додека клетката мора само да биде снабдена со храна и/или прекурсори за саканиот производ. Квасецот беше конструиран да произведува неколку ензими првично пронајдени во растенија и бактери и успешно го конвертира почетното соединение ванилин во ванилиламин и конечно во производот нонивамид. Наидовме на неколку предизвици како формирањето

на непотребни нуспроизводи и инхибиторни интеракции помеѓу клетката и производот, како и неговиот прекурсор.

Микробите имаат голем потенцијал да станат фабрики за производство на низа соединенија пронајдени или инспирирани од природата. Тие нудат еколошка алтернатива во споредба на другите начини на производство. Овој систем за производство во клетки заедно со употребата на обновливи суровини како храна за клетката, може да донесе огромни придонеси во денешниот дизајн и производство на корисни природни соединенија.

List of papers

This thesis is based on the following research papers and manuscripts, found at the end of this thesis. They will be referred to in the text by using Roman numerals.

I. **Engineering *Saccharomyces cerevisiae* for production of the capsaicinoid nonivamide**
Muratovska, N., Grey, C. & Carlquist, M. (2022) In: *Microbial Cell Factories.* 21, 106. doi: 10.1186/s12934-022-01831-3

II. **Recombinant yeast for production of the pain receptor modulator nonivamide from vanillin**
Muratovska, N., & Carlquist, M., 2023, In: *Frontiers in Chemical Engineering*. 4, 1097215. doi: 10.3389/fceng.2022.1097215

III. **Metabolic engineering of *Pseudomonas putida* for production of vanillylamine from lignin-derived substrates**
Heitor Colombelli Manfrão-Netto, J., Lund, F., Muratovska, N., Larsson, E. M., Skorupa Parachin, N. & Carlquist, M. (2021) In: *Microbial Biotechnology*. 14, 6. doi: 10.1111/1751-7915.13764

IV. ***In vivo* assay for measuring modulation of mammalian TRPV1 recombinantly expressed in yeast**
Muratovska, N., Johanson, U., Carlquist, M. *Manuscript*

I have contributed to the following review and paper, which are not included in the thesis:

1. **Towards engineered yeast as production platform for capsaicinoids**
Muratovska, N., Silva, P., Pozdniakova, T., Pereira, H., Grey, C., Johansson, B. & Carlquist, M. (2022) In: *Biotechnology Advances*. 59, 107989. doi: 10.1016/j.biotechadv.2022.107989

2. ***Saccharomyces cerevisiae* remain competent after one month on ice**
Silva, P., Muratovska, N., Pereira, H., Carlquist, M., Johansson, B. (2023) *Submitted and under review*

My contribution to the papers

I. I participated in the design of the study established by Magnus Carlquist, carried out the experimental work and analysis of data and drafted the manuscript

II. I contributed to the design of the study, performed all the experimental work, data analysis and drafted the manuscript.

III. I assisted in performing and analysing the bioconversion experiments, developed the HPLC setup for analysis and revised the manuscript.

IV. I contributed to the design of the study, performed all the experimental work together with a student, data analysis and drafted the manuscript.

Abbreviations

ACP – Acyl carrier protein

ACS – Acyl-CoA synthetase

ADH – Alcohol dehydrogenase

AlaDH, L-ADH – Alanine dehydrogenase

AT3 – Putative acyltransferase

ATA – Aminotransaminase

ATP – Adenosine triphosphate

BC – Branched chain

BCFA – Branched chain fatty acid

BCFAP – Branched chain fatty acid pathway

CL – CoA-ligase

CoA – Coenzyme A

CRISPR/Cas9 – Clustered Regularly Interspaced Short Palindromic Repeats and the Cas9 nuclease

CS – Capsaicin synthase

DNA – Deoxyribonucleic acid

E.g. - Example

TA- Transaminase

FA – Fatty acid

FAS – Fatty acid synthase

GFP – Green fluorescent protein

GPCR – G-protein coupled receptors

HTS – High throughput screening methods

LDH – Lactate dehydrogenase

MCFA – Medium-chain fatty acids

NAD(P)H, NAD(P)+ Nicotinamide adenine dinucleotide (phosphate) + hydrogen

NAT – *N*-acyltransferase

PLP – Pyridoxal 5'-phosphate

RNA – Ribonucleic acid

TRPV1 – Transient receptor potential vanilloid type 1

VAMT, or Cc-ATA – *Capsicum chinense* vanillin aminotransferase

Contents

Chapter 1
Introduction

The value of natural products

Natural products are small bioactive compounds produced by living organisms, both prokaryotes and eukaryotes, and have a large structural diversity, comparable to the vast diversity of existing organisms. To generate such diverse compounds, nature has developed many complex biosynthetic routes driven by enzymes. Cells produce these compounds as primary or secondary metabolites, depending if they are essential to the survival of the producing organism, or are used to affect other organisms.

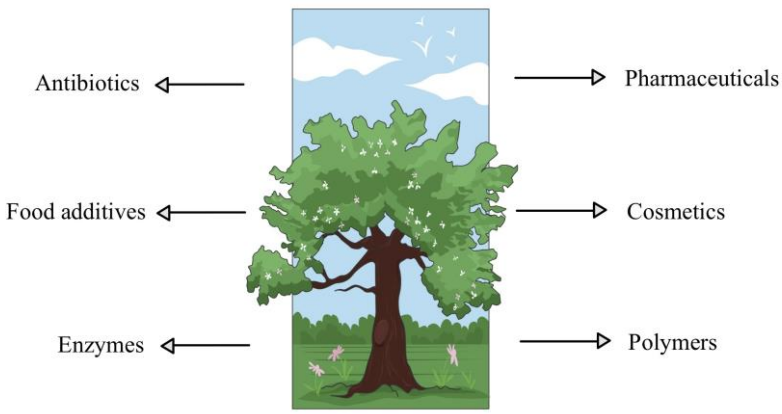


Figure 1. Examples of natural products of value for society[1].

Society has been greatly influenced by the bioactive compounds found in nature (Figure 1). For decades, most of these natural products have been discovered and researched due to their pharmaceutical or therapeutic benefits (Newman and Cragg, 2020). Some key examples of microbial-derived natural products involve antibiotics and therapeutics, enzymes as biocatalysts, food additives, biosurfactants and biopolymers (Willey et al., 2017). For instance, a large portion of all known

[1] *Spring icon by Servier https://smart.servier.com/ is licensed under CC-BY 3.0 https://creativecommons.org/licenses/by/3.0/I*

antibiotics (>65%) are microbially synthesised, with examples including mycophenolic acid, penicillin, cephalosporin, tyrotricin, streptomycin, tetracycline, chloramphenicol (Nicolaou and Rigol, 2018). Plants are another valuable source of bioactive compounds and besides their nutritional value, they have been used for medicinal purposes since ancient times (Atanasov et al., 2015). Important applications for plant-derived compounds, such as alkaloids, terpenes, aromatics and polyketides, have been largely found in the textile, food, cosmetics and pharmaceutical industries (Romero-Suarez et al., 2022). Because of this recognised value of natural products, large collections of compounds are now available as part of online databases and has thus become a resource that can be utilised in research (Sorokina and Steinbeck, 2020).

Natural product synthesis through biotechnology

Chemical synthesis of natural products has been studied a great deal and there is a lot of interest in developing novel strategies for reaching total synthesis of these compounds. In some cases, synthesis has even preceded detection of a compound in nature (Hetzler et al., 2022). However, the structural complexity and stereochemical elements present in natural products makes purely chemical synthesis in many cases challenging, though still achievable (Mohr et al., 2008).

Newer efforts have been directed towards combining chemical synthesis with biocatalysis, either with the help of enzymes or whole-cells (Vanable et al., 2022). Almost all compounds produced by the cellular metabolism are generated in processes catalysed by enzymes, and as such the potential for enzymes to be utilised for high diversity of chemical applications is very high.

Three waves of biocatalysis development have been described (Bornscheuer et al., 2012). The first wave began about a century ago with the recognition that enzymes (from cell extracts or immobilised) could be applied for chemical synthesis. The second wave was enabled by structure-based protein engineering strategies, which were used to improve existing enzymes or create new ones. These could catalyse reactions that do not even exist in nature, with applications for pharmaceuticals and fine chemicals. Currently, in the third wave of development, advanced protein engineering strategies such as directed evolution of enzymes is applied, using molecular biology and selection and screening, to tailor-design enzymes with better properties like stability, substrate specificity and enantioselectivity (Bornscheuer et al., 2012). Due to these developments, the application of enzyme cascades and/or processes has gained much attention, particularly in production of pharmaceuticals because of the possibility of coupling multiple reactions (cascades), producing pure chiral compounds, use of mild conditions and their biocompatibility and biodegradability (Benitez-Mateos et al., 2022).

In addition to this, advances in biocatalysis and industrial biotechnology have led to emergence of the field of synthetic biology and its applications for production of therapeutic natural products (Awan et al., 2016). Synthetic biology, as a term can sometimes be used interchangeably with the concept of metabolic engineering, and involves engineering of organisms using molecular biology for performing new tasks. An application can be enabling complete biosynthetic production of a bioactive compound, using live whole-cells or cell extracts. An early important example of a natural product that was successfully produced using synthetic biology is the anti-malarial drug precursor artemisinic acid (Ro et al., 2006).

The engineering cycle in metabolic engineering and synthetic biology in general is iterative and consists of four parts: Design, Build, Test and Learn (Figure 2). Initially, the engineering goal is identified and a suitable host cell and metabolic pathway is chosen (Design). Then, the host is engineered (Build), validated through experiments and analysis methods (Test). The generated data is processed and the generated knowledge (Learn) is further applied in the next cycle.

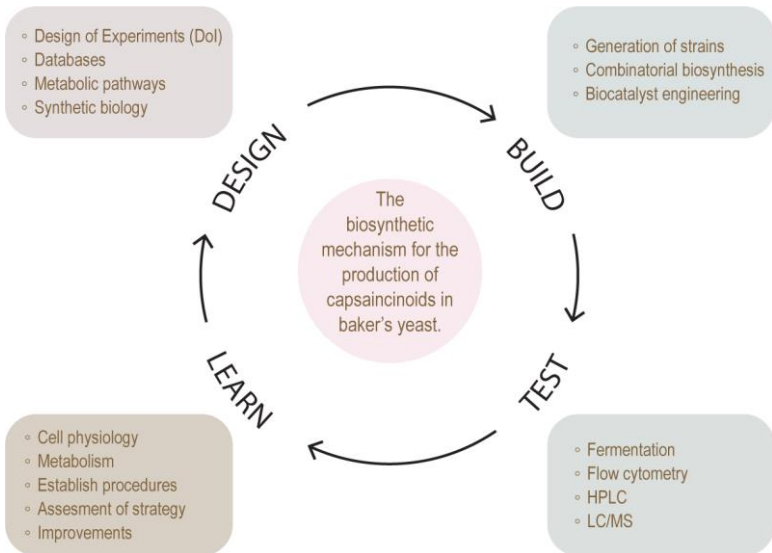


Figure 2. Design, Build, Test, Learn cycle in synthetic biology applied in the project.

Choosing the proper platform host is one of several important tasks in metabolic engineering and synthetic biology when developing a production process. Features such as availability of genetic tools, survival under desired conditions, possibility of gene expression and stability need all be considered. Using the natural host and optimizing its production pathways by metabolic engineering has been a successful strategy, for example with *Streptomyces* and *Aspergillus*, which are producers of many natural products. Often, genes of importance are transferred from the natural host to heterologous hosts, which generally are easier to grow and engineer.

Well-characterised microbial hosts are most commonly used, especially in industrial processes e.g. *Saccharomyces cerevisiae*, *Escherichia coli*, *Bacillus subtilis*, *Streptomyces coelicolor* due to their fast growth rate, cultivation conditions, availability of tools for genetic engineering and robustness (Keasling, 2010). In this thesis, *Saccharomyces cerevisiae* was used as a production host. Additionally, *Pseudomonas putida* was utilised for its ability to catabolise several aromatic compounds such as vanillin and ferulic acid, and is commonly used a platform host in studies on the valorisation of depolymerised lignin to value added compounds, due to its high tolerance to aromatic compounds (Luengo and Olivera, 2020). Production of compounds in a heterologous host organism, requires optimised expression of one or several heterologous genes coding for particular enzyme(s), and utilizing knowledge of metabolic pathways in order to balance production yields and growth (Awan et al., 2016).

Metabolic engineering has the potential to result in more sustainable production processes compared with synthetic chemistry for a range of valuable natural products. Biosynthesis of active pharmaceutical ingredients (APIs), such as alkaloids (e.g. isoquinoline alkaloids (BIAs)), polyketides (e.g. erythromycin) and nonribosomal peptides (e.g. vancomycin) and isoprenoids (e.g. artemisinic acid), can be more advantageous over synthetic chemistry particularly due to the compounds complexity (Keasling, 2010). Further developments in synthetic biology could in the future complement chemical synthesis reactions by substituting the use of organic solvents and heavy metals to reach more sustainable production processes for even more complex compounds.

Baker's yeast as a platform host for biosynthesis of plant natural products

S. cerevisiae (baker's yeast) has been the organism of choice for the complete synthesis of many plant-derived natural products (Table 1.) Plant natural products (PNPs) produced in yeast can be divided in several main categories: alkaloids, terpenes and terpenoids, and phenolic compounds. These compounds all derive from four major synthetic pathways: (1) shikimic acid pathway, (2) malonic acid pathway, (3) mevalonic acid pathway and (4) MEP (non-mevalonate) pathway (Azmir et al., 2013). *S. cerevisiae*'s metabolism has been engineered so that they are able to produce several representative products of each PNP category. Many more proof of concept products have been described using baker's yeast as a host for synthesising precursors or parts of important metabolic pathways.

Table 1. *De novo* synthesised plant natural products (PNPs) in *S. cerevisiae.*

Compounds			Reference
Alkaloids	Papaverine and (*S*)-tetrahydropapaverine (THP)	Benzylisoquinoline alkaloids (BIAs)	(Jamil et al., 2022)
	Vindoline and catharanthine	Monoterpene indole alkaloids	(Zhang et al., 2022)
	Guattegaumerine and berbamunine	Benzylisoquinoline alkaloids (BIAs)	(Payne et al., 2021)
	Tropine, intermediate to scopolamine	Tropane alkaloids	(Srinivasan and Smolke, 2019)
	Noscapine	Halogenated alkaloid	(Li et al., 2018)
	Thebaine and hydrocodone	Opioids (BIAs)	(Galanie et al., 2015)
Terpenoids	Cannabigerolic acid, Δ9-tetrahydrocannabinolic acid, Cannabidiolic acid, Δ9-tetrahydrocannabivarinic acid and Cannabidivarinic acid	Cannabinoids	(Luo et al., 2019)
	Artemisinic acid, precursor to artemisinin	Sesquiterpene lactone derivative	(Ro et al., 2006)
Phenolic compounds	Pelargonidin-3- Delphinidin-3-*O*-glucoside *O*-glucoside and Cyanidin-3-*O*-glucoside	Antocyanins	(Eichenberger et al., 2018)
	Naringenin, liquiritigenin, kaempferol, resokaempferol, quercetin, and fisetin	Flavonoids	(Rodriguez et al., 2017)
	Cinnamaldehyde, cinnamyl alcohol, hydroxcinnamyl alcohol	Trans-cinnamic acid derivatives	(Gottardi et al., 2017)
	Vanillin and vanillin-*β*-glycoside	Phenolic aldehyde	(Hansen et al., 2009, Strucko et al., 2015)

Genetic engineering of *S. cerevisiae*

S. cerevisiae is a preferred platform host for production of a range of compounds due to its well understood metabolism and growth conditions, but most importantly due to the engineering tools available. Here, the use of CRISPR/Cas9 system for genome engineering has enabled multi-target engineering in yeast (Stovicek et al., 2017). The mechanism behind is that the Cas9 nuclease is guided to a specific DNA region where it performs a double-stranded DNA break, three nucleotides upstream of a protospacer adjacent motif (PAM) site NGG. The Cas9 is recruited and guided by a guiding RNA (gRNA) consisting of two RNA molecules fused together: the CRISPR RNA (crRNA) and transactivating crRNA (tracrRNA) forming a loop. The double stranded DNA break must be repaired by the cells for survival through

homologous recombination or non-homologous end joining. By supplying the cells with a DNA template for repairment, various modifications can be made (Stovicek et al., 2017). The advantage of using CRISPR/Cas9 is the precision to the engineering site, there is no need of selective markers and multiple locations can be targeted simultaneously. In parts of this work CRISPR/Cas9 was used in combination with homologous recombination, by using *in vivo* combinatorial assembly of five fragments as a template for the repair (Paper I, Paper II).

Active compounds from chilli peppers - capsaicinoids

The genus *Capsicum*, to which chilli peppers belong, is part of the *Solacenae* family of plants (nightshade family) originating from Central and South America (Suzuki and Iwai, 1984). There are around 35 species of chilli peppers grouped into 11 clades; however, the most economically important belong to the *Capsicum annum* complex, comprising of *C. annum*, *C. chinense* and *C. frutescens* species (Carrizo Garcia et al., 2016). Their fruits appear in different shapes, elongated or bell shaped and in various colours (red, green, orange and yellow).

Sweet and hot chilli peppers are used worldwide as vegetables, spices, pigments, but also for medicinal purposes (e.g. analgesics and anti-inflammatory agents). It is believed that the evolutionary drive for the plant to develop its spiciness was as a chemical defence against pathogens (Tewksbury et al., 2008). Chilli peppers contain various active compounds such as carotenoids and flavonoids, but most known are the phenolic compounds capsaicinoids (and capsinoids), currently known to be uniquely present only in the *Capsicum* genus (Figure 3). The two former are responsible for the colour of the pepper, while the latter for the characteristic pungency (Antonio et al., 2018).

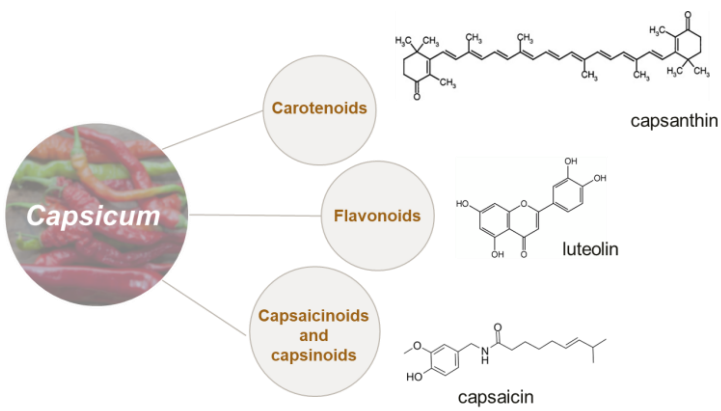


Figure 3. Active compounds in chilli peppers and an example of each group.

The structure of capsaicinoids consists of a vanillyl group and an acyl chain connected with an amide bond and have been shown to determine the pungency (Castillo et al., 2007). Variations can occur in the acyl chain, which can be saturated or unsaturated, branched or linear (Figure 4). The amphiphilic structure of capsaicinoids is important as the bioactivity in these compounds is credited to the vanillyl group. This is due to its reactivity with enzymes and the nervous receptor Transient Receptor Potential Vanilloid type 1 (TRPV1), while the lipophilic acyl tail interacts and directs the compound to cell membranes (Antonio et al., 2018). Capsaicinoids are practically insoluble in water, but are soluble in solvents with low polarity like methanol, ethanol and acetonitrile which are used for their extraction.

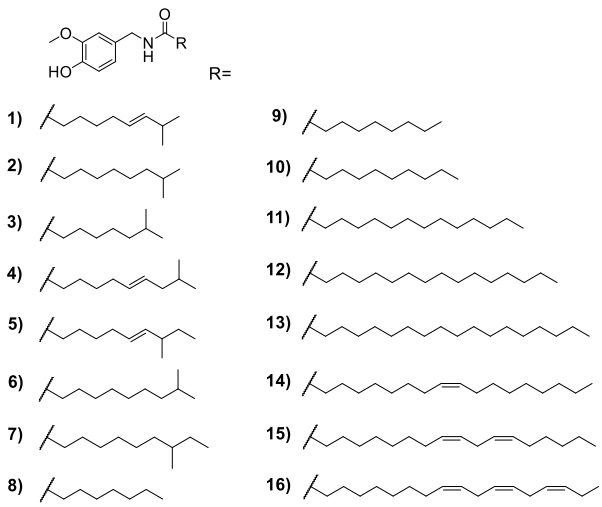


Figure 4. Plant derived capsaicinoids. 1) capsaicin, 2) dihydrocapsaicin, 3) nordihydrocapsaicin, 4) homocapsaicin I, 5) homocapsaicin II, 6) homodihydrocapsaicin I, 7) homodihydrocapsaicin II, 8) *N*-vanillyl octanamide, 9) *N*-vanillyl nonamide (nonivamide), 10) *N*-vanillyl decanamide, 11) *N*-vanillyl tetradecanamide (myrvanil), 12) *N*-vanillyl hexadecanamide (palvanil), 13) *N*-vanillyl octadecanamide (stevanil), 14) olvanil, 15) linivanil, 16) linvanil.

Early analysis of extracts of capsaicinoids from *C. annum*, indicated that the natural pungent material was a mixture of at least five closely related amides. The approximate composition was mostly capsaicin (69%), followed by dihydrocapsaicin (22%), nordihydrocapsaicin (7%), homocapsaicin (1%) and homodihydrocapsaicin (1%) (Bennett and Kirby, 1968). Others have reported that that *C. frutescens* peppers contain approximately equal fractions of capsaicin (47%) and dihydrocapsaicin (53%) (Leete and Louden, 1968). Later, other determinations of capsaicinoids confirmed that the composition and content of capsaicinoids varies greatly in different *Capsicum* species (Suzuki and Iwai, 1984). Overall, the content of the major components capsaicin and dihydrocapsaicin can correspond to 89-98%, and the rest consists of other reported minor capsaicinoids such as nordihydrocapsaicin, homodihydrocapsaicin, homocapsaicin and nonivamide (Antonio et al., 2018) (Figure 4).

Capsaicinoids and their analogues have been reported to have antimicrobial and antivirulence properties (Fuchtbauer et al., 2021, Marini et al., 2015), insecticidal properties (Claros Cuadrado et al., 2019) antioxidant activity (Materska and Perucka, 2005), and are agonists to the TRPV1 receptor (pain desensitization), with applications in treating neuropathic pain (Peppin and Pappagallo, 2014) and as anticancer agents (Friedman et al., 2018). They also have applications in the food industry, cosmetics, as self-protection aerosol sprays and bio-pesticides (Aza-Gonzalez et al., 2011).

Scope and outline of the thesis

The work in this thesis is focused on the biosynthesis of capsaicinoids, particularly nonivamide, in a microbial platform strain *Saccharomyces cerevisiae*, through the expression of several heterologous genes coding for amine and amide forming reactions for the capsaicinoid biosynthesis pathway. As such, this work aims to open up the possibility of achieving efficient whole-cell biocatalytic production, as an alternative to plant- or organic chemistry-based production strategies. This work contributes to the field by (i) exploring conditions for efficient *in vivo* transamination of vanillin to vanillylamine, without the addition of alanine as an amine donor and decreasing by-product levels, as well as (ii) identifying amide-forming enzymes for nonivamide production using whole-cells. Challenges that were addressed included: the toxicity of chosen substrates and products to the host cell, conditions for effective transamination, the choice of enzyme cascade for the amidation steps, as well as the potential of utilizing *S. cerevisiae* as a biosensor for TRPV1 receptor modulators.

Paper I evaluated the potential of *S. cerevisiae* to produce nonivamide from supplemented precursors. Several plant- and bacterial-derived heterologous genes were expressed for enzymes catalysing the amide-forming reactions and successful whole-cell nonivamide production was achieved.

Paper II demonstrates engineering of recombinant *S. cerevisiae* for catalysing reductive amination of vanillin to vanillylamine. This was combined with the amide-forming reactions from Paper I and nonivamide production from supplemented vanillin and a fatty acid was achieved.

Paper III explores the potential of engineered *P. putida* for transamination of vanillin and ferulic acid to vanillylamine, and a method for screening of recombinant transaminases was developed.

Paper IV addresses the potential of *S. cerevisiae* as a biosensor for studying TRPV1 modulation *in vivo,* with the use of flow cytometry and a calcium binding dye as a tool for strain development.

Chapter 2
Production of capsaicinoids

There are different ways of producing capsaicinoids (Figure 6), such as extraction from the chilli plant itself, chemical synthesis or bio-based synthesis, either by enzymes or whole-cells, and each has their advantages and disadvantages. Natural products are not usually produced at levels that are directly commercially feasible, and extractions from the plant can give low yields. Environmental conditions additionally impact the accumulation of capsaicinoids in the plant. Capsaicin analogues, natural and unnatural, could be synthesised chemically from vanillin through reductive amination to vanillylamine and chemical amidation of vanillylamine to capsaicin, but this could involve strong conditions and handling of chlorinated compounds, metals and organic solvents. Biotechnological advances have allowed the use of various methods for synthesis of natural products, such as the use of enzyme like lipases, and recombinant production hosts.

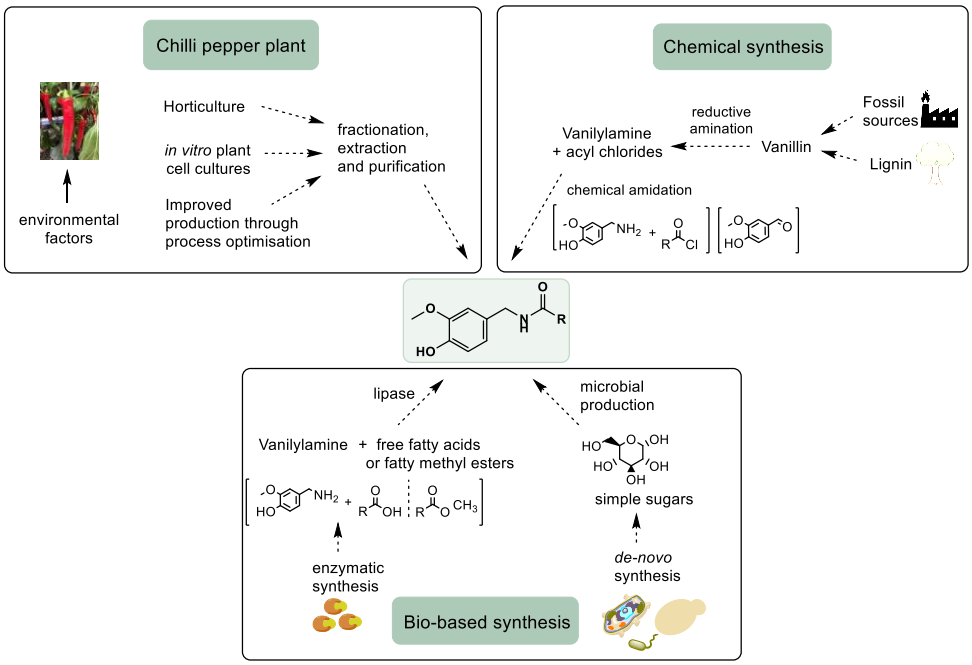


Figure 6. Ways of synthesizing capsaicinoids.

Chilli pepper plant

The capsaicinoids (and capsinoids) are synthesised in the epidermal cells of the interlocular septum, formed from the tissue connecting the placenta and pericarp of the pepper fruit in specific compartments called blisters, only present in pungent fruits (Stewart et al., 2007).

The biosynthesis of these compounds in the plant involves two main pathways: the phenylpropanoid pathway and the branched chain fatty acid pathway (BCFAP) (Figure 7). The phenylpropanoid pathway determines the structure of the vanillyl moiety, while the BCFAP determines the fatty acyl chain length and branching. Starting from phenylalanine through the phenylpropanoid pathway, vanillylamine is produced from vanillin in a reaction catalysed by a vanillin aminotransferase (VAMT) [EC Number: 2.6.1.]. On the other side, from valine, leucine or isoleucine (depending of branching) through the branched chain fatty acid pathway, a branched fatty acid (BCFA) is formed and activated by a CoA ligase enzyme - Acyl-CoA synthetase (ACS) [EC- 2.3.1.86]. Finally, capsaicinoids are produced in a condensation step between vanillylamine and the activated BCFA, catalysed by an acyltransferase enzyme named capsaicin synthase (CS) [Accession number: Q58VT0] (Arce-Rodriguez and Ochoa-Alejo, 2019).

Vanillin can also be converted to vanillin alcohol which then through a condensation reaction with a CoA-activated BCFA catalysed by the CS leads to the formation of the ester. In this case, the ester (e.g. capsiate) is another type of compound in *Capsicum*, representing the capsinoids (Kobata et al., 2013b). Even though the capsaicinoid biosynthesis pathway has been researched extensively for a long time, some questions regarding the function of uncharacterised putative enzymes still remain (Arce-Rodriguez and Ochoa-Alejo, 2019).

The plant produces a mixture of capsaicinoids, therefore extraction of separate compounds in high purity from the chilli plant could prove challenging. In the case of plant derived products, conventional extraction techniques involve the use of organic solvents combined with heat treatment and mixing. There are also non-conventional methods which are: ultrasound-assisted extraction, pulse-electric field extraction, enzyme-assisted extraction, microwave assisted extraction, pressurised liquid extraction and supercritical fluid extraction. However, the sensitivity, selectivity and separation of the methods, and complexity of the sample matrix remain as challenges to be addressed (Azmir et al., 2013, Lefebvre et al., 2021). For large-scale production of pure bioactive compounds, extraction is not the most optimal way of production due to low yields, higher costs and complex purification steps. Additionally, due to the side-effects exhibited by capsaicin in clinical trials (Wong and Gavva, 2009), less-potent analogues or unnatural capsaicinoids are of pharmaceutical interest for synthesis, which cannot be extracted from the plant.

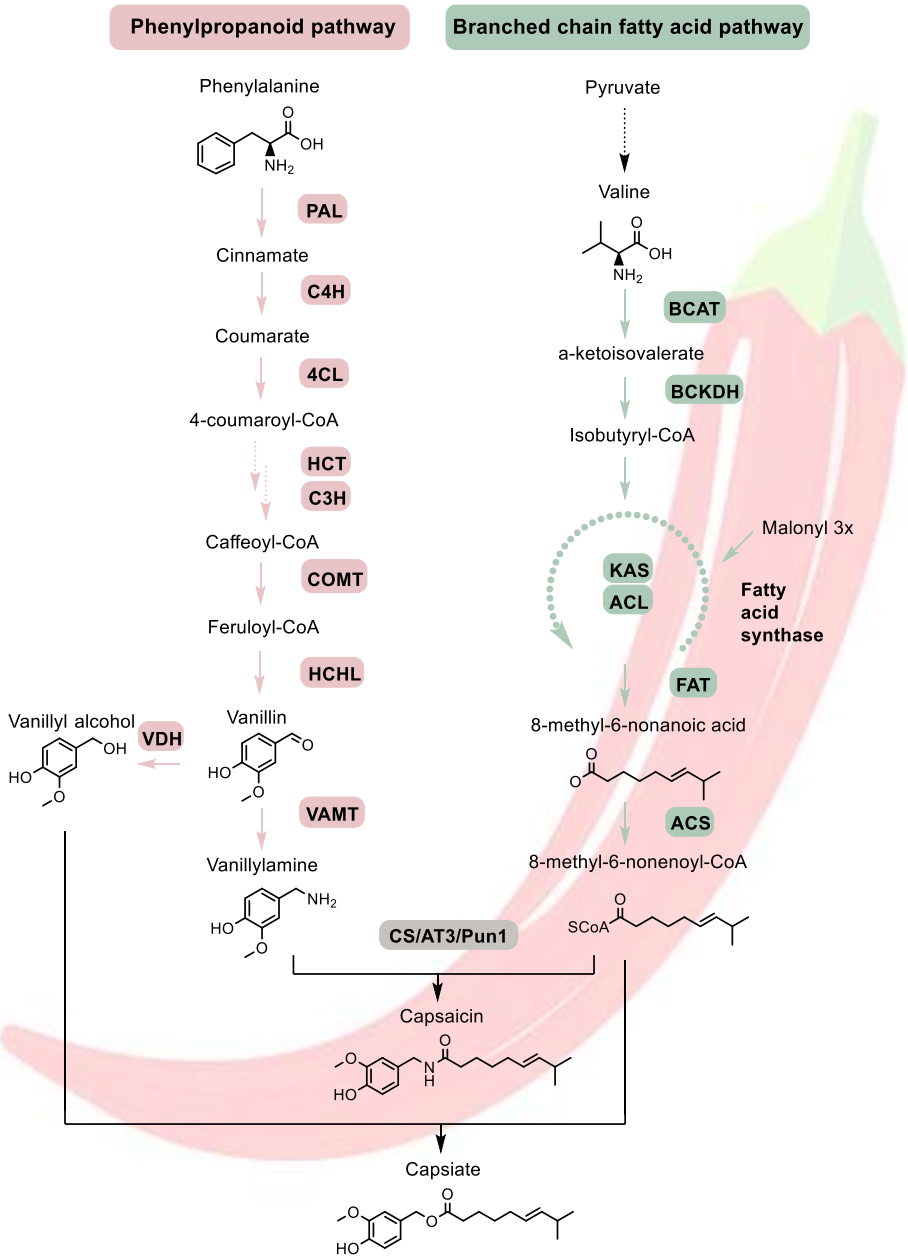


Figure 7. Synthesis of capsaicinoids in the chilli plant is through the phenylpropanoid pahtway (marked in pink) and the branched chain fatty acid pathway (marked in green). PAL (phenylalanine ammonia-lyase), C4H (cinnamate-4-hydroxylase), 4CL (4-coumaroyl-CoA ligase), HCT (hydroxycinnamoyl transferase), C3H (coumaroyl shikimate/quinate 3-hydroxylase), COMT (caffeoyl-CoA O-methyltransferase), HCHL (hydroxycinnamoyl-CoA hydratase/lyase), VDH (vanillin dehydrogenase), VAMT (vanillin aminotransferase), BCAT (branched-chain amino acid transferase), BCKDH (branched-chain α-ketoacid dehydrogenase/decarboxylase), KAS (ketoacyl-ACP synthase), ACL (acyl carrier protein), FAT (acyl-ACP thioesterase), ACS (acyl-CoA synthetase), CS (capsaicinoid synthase)/ AT3 (acyltransferase)/ Pun1 (putative acyltransferase).

Moreover, capsaicin and dihydrocapsaicin are the most prevailing capsaicinoids (~90%) in *Capsicum* sp., differing only by one double bond in the acyl group (Bennett and Kirby, 1968, Leete and Louden, 1968), which limits the extraction to predominately those two compounds. Additionally, environmental factors greatly affect the capsaicinoid biosynthesis and accumulation. Genotype and maturity stage, as well as external factors such as soil quality, location, humidity, water availability, atmospheric CO_2, temperature and light exposure, all influence the activity of enzymes, content and levels of capsaicinoids (Arce-Rodriguez and Ochoa-Alejo, 2019, Uarrota et al., 2021). To reach high titres, a lot of resources for farming and use of arable land would be required.

An alternative could be plant tissue culture techniques (Wawrosch and Zotchev, 2021). In plant biotechnology, chilli pepper cells, tissues or organs have been engineered using different strategies for enhanced *in vitro* biosynthesis of capsaicinoids, such as: treatments with elicitors of secondary metabolites, light regimes, stress (osmotic, pH, nutrient), immobilization of cells, precursor or intermediate feeding (Kehie et al., 2015). Osmotic stress and precursor feeding have given high product accumulation when compared to control conditions (Kehie et al., 2015). More recently, in a study on cell suspension cultures of *C. chinense* upon elicitation using chitosan, 2.9 mg g^{-1} fresh weight (FW) capsaicin and 1.0 mg g^{-1} FW dihydrocapsaicin have been obtained (Kabita et al., 2020). However, reached levels were still lower than the levels found in the fruit, and it is questionable if *in vitro* plant synthesis of capsaicinoid could outcompete plant extraction (Wawrosch and Zotchev, 2021). Further developments are therefore needed involving genetic and metabolic engineering as well as large-scale bioreactor cultivations (Kehie et al., 2015).

Chemo-enzymatic synthesis

An alternative to extraction from the chilli pepper fruit is chemical synthesis, allowing preparation of less common and unnatural capsaicinoids. In this case, capsaicinoids are prepared from vanillin, derived from guaiacol, coming from fossil resources or lignin as a more sustainable alternative (Pelckmans et al., 2017). Vanillin is a commercially valuable aromatic compound used mainly in flavouring and fragrance. The global market for vanillin in 2019 was estimated at around 480 million dollars and rising with an annual growth rate of around 7% (Global industry report ID: GVR-1-68038-836-7, source www.grandviewresearch.com, accessed 11/2022). Naturally vanillin comes from the vanilla orchid beans (*Vanilla planifolia*); however, production from the plant is expensive, time-consuming and gives low yields. Synthetic vanillin for commercial purposes can be produced from petroleum-derived guaiacol, reaching much cheaper prices ($15/kg) compared to plant-derived natural vanillin ($1.200-4.000) (Walton et al., 2003). Recently as a more sustainable way of production, a by-product from pulp and paper industry, industrial kraft lignin has been utilised for production of valuable chemicals. One method has been vanillin production through batch oxidation of lignin (3.5-7.6% yields) (Araújo et al., 2010). More recently, vanillin and other valuable phenolics were produced by base-catalysed (NaOH) depolymerisation of industrial lignin in continuous mode (Abdelaziz et al., 2017).

Capsaicin prepared by chemical synthesis can be present both as *E* and *Z* isomers due to the double bond in the acyl chain, while the latter are not found in nature. This issue has been addressed by development of stereoselective synthetic routes for the acylation process. An example is by using orthoester Claisen rearrangement where mostly *E* isomer alkanoates (ester precursors to capsaicinoids) were obtained from allylic alcohols (Kaga et al., 1996). The Claisen rearrangement involved heating triethyl orthoacetate at 138ºC for 3 hours, in the presence of propionic acid. The esters were then hydrolysed to obtain carboxylic acids, which were treated with thionyl chloride leading to formation of acyl chlorides (Kaga et al., 1996). Vanillylamine can be obtained by reducing vanillin oxime solution in methanol using zinc dust and ammonium formate (Abiraj and Gowda, 2003). Reductive amination of vanillin to vanillylamine can be also performed in a solvent-free method with Rh/Al_2O_3 catalyst at 80°C and under 20 bar H_2 pressure for 2 hours, reaching yields of 91% vanillylamine with 9% of vanillyl alcohol as by-product (Chatterjee et al., 2016).

Finally, an early method for capsaicinoid synthesis was involving vanillylamine in dry ether (diethyl ether) and acyl chlorides (Nelson, 1919). This is known as Schotten-Baumann reaction by using acyl chloride, an amine and base, used for production of amides, and has been shown to give yields of 48-63% for capsaicinoid synthesis (Barbero et al., 2010). Other protocols have been developed for long-chain

polyunsaturated acids with good results by acylation of vanillylamine with mixed phosphoric anhydrides instead of acyl chlorides (Appendino et al., 2002).

The interest of capsaicin and its analogues for the food and pharmaceutical industry has led to developments in their synthesis procedures, using milder conditions and less hazardous compounds by introducing enzyme catalysis. Enzymatic amidation using lipases has been demonstrated to catalyse the condensation reaction between vanillylamine and free fatty acids or methyl/ethyl esters in an organic solvent (Castillo et al., 2007). A more sustainable approach for synthesis of capsaicinoids in one pot involving multi-enzyme cascade has been described from vanillyl alcohol and vanillin, derived from lignin (Figure 8) (Anderson et al., 2014). This work demonstrated an enzymatic approach for the catalysis of vanillin from vanillylamine using an aminotransaminase (ATA), *L*-alanine as amine donor and an *L*-alanine dehydrogenase (L-ADH) to recycle the amine donor, with ammonia as nitrogen source, reaching 95% conversion after 17 hours. Vanillin could be acquired from vanillyl alcohol using palladium nanoparticles on controlled pore glass as catalysts for aerobic oxidation of vanillyl alcohol reaching >99% conversion after 2 hours. An enzymatic alternative to Pd has been shown using an alcohol dehydrogenase (ADH) in combination with the ATA enzyme cascade to oxidise vanillyl alcohol to vanillin, which is then converted to vanillylamine, with 61% conversion after 22 hours (Anderson et al., 2014). Pure vanillylamine was then combined with acyl chlorides under Schotten-Baumann conditions (>91% yields) or lipase catalysed amidation (52% nonivamide yield), to reach synthesis of capsaicinoids (Anderson et al., 2014).

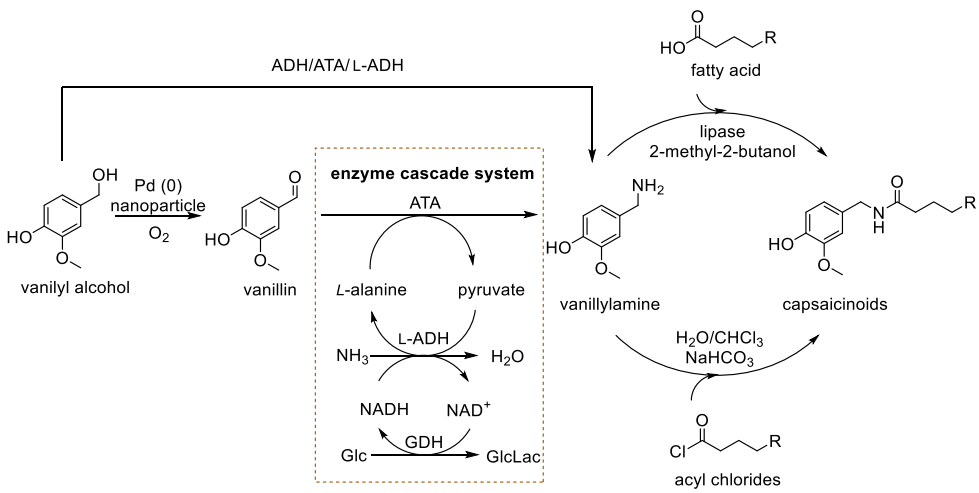


Figure 8. Synthesis of capsaicinoids using a chemo-enzymatic or a multi-enzymatic approach in one pot, *in vitro*. Figure adapted from (Anderson et al., 2014). ADH (alcohol dehydrogenase), ATA (aminotransaminase), L-ADH (L-alanine dehydrogenase), GDH (glucose dehydrogenase).

Microbial biosynthesis

De novo microbial biosynthesis of capsaicinoids has not been achieved yet. Microbial production of the capsaicinoid precursor vanillin and vanillin *β*-glycoside from glucose has already been established successfully in yeast (Figure 9) (Hansen et al., 2009, Brochado et al., 2010). Baker's yeast can produce 3-dehydroshikimate from glucose through the shikimate pathway. This metabolite was then directed towards vanillin through a heterologous enzyme cascade involving 3-dehydroshikimate dehydratase (3DSD) (*Podospora pauciseta*; EC 4.2.1.118), aryl carboxylic acid reductase (ACAR) (*Nocardia* sp.*;* EC 1.2.1.30), and two transferases (EC 2.1.1.6) phosphopantetheine transferase (PPT) (*Corynebacterium glutamicum*) and *O*-methyltransferase (OMT) (*Homo sapiens*). Fermentative production from glucose with recombinant yeast expressing the four heterologous was reported to have reached 45 mg/L vanillin (Hansen et al., 2009). Vanillin can be inhibitory to the cells, but glycosylating it has been shown to reduce the inhibitory effect, possibly due to better solubility and secretion. For this reason, a UDP-glucosyltransferase (UGT) (*Arabidopsis thaliana*; EC 2.4.1.35) has been overexpressed for the production of vanillin-β-glucoside, increasing the tolerance from 0.5 g/L vanillin to 25 g/L vanillin β-glucoside (Hansen et al., 2009). The yeast carries endogenous *β*-glucosidase activity, but a deletion of the BGL1 (*β*-glucosidase 1) has been shown to reduce this activity; although some hydrolysis (5%) has been seen likely due to remaining glycosidase or promiscuous enzymes (Hansen et al., 2009).

A major challenge to produce capsaicinoids in yeast is to achieve the amination of vanillin to vanillylamine (Figure 9). ATAs can catalyse this reaction; however, due to the reversible nature of the reaction, it can prove difficult. This will be thoroughly discussed in the following Chapter 3 as well as the solutions to the challenges with this reaction will be presented. The final and key enzymes in the formation of capsaicinoids are CoA- ligase (CL) - performing the activation of the free fatty acid and *N*-acyltransferase (NAT) - catalysing the formation of the amide bond (Figure 9). A combination of these enzymes was used in this thesis for the production of a model capsaicinoid (nonivamide). These reactions will be further addressed in Chapter 4.

Another challenge in the production of capsaicinoids is related to the aliphatic side chain (Figure 9). The carbon-chain length, branching and saturation of the FA precursor of capsaicin (8-methyl-6-nonanoic acid) makes synthesis of the compound by the yeast rather complex; however, strategies for achieving this have been previously discussed (Muratovska et al., 2022). Yeast can produce α-ketoisovalerate from pyruvate involving several enzymes (encoded by *ILV2, ILV5, ILV3*) (Lee et al., 2012). α-Ketoisovalerate is a branching compound that can be converted to valine [by branched-chain amino acid aminotransferase–BCAT (EC Number: 2.6.1.42)], or used for BCFA synthesis. In *S. cerevisiae* BCAT has two

homologues, Bat1 and Bat2 (Takpho et al., 2018). This enzyme is part of the branched-chain amino acid (BCAA) breakdown pathway (Ehrlich pathway) (Hazelwood Lucie et al., 2008). Valine can be deaminated to form α-ketoisovalerate, which is followed by a decarboxylation producing a fusel aldehyde and further to a fusel alcohol. The oxidative *α*-keto acid decarboxylase activity in yeast would need to be suppressed in order to accumulate α-keto acids (such as α-ketoisovalerate) as precursors for medium or long-chain branched-chain (BC) FA (Muratovska et al., 2022). The next reaction to isobutyril-CoA by isovalerate dehydrogenase has not been annotated in *S. cerevisiae*. The BC *α*-keto acid dehydrogenase (BCKDH) complex in eukaryotes is located in the mitochondria (Mazourek et al., 2009) and it requires lipoylation in one of the subunits for proper function. Heterologous expression together with a lipolyation system in the yeast cytosol might be needed in order to achieve the reaction to reach isobutyril-CoA (Muratovska et al., 2022).

S. cerevisiae expresses a hexameric FAS system in the cytosol producing mainly straight-chain 16 and 18 carbon chain lengths (Tehlivets et al., 2007). The cytosolic FAS consists of the subunits Fas1 and Fas2, with each performing several enzymatic activities. Fas1 carries acetyl transferase, enoyl reductase, dehydrogenase, malonyl-palmitoyl transferase activities and Fas2 with acyl carrier protein, 3-ketoreductase, 3-ketosynthase and phosphopantheteine transferase activities (Tehlivets et al., 2007, Klug and Daum, 2014). There is also a mitochondrial FAS II system in yeast, where the enzymatic activities are carried by individual proteins (Tehlivets et al., 2007). The elongation (by Elo2p and Elo3p elongases) and desaturation (by acyl-CoA Δ9-desaturase Ole1p) happens in the endoplasmic reticulum (Klug and Daum, 2014).

To reach branching of fatty acids in *S. cerevisiae* it would be required to engineer the FAS system to accept starting precursors for BCFA synthesis. An β-ketoacyl synthase (KAS) condenses a branched acyl-CoA to malonyl-ACP for the extension of the acyl chain. Bacterial KASIII (FabH) (Choi et al., 2000) could initiate the synthesis by condensing a branched acyl-CoA with malonyl-ACP, controlling the methylation, but has been reported not able to incorporate precursors for BCFA synthesis in *S. cerevisiae* (Kaneda and Smith, 1980). For controlling the chain length in *S. cerevisiae*, heterologous thioesterases (TE) can be expressed. The activity of various thioesterases has been greatly studied for medium-chain fatty acid production in *E. coli* (Zheng et al., 2012, Hernandez Lozada et al., 2018, Deng et al., 2020). Synthesis of medium chain FA (C6-C12) in *S. cerevisiae* has been achieved through a complex approach by engineering of both the yeast-native FAS and the bacterial FAS I, adaptive laboratory evolution of the strain and directed evolution of a membrane transporter Tpo1 (Zhu et al., 2020, Zhu et al., 2017).

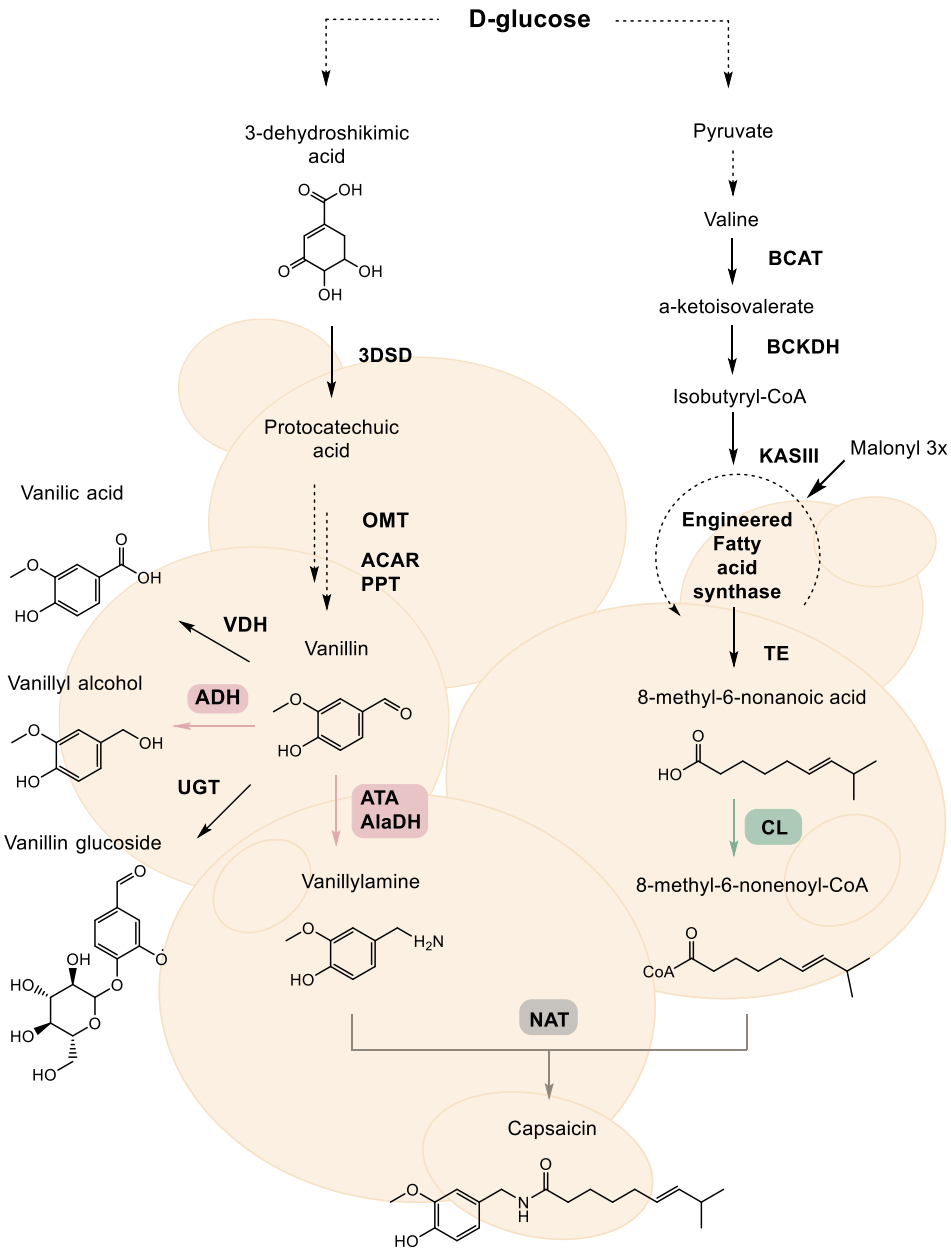


Figure 9. Synthesis of capsaicinoids and potential reactions involved in *de novo* production from glucose in *S. cerevisiae*. The enzymes marked with pink, green and gray boxes are of interest in in this thesis. 3DSD (3-dedhydroshikimate dehydratase), OMT (*O*-methyltransferase), ACAR (aryl carboxylic acid reductase), PPT (phosphopantetheine transferase), VDH (vanillin dehydrogenase), ADH (alcohol dehydrogenase), UGT (UDP-glucosyltransferase), ATA (aminotransaminase), AlaDH (alanine dehydrogenase), BCAT (branched-chain amino acid transferase), BCKDH (branched-chain α-ketoacid dehydrogenase/decarboxylase), KASIII (ketoacyl-ACP synthase III), TE (acyl-ACP thioesterase), CL (CoA ligase), NAT (*N*-acyltransferase).

In this thesis, the steps which have been addressed for reaching the complete synthesis of capsaicinoids are: (i) the transamination of vanillin to the direct precursor vanillylamine, (ii) preventing by-product formation and (iii) the amide bond formation with an activated free fatty acid. These parts of the pathway will be expanded further in the following two chapters. Using a whole-cell biocatalytic system for production from simple sugars in a fermentation setup has the potential to become an economically and environmentally-friendly competitive method of production. Through the use of heterologous enzymes with the right activity, a variety of novel capsaicinoids could be produced in the future, having potentially new and improved bioactivity.

Chapter 3
In vivo transamination of vanillin to vanillylamine

ω-Transaminases

Compounds containing an amine group have various functions (e.g. amino acids, alkaloids, polymers) and are important building blocks for pharmaceuticals and agricultural compounds (Breuer et al., 2004, Roughley and Jordan, 2011). ω-Transaminases (TAs) or aminotransaminases (ATAs) are valuable enzymes that catalyse the production of amino acids and amines, using a ping-pong mechanism by transferring an amine group from an amino donor to an amino acceptor in a two-step process (Guo and Berglund, 2017). In the first part of the reaction, the amine donor is deaminated and released as a co-product, while in the second part, an amine acceptor (keto acid, ketone, aldehyde) is aminated producing an amino acid or amine product (Figure 10). A co-factor pyridoxal 5'-phosphate (PLP), i.e. the phosphorylated form of vitamin B_6, is assisting the transamination reaction by carrying the amine group. An important advantage of PLP is that it is being recycled in the process, so there is no need for addition of co-factors. These enzymes have high stereoselectivity, and can operate in aqueous conditions, ambient temperature and pH. They have therefore been found very useful for biocatalytic production of several chiral amines in the pharmaceutical industry, with an important example being the antidiabetic drug sitagliptin (Savile et al., 2010, Kelly et al., 2018).

Due to the reversibility of the reaction, a common challenge with transaminases is the direction of the thermodynamic equilibrium. This is dependent on the structure of the substrate and products, and can be predicted by a group contribution model (Jankowski et al., 2008). In the case for the conversion of vanillin to vanillylamine, the thermodynamic equilibrium lies in the direction towards vanillin if amino acids (e.g. alanine) are used as amine donors (Weber et al., 2014b). There are ways of impacting the reaction equilibrium by the choice of amine donor substrate, addition of excess amino donor, *in situ* product removal and preventing (co-)product inhibition by multi-enzyme systems (Koszelewski et al., 2010b, Shin and Kim, 1999, Hohne et al., 2008, Truppo et al., 2009, Tufvesson et al., 2011).

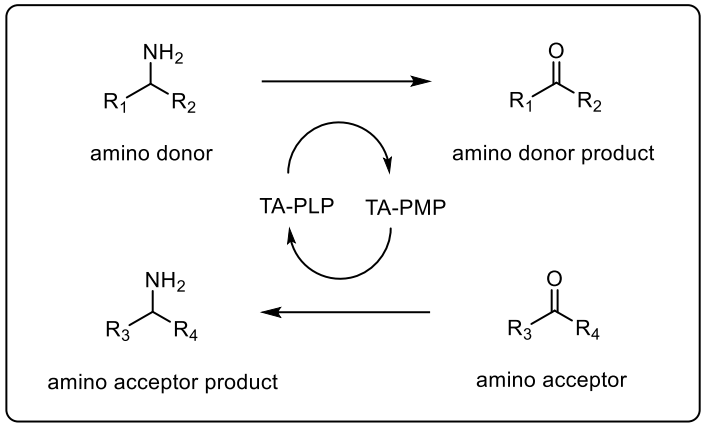


Figure 10. Transamination by a transaminase enzyme with pyridoxal 5'-phosphate (PLP). TA-PLP, transaminase with covalently bound PLP; TA-PMP, transaminase with non-covalently bound pyridoxamine 5' phosphate (PMP). Figure adapted from review (Guo and Berglund, 2017).

Alanine has been a commonly used amine donor in ATA catalysed reactions due to its price and acceptance by many enzymes; however, it can impact the reaction equilibrium towards the substrate (Kelly et al., 2018). Other amine donors such as isopropylamine (IPA), 1-phenylethylamine (or (*R*)-(+)-α-methylbenzylamine (MBA)), and co-called "smart" diamine donors (e.g. cadaverine) whose product can dimerise or cyclise, have been utilised to improve the reaction equilibrium (Kelly et al., 2018, Gomm et al., 2016). Some of the unfavourable equilibrium issues have been addressed by the addition of another enzyme in the reaction (Sánchez-Moreno et al., 2012) when *L*-alanine was used as an amine donor, in order to remove the co-product pyruvate. Combining the amino transaminase together with a lactate dehydrogenase (LDH) to reduce the pyruvate to *L*-lactate has been shown to significantly improve the reaction rate (Shin and Kim, 1999). The LDH requires an NADH-recycling system though, so combining LDH with a NADH recycling step by a glucose dehydrogenase (GDH) has also been done (Fuchs et al., 2010, Truppo et al., 2009). Instead of the LDH, using an amino acid dehydrogenase (AADH) coupled with the GDH co-factor recycling to convert pyruvate back to *L*-alanine, has been shown to have the advantage of generating the amine donor *in situ* (Truppo et al., 2009). Alternatively, pyruvate decarboxylase (PDC) has been proposed for the same purpose of removing pyruvate by converting it to the volatile acetaldehyde and carbon dioxide (Hohne et al., 2008). When having several enzymes in the reactions and multiple products formed, the pH of the reactions needs to be considered and the reaction conditions have to be compatible. The approaches above propose using enzyme cascades *in vitro*, so overexpression of a range of enzymes in whole-cells for efficient transamination could be an alternative (Tufvesson et al., 2011), as it removes the need for enzyme purification and co-factor addition and will be discussed further in this chapter.

Vanillin aminotransaminases

Vanillin aminotransferase from Capsicum (VAMT)

Studies on this aminotransferase (EC 2.6.1.-) from the chilli pepper plant have been mostly directed towards studying the gene expression and its positive correlation to capsaicin accumulation in the plant (Burgos-Valencia et al., 2020). The *Capsicum chinense* vanillin aminotransferase (VAMT, or Cc-ATA) has been previously characterised using a purified enzyme resulting in high transaminase activity catalysing the conversion of vanillylamine to vanillin (Weber et al., 2014b). In the case of vanillin as a substrate, almost no vanillylamine from vanillin had been detected with an excess of *L*-alanine as amine donor (Weber et al., 2014b). Regarding its use in whole-cells, the Cc-ATA was shown to have *in vivo* transaminase activity when used for kinetic resolution of racemic 1-phenylethylamine in *S. cerevisiae* (Weber et al., 2014a).

Chromobacterium violaceum transaminase (Cv-ATA)

The *Chromobacterium violaceum* transaminase (Cv-ATA) has been previously reported to carry activity towards vanillin, among several other ketoacids and aldehydes (Kaulmann et al., 2007). This Cv-ATA and several other ATAs have also been assessed for amine synthesis using lyophilised recombinant *E. coli* cells, where the Cv-ATA showed the highest activity (Koszelewski et al., 2010a). As part of Paper III the Cc-ATA and Cv-ATA enzymes were both characterised for whole-cell vanillin bioconversion in *Pseudomonas putida*, resulting in some vanillylamine produced, mainly from Cv-ATA (Manfrao-Netto et al., 2021), demonstrating superior activity compared to the chilli peppers Cc-ATA. Furthermore, in *P. putida* several native transaminases were identified (Pp-SpuC-I, Pp-SpuC-II and Pp-ATA) and evaluated for activity together with the heterologous Cv-ATA and Cc-ATA. The highest specific transaminase activity against vanillylamine was again detected for Cv-ATA, as well as Pp-Spuc-II (Paper III). Due to its high transaminase activity, the Cv-ATA was introduced in a *S. cerevisiae* strain and in order to drive the reaction equilibrium towards the product, the strain was engineered to express multiple copies of a Cv-ATA (6x). This yeast strain demonstrated successful reductive amination of vanillin to vanillylamine (Paper II).

Growth-based screening and sequence-based analysis

Vanillin is one of the aromatic compounds that *P. putida* can use as a carbon source, through the activity of vanillin dehydrogenase and reductases it can be metabolised to vanillic acid or vanillyl alcohol and assimilated (Luengo and Olivera, 2020). It has been previously seen that vanillylamine can also be used as a carbon source, converted to vanillin by native aminotransferases and then further catabolised (Flagan and Leadbetter, 2006). This knowledge was used for developing a growth-based method for screening for transaminases with activity towards vanillin. The

thermodynamic equilibrium is towards vanillin, so the transamination reaction should be readily performed. Laboratory strain KT2440 was grown in liquid M9 medium with glucose, vanillin or vanillylamine as carbon sources (Paper III). The strain grew with glucose and vanillin but no growth was measured with vanillylamine as the only carbon source during 48 hours. This indicated that there was low level or no expression of endogenous ATAs that carry activity towards vanillylamine to enable growth on it as sole carbon source. Bioinformatics sequence-based analysis was used to identify several endogenous ATAs (Pp-SpuC-I, Pp-SpuC-II and Pp-ATA) and these were individually overexpressed in *P. putida* KT244. From the three native transaminases, overexpression of the native Pp-SpuC-II enabled growth of the KT2440 on vanillylamine as a single carbon source, showing that it carries the desired activity. The growth-based screening method was also used on solid agar media, with vanillylamine as a sole carbon source, showing potential to be coupled directly with the transformation protocol. It may be used in high-throughput screenings and evaluation of TAs by testing growth on specific amines as a carbon source (Paper III).

Depending on the product of interest, finding enzymes with specificity for a particular substrate could be challenging. Screening for novel enzymes (*in silico* or activity-based) or modifying existing enzymes (random mutagenesis or rational design) could be viable approaches for finding specific transaminases.

Whole-cell transamination

The use of microbial cells for the conversion of a substrate to a product by either innate or heterologous enzyme or enzyme cascade is referred to as whole-cell biocatalysis. This process can occur as part of a fermentation from simple sugars or in a bioconversion setup with non-growing or growing cells. The choice of host organism should be based on several criteria, such as cultivation requirements, metabolism, tolerance to substrate and product in question, tolerance to stress, availability of genetic engineering tools, and safety. In this work, the potential of both bacteria (*Pseudomonas putida*) and yeast (*Saccharomyces cerevisiae*) for reductive amination of vanillin to vanillylamine was demonstrated.

Using whole-cells as biocatalysts has several advantages due to the easy production of enzymes (e.g. in a fermentation process), lower cost, good enzyme stability in host organism as well as availability of co-factors and energy (Weber et al., 2017, Tufvesson et al., 2011). Due to the complex metabolism in the cell, the occurrence of potential side reactions and by-products should be prevented, as well as issues with competing pathways, toxicity and localisation should be considered. Some of these main challenges are addressed in this work.

Challenges addressed in the *in vivo* transamination of vanillin to vanillylamine

Effect of bioconversion reaction conditions

In Paper II using a *S. cerevisiae* strain overexpressing the Cv-ATA, it was shown that the conditions in which the whole-cell bioconversion is performed have a notable impact on the transamination reaction, as it impacted the activity of promiscuous native enzymes competing with the transamination reaction and affected the co-factor (NAD(P)H and NAD(P)+) balance in the cell (Figure 11). The reaction conditions tested in a strain expressing six copies of the Cv-ATA, were: (1) aerobic bioconversion with glucose as co-substrate, (2) anaerobic bioconversion with glucose as co-substrate, (3) aerobic bioconversion with ethanol as co-substrate and (3) anaerobic bioconversion with ethanol as co-substrate.

Aerobic conditions proved to be beneficial for the vanillin consumption rate, with all vanillin being consumed within 20 hours, but much of it was directed towards the by-products vanillyl alcohol and vanillic acid (Paper II). This is likely due to co-factor availability and regeneration during respiration, and high activity of native reductases and dehydrogenases. Both glucose and ethanol as co-substrates were consumed aerobically, although the latter at a slower rate. Glucose as a co-substrate positively affected the production of by-products and likely limited the transamination by the presence of higher levels of pyruvate generated both during glycolysis and in the transamination reaction from the deamination of *L*-alanine.

During anaerobic conditions the transamination reaction was slower and a large fraction of the vanillin was left in the medium; however, the molar yields of vanillylamine produced per vanillin consumed were higher than when compared to aerobic conditions (Paper II, Table 2). Almost no vanillic acid was produced anaerobically. Glucose was readily consumed anaerobically, while ethanol levels stayed stationary. In general, ethanol as a co-substrate gave higher yields of vanillylamine produced per vanillin consumed when compared to glucose as a co-substrate, as well as less by-product formation. The highest yield of vanillylamine per consumed vanillin and the least amount of by-products were obtained during anaerobic conditions with ethanol as a co-substrate, when the cells were not growing, which also corresponds to the resting cells bioconversion with *P. putida*.

In *P. putida*, the bioconversion of vanillin to vanillylamine was performed with resting cells in a buffer and addition of *L*-alanine as amine donor (Paper III). This proved to decrease the formation of by-products when compared to the growing-cells condition, possibly due to limited regeneration of co-factors NAD(P)+ with uncoupled bioconversion to cell growth. In future work, the levels of co-factors (NAD(P)H and NAD(P)+) should be quantified, as well as comparing the metabolite profiles (e.g. using metabolomics) during the various conditions on ethanol and glucose with or without oxygen.

Recycling of amine donor and co-product removal

The low titres of vanillylamine, re-assimilation and by-product formation in *P. putida* were likely because of the unfavourable transamination reaction equilibrium in the cell (Paper III). Co-expression of an NADH-dependant alanine dehydrogenase from *Bacillus subtilis* (Bs-AlaDH) together with Cv-ATA was evaluated, as to whether this can help with removal of possibly accumulated pyruvate and for *L*-alanine recirculation. It has been shown previously that ATA/AlaDH enzyme cascade (*in vitro*) shifts the equilibrium by recycling the amine donor (Anderson et al., 2014, Truppo et al., 2009). Strains with and without Bs-AlaDH and with and without of addition of *L*-alanine were compared. The Bs-AlaDH gave a slight impact on the vanillylamine titres (0.70 with vs. 0.84 mM without). Nonetheless, removing *L*-alanine supplementation and using only ammonium as the sole nitrogen source, decreased the re-assimilation of vanillylamine and more vanillylamine was observed after 24h in this case (Paper III). The combination of Cv-ATA with Bs-AlaDH, was later also demonstrated for vanillylamine production in *E. coli* (Fu et al., 2021).

In order to drive the reaction equilibrium towards vanillylamine in a reductive amination of vanillin in *S. cerevisiae*, a strain was engineered to express multiple copies of a Cv-ATA (6x), together with the NADH-dependant alanine dehydrogenase from *Bacillus subtilis* (Bs-AlaDH) (Paper II) (Figure 11). In *S. cerevisiae* only expressing Cv-ATA, similarly to *P. putida*, the transamination reaction was difficult, especially during the anaerobic condition, likely due to inhibition by pyruvate. Introducing the Bs-AlaDH in *S. cerevisiae* indeed improved transamination and led to all vanillin being consumed in the anaerobic conditions. Additionally, omitting the amine donor in this strain overexpressing Cv-ATA with Bs-AlaDH in anaerobic conditions with ethanol as a co-substrate, improved the vanillylamine titre and yield (Paper II), as seen also with *P. putida* (Paper III). This indicates that Bs-AlaDH effectively removes pyruvate and converts it to *L*-alanine which drives the transamination reaction.

Preventing side reactions and by-products

In *P. putida*, formation of by-products vanillyl alcohol and vanillic acid and re-assimilation of the vanillylamine was detected at different levels in the strains after the transamination phase (~6 h). This indicates the presence of native activity on the substrate vanillin, even after the deletion of several dehydrogenases (Paper III).

There are native promiscuous alcohol dehydrogenases and reductases in *S. cerevisiae* competing with the aminotransaminase, that act on vanillin and either oxidise it to vanillic acid or reduce it to vanillyl alcohol (Wang et al., 2016). Their activity was impacted by availability of oxygen and choice of co-substrate, likely impacting the balance of co-factors NAD(P)H and NAD(P)+ present in the cell during the bioconversion reaction. In glucose-free anaerobic conditions, very little to no vanillic acid was produced. This could indicate of a lack of available NAD+,

likely due to inactive electron transport chain. However, there was still a significant amount of vanillyl alcohol produced (Paper II). ADH6 is an NAD(P)H-dependant alcohol dehydrogenase, found previously to reduce vanillin to vanillyl alcohol in yeast and to be responsible for aiding with vanillin resistance (Wang et al., 2016, Larroy et al., 2002). In agreement with a previous study (Hansen et al., 2009), a deletion of *adh6* proved to be beneficial for decreasing the level of vanillyl alcohol formation in both the condition with glucose and the condition with ethanol as co-substrate, especially with ethanol in anaerobic conditions (Paper II).

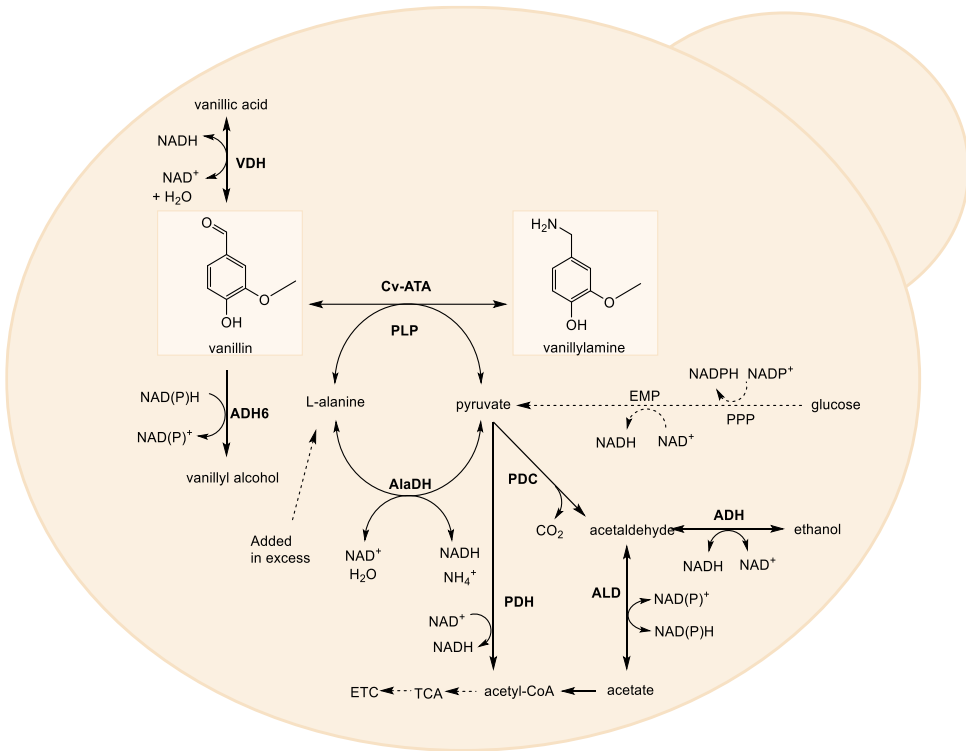


Figure 11. Reactions of interest in the transamination of vanillin to vanillylamine in yeast. VDH (vanillin dehydrogenase), ADH6 (alcohol dehydrogenase), Cv-ATA (*Chromobacterium violaceum* aminotransaminase), PLP (pyridoxal 5'-phosphate), AlaDH (alanine dehydrogenase), EMP (Embden-Meyerhof pathway), PPP (Pentose phosphate pathway), PDC (pyruvate decarboxylase), PDH (pyruvate dehydrogenase), ADH (alcohol dehydrogenase) ALD (acetaldehyde dehydrogenase).

Chapter 4
Amide formation by recombinant *S. cerevisiae*

N-acyltransferases and CoA-ligases

One of the ten key research areas in green chemistry is finding "general methods for catalytic/sustainable (direct) amide or peptide formation" (Bryan et al., 2018). Amide-bond formation is an important reaction in organic chemistry for production of many bioactive compounds and there is a requirement to perform it more efficiently and more environmentally-friendly. There is a need for finding efficient amide-forming enzymes with activity on a range of substrates. One biocatalytic approach for amide bond formation is by combining *N*-acyltransferase enzymes (NAT) and CoA-ligases (CL) in a whole-cell system (Figure 12), as described previously (Philpott et al., 2018). CLs use ATP and CoA which can be acquired from the cell metabolism, mainly from the glycolysis (Andexer and Richter, 2015). The CoA, besides synthesised in the cell, is also recycled during the amidation by NAT. In this work, heterologous NAT and CL enzymes were evaluated for their amide-bond forming activity in the production of capsaicinoids *in vivo* in *S. cerevisiae* (Paper I, Paper II).

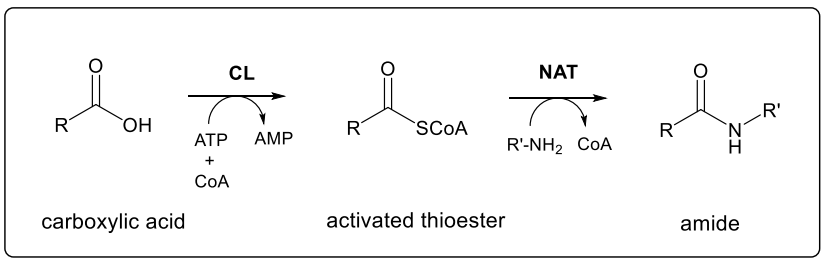


Figure 12. Enzymatic amide formation can be catalysed by two enzymes, a CoA-ligase (CL) and an *N*-acyltransferase (NAT). Figure adapted from (Philpott et al., 2018).

N-acyltransferases – for condensation of vanillylamine and Acyl-CoA to form capsaicinoids

Capsaicin synthase from Capsicum

In the chilli pepper plant, the final amide-forming step in capsaicinoid synthesis is believed to be performed by a capsaicin synthase (CS) encoded by *AT3* or *Pun1*, an *N*-acyltransferase (NAT) (EC 2.3.1.-), acting on a CoA-thioester and an amine. Initial experiments on *Capsicum* species had indicated that the accumulation of capsaicinoids in the placental tissue was correlated with the degree of pungency and the transcription of the enzymes studied in connection to the branched-fatty acid biosynthesis: vanillin aminotransferase (*vAmt)*, acyl carrier protein (*Acl*) and *β*-keto-acyl-ACP synthase (*Kas*) (Aza-Gonzalez et al., 2011, Aluru et al., 2003, Curry et al., 1999). Additionally, early results from a cDNA library of *C. chinense* genes showed SB2-149 and SB1-158 to be similar to *vAmt* and *Kas* genes, while SB2-66 and SB2-115 were related to the capsaicinoid biosynthesis and a hypothesis was made that the SB2-66 could be the capsaicinoid synthase (Minwoo et al., 2001, Aza-Gonzalez et al., 2011). Molecular mapping of pungent and non-pungent cultivars showed that locus C on chromosome 2 was essential for capsaicinoid production and quantitative trait loci (QTL) named *cap* regulated the quantity of capsaicinoids, but no genes were correlated with those loci (Blum et al., 2002, Blum et al., 2003). Until later, when the SB2-66 (also known as *AT3/Pun1*) was found to be in close proximity with the mapped QTL (Stewart et al., 2005). Furthermore, the recessive trait of non-pungency had been shown to be dependent on the presence of a gene named *Pun1,* coding for a putative acyltransferase (capsaicin synthase) in *Capsicum* plants and is therefore crucial for the production of capsaicinoids (Stewart et al., 2005). The *Pun1* gene was also connected to capsinoid synthesis (Han et al., 2013, Kobata et al., 2013a). Newer *in vitro* experiments using *C. annuum* protoplasts and anti-*Pun1* antibodies, that inhibit the biosynthesis, have confirmed that the gene product from *Pun1* is indeed a part of the capsaicin synthesis (Ogawa et al., 2015). However, a purified capsaicin synthase enzyme has not been structurally or biochemically assayed.

BAHD family of acyltransferases

The *Pun1* encoded enzyme belongs to the BAHD superfamily of acyltransferases (Stewart et al., 2005). These enzymes, which are specific for plants, use acyl-CoA donors to catalyse the transfer of an acyl group to an acceptor molecule. They are involved in production of plant secondary metabolites and achieving diversity of these compounds (D'Auria, 2006). It has been shown that even the biosynthesis of one of the most known alkaloid in plants, cocaine, involves a BAHD acyltransferase (cocaine synthase) in one of the final steps in the biosynthesis (Schmidt et al., 2015). The current knowledge suggests that these enzymes are located in the cytosol and they can acylate a wide range of substrates (D'Auria, 2006). With the first crystal

structure of a BAHD acyltransferase, vinorine synthase, light was shed on the catalytic mechanism and the significance of conserved regions (the HXXXD and DFGW motifs) (Ma et al., 2005) (Figure 13). Initially, many BAHD enzymes were recombinantly expressed mainly in *E. coli* with only a few in yeast strains, until one study expressed dozen of members of the BAHD family in *S. cerevisiae* for the synthesis of hydroxycinnamate and benzoate conjugates (Eudes et al., 2016). A more recent study showed that expressing a BAHD acyltransferase named HDT1, isolated from a red clover, together with a 4-coumarate:CoA ligase (4CL), led to the biosynthesis of clovamide and other analogues in *S. cerevisiae* and *L. lactis* (Bouchez et al., 2019). These studies show that this family of enzymes has potential for synthesising valuable compounds in microbial systems used in many applications.

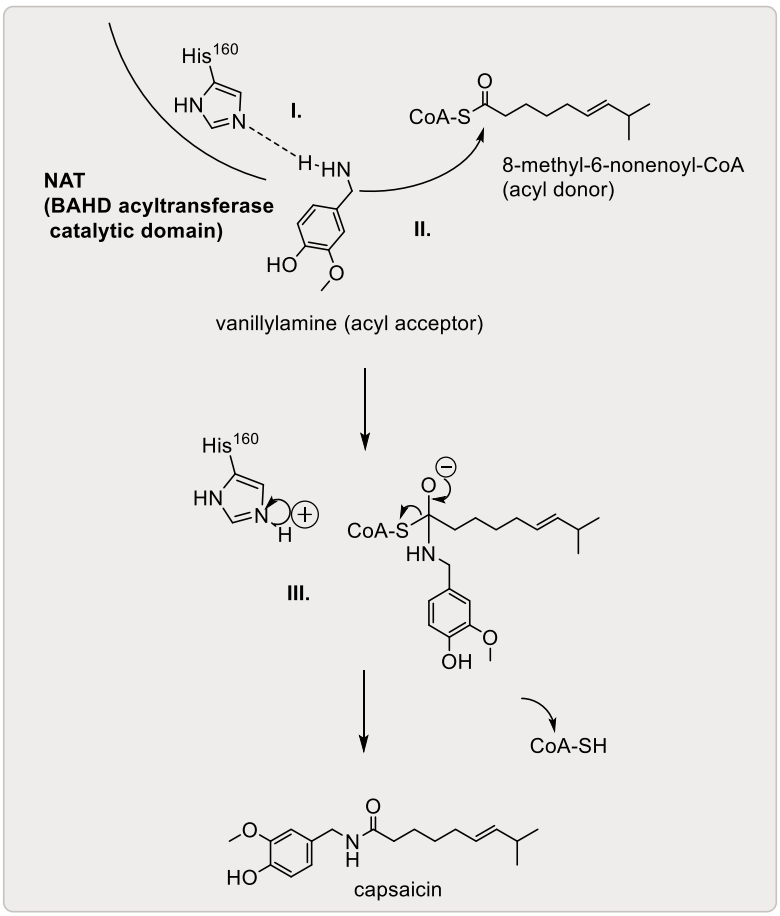


Figure 13. Schematic on the acyl transfer mechanism of BAHD enzymes and theory on how it would work for capsaicin synthase. I. The acyl acceptor (vanillylamine) is deprotonated by the catalytic region of the enzyme. II. Next, the acyl acceptor carries out a nucleophilic attack on the carbonyl carbon of the acyl donor (acyl-CoA). III The amide bond is formed between the acyl donor and acceptor and CoA is released (Ma et al., 2005).

CoA-ligases – for CoA activation of free fatty acids

Acyl-CoA synthetases (ACS) (EC 6.2.1.-) or CoA-ligases are ATP-dependant enzymes which activate free fatty acids in the cell into acyl-CoA thioesters. Their role in yeast cells has been found in lipid metabolism (Black and DiRusso, 2007). In the chilli plant, for the production of capsaicin for example, the branched-chain 8-methyl-6-nonanoic acid is activated with a coenzyme A by an ATP-dependant acyl-CoA synthetase (EC 6.2.1.3). It is encoded by *ACS* and is found to be continuously expressed throughout capsaicinoids accumulation and fruit development in the plant (Burgos-Valencia et al., 2020). These enzymes belong to an ANL superfamily of adenylating enzymes, consisting of three groups: Acyl-CoA synthetases, Non-ribosomal peptide synthetase adenylating domains and Luciferase enzymes. The reaction they perform is catalysed in two parts (Figure 14), first an adenylation of a carboxylic acid or carboxylate to form an intermediate acyl-AMP, then a second reaction to form the thioester (Gulick, 2009).

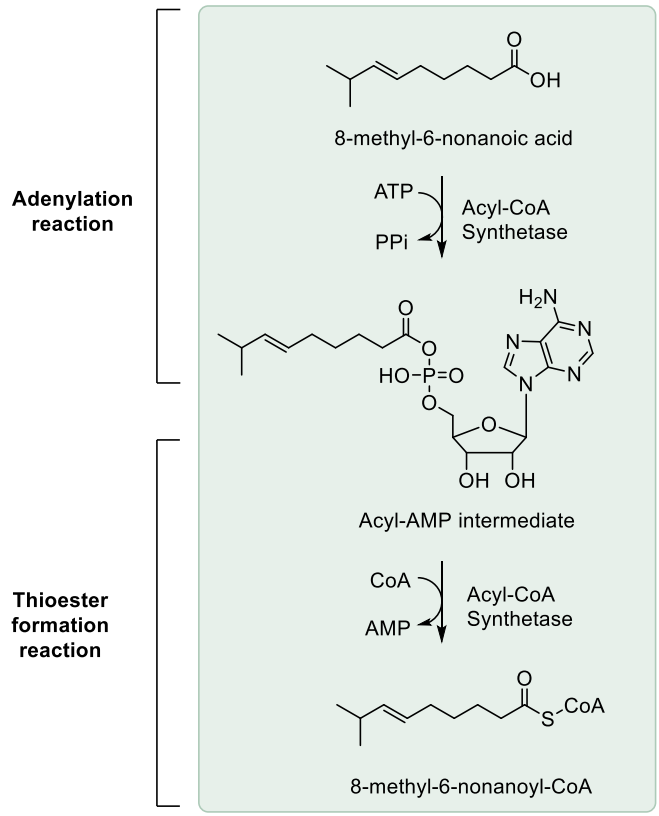


Figure 14. Shematic mechanism of carboxylic acid activation by CoA-ligases, examplified here for 8-methyl-6-nonanoic acid. The reaction consists of two parts, first an adenylation of the 8-methyl-6-nonanoic acid to form the Acyl-AMP intermediate using ATP, and a second thioester formation reaction using CoA where the intermediate is converted into a thioester 8-methyl-6-nonanoyl-CoA. Figure adapted from (Starai et al., 2002).

Biosynthetic production of nonivamide

Screening for amide-forming NAT and CL enzyme cascade

The model capsaicinoid chosen for whole-cell biosynthesis in this work was nonivamide. The structure of nonivamide is similar to that of capsaicin, but the fatty acyl chain is linear and unbranched. Production of nonivamide from direct precursors (vanillylamine and nonanoic acid) is achieved through the action of the CL activating nonanoic acid to nonanoyl-CoA and the NAT catalysing the formation of the amide bond between the amine vanillylamine and the thioester nonanoyl-CoA. A panel of heterologous NAT and CL enzymes from bacteria and plants was identified in a literature search to have amide-forming activity on substrates of interest, such as aromatic amines and thioesters (Philpott et al., 2018). These, including the *Capsicum* capsaicin synthase (encoded by *Pun1* or *AT3*) and acyl-CoA synthetase (encoded by *ACS*) were screened for nonivamide production in yeast (Paper I) (Figure 15).

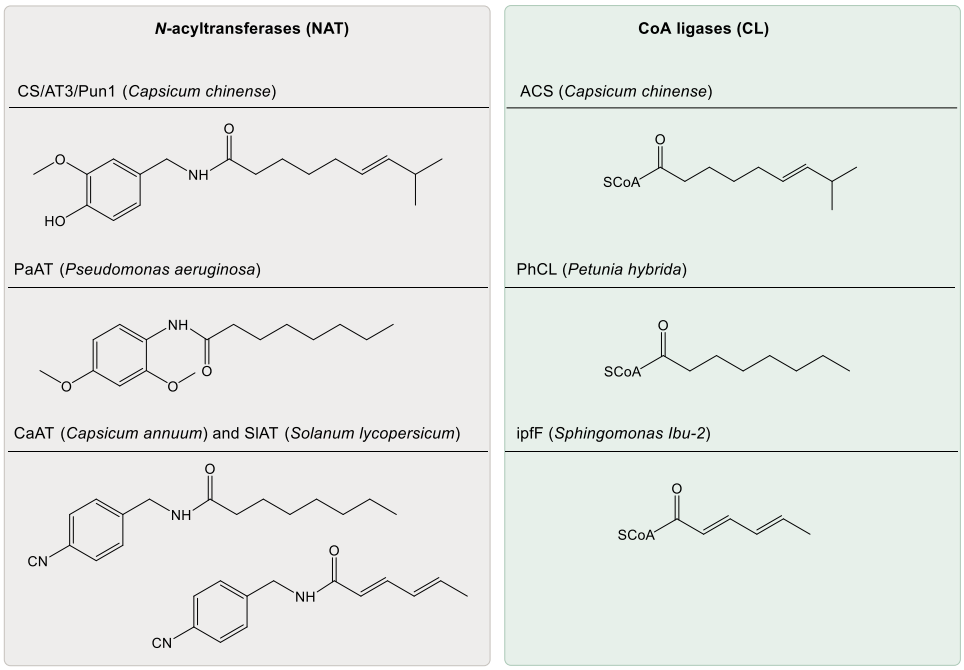


Figure 15. Chosen enzymes for expression in yeast, the respective organism they come from and a selection of compounds they have been shown to produce previously (Arce-Rodriguez and Ochoa-Alejo, 2019, Philpott et al., 2018).

A set of twelve strains was created using a combinatorial gene assembly method, where different NAT and CL coding gene combinations were expressed. All of the NAT and CL combinations showed activity to vanillylamine and nonanoic acid in varying levels (Paper I). The NAT/CL combination from *Capsicum* (*AT3/ACS*) actually gave the lowest nonivamide production in the tested conditions (Paper I). The low production could be due to weak expression in yeast, possibly due to presence of plant-specific localising sequences like a plastid targeting sequence, which could affect the localisation or activity of the enzyme in yeast (Williams et al., 1998). The protein levels in the cell should be quantified to investigate if low expression is the cause of the low nonivamide levels.

The best performing combination of enzymes was IpfF and CaAT, which were later also tested in a well-controlled bioreactor setup reaching 10.6 mg L^{-1} nonivamide from supplemented precursors (Paper I). Individually, the CaAT enzyme, a tyramine *N*-hydroxycinnamoyl transferase (THT) from *Capsicum annuum* achieved high nonivamide levels in combination with any CL enzyme. After a sequence analysis performed as part of this work, this enzyme was found to be present in several chilli pepper species and could therefore have an underestimated role in amide formation in capsaicinoid synthesis in the plant. Previously, this enzyme has been purified and confirmed to use tyramine, an aromatic amine as an acyl acceptor (Kang et al., 2006). Another THT with activity for nonivamide synthesis in yeast was the SlAT from tomato (*Solanum lycopersicum*). THT enzymes have been found in potato, tobacco, opium poppy and have been reported to have wide substrate specificity (Von Roepenack-Lahaye et al., 2003). Additionally, they have been described to have a role in synthesis of phenolic compounds derived from the phenylpropanoid pathway by catalysing the condensation of p-coumaric acid with octopamine and noradrenaline (forming *p*-coumaroyloctopamine and *p*-coumaroylnoradrenaline), involved in plant resistance to pathogens (Von Roepenack-Lahaye et al., 2003). From the CL group of enzymes, the PhCL (4-coumarate: CoA ligase 1) from *Petunia hybrida*, followed by the IpfF (Ibuprofen CoA ligase) from *Sphingomonas sp. Ibu-2* had the highest activity towards nonanoic acid (Paper I), even though their previous characterization showed strong activity on aromatic acids (Philpott et al., 2018, Klempien et al., 2012, Murdoch and Hay, 2013). The broad substrate recognition of both of these NAT and CL enzymes could be beneficial for exploring synthesis of uncommon and synthetic capsaicinoids.

Whole-cell production of nonivamide from vanillin

Combining the knowledge for reductive amination of vanillin from the previous chapter and the amide-forming NAT/CL cascade, a *S. cerevisiae* strain was constructed overexpressing multiple enzymes Cv-ATA, Bs-AlaDH, CaAT, IpfF and with deleted ADH6, and was evaluated for nonivamide production (Figure 16) from vanillin and nonanoic acid in a bioreactor setup (Paper II). Bioconversions were

performed either with low aeration or no aeration in order to investigate the effect of oxygen on the combined transamination and amidation reactions. During oxygen limited conditions, the highest molar yields of produced vanillylamine from consumed vanillin were 0.74±0.02 after 48 hours and without the addition of an amine donor. Nonivamide titre reached 0.19 µmol L^{-1} OD^{-1} after 68 hours. By-products vanillic acid and vanillyl alcohol were also produced, which indicates that the *adh6* deletion is not enough to prevent their production. The available oxygen may affect the co-factor ratio as to favour side-reactions instead of the transamination. In the non-aerated condition, molar yields of vanillylamine from consumed vanillin were higher, reaching 0.89±0.07 after 48 hours; however, nonivamide production was slightly lower reaching 0.12 µmol L^{-1} OD^{-1} after 68h. In this case, no vanillic acid was detected and lower levels of vanillyl alcohol were produced (Paper II).

Anaerobic conditions are beneficial for the transamination reaction to decrease by-product formation, but on the other hand respiration is beneficial for the regeneration of ATP (Andexer and Richter, 2015) and CoA-SH. Thus, aerobic conditions would be better for the amidation steps. These results indicate that the amidation step is the bottleneck in the production of nonivamide from vanillin (Paper II). However, the results also demonstrated that the desired enzymatic activity was present and that there is potential for optimizing the enzymes and the biosynthesis process further to reach higher levels of capsaicinoids.

Challenges in the amidation step

Even though the NAT and CL enzyme cascade effectively produced nonivamide, it is clear that the titres and yields of the process can be largely improved by metabolic engineering and bioprocess optimization. The toxicity of the products to the host cell can be addressed by transporter engineering through overexpression of a native or heterologous transmembrane transporter to export the product extracellularly (Lv et al., 2016), also aiding in downstream processing. Alternatively, plant transporters can be used to aid in intracellular transport in order to reconstruct sub-cellular compartmentalization (Srinivasan and Smolke, 2020). An underestimated challenge for production of various naturally-derived compounds is the chemical analysis for strain assessment, since many compounds are not readily available as standards and might be difficult to synthesise (Romero-Suarez et al., 2022). However, the emergence of open-access reference libraries for mass spectra data could aid in the future. Going from a proof of concept to development of a yeast factory may take several years to decades; however novel high-throughput and computer based technologies positively impacting the Design-Build-Test can greatly optimise the development time. Remaining challenges and future work will be discussed in the concluding Chapter 6.

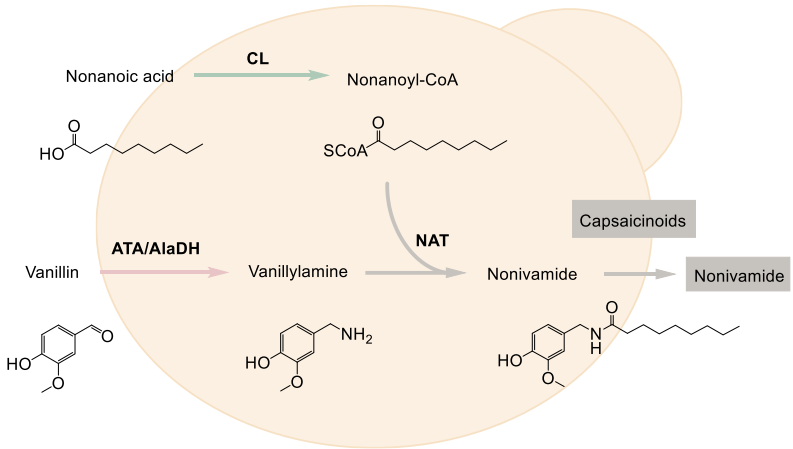


Figure 16. Production of nonivamide from vanillin and nonanoic acid in yeast is catalysed by the activty of ATA (aminotransaminase), AlaDH (alanine dehydrogenase), CL (CoA ligase) and NAT (*N*-acyltransferase).

Chapter 5
Yeast as a biosensor to screen for TRPV1 modulators

Role of capsaicinoids in nociception

We sense pain in the body when specific sensory neurons, nociceptors, are activated by harmful chemical, mechanical or thermal stimuli. The "capsaicin receptor" or Transient Receptor Potential Vanilloid type 1 (TRPV1) is a receptor found on sensory neurons, and targeted by capsaicin to cause the characteristic or burning pain of chilli peppers (Caterina et al., 1997). TRP channels were first discovered more than 50 years ago, when a *Drosophila* mutant displayed a transient receptor potential (TRP) instead of the expected continuous response after light exposure. The reason for this was the lack of a gene (named *trp*) coding for an ion channel (Cosens and Manning, 1969). TRP channels are present in many tissues and most are polymodal, which are activated by both physical and chemical stimuli. The superfamily of TRP channels has 28 members, but based on sequence homology analysis they are divided in six groups: canonical (short TRPCs), vanilloid (TRPVs), melastatin (TRPMs), ankyrin (TRPAs), mucolipins (TRPMLs) and polycystins (TRPPS). However, even though sequence homology has grouped some of these receptors together, their functions can be very different even within the same group (Koivisto et al., 2022).

The discovery of the TRPV1 heat receptor was recently awarded the Nobel Prize in Physiology or Medicine (2021). The structure of this receptor has been determined by cryogenic electron microscopy (Liao et al., 2013). It is a transmembrane tetrameric protein where the subunits are folded around a central ion permeation path. Each subunit has six transmembrane α-helices placed in the lipid bilayer and a loop with a pore helix between the 5^{th} and 6^{th} segment (Liao et al., 2013). It is a nonselective cation channel with high calcium permeability. Besides capsaicin, this receptor is modulated by physical and chemical stimuli such as heat (>43ºC *in vitro*), capsaicin, protons, bradykinin, anandamide, arachidonic acid olvanil, capsazepine etc (Caterina and Julius, 2001). Interestingly, birds are insensitive to the painful stimuli of capsaicin and by cloning of the avian receptor orthologue and comparing to the native mammalian one, it was found that it is not activated by capsaicin. In

this comparison, a segment in the transmembrane part of the receptor which was modelled to interact with vanilloid compounds, was found to be a rudimentary vanilloid binding site (S512Y) (Jordt and Julius, 2002). This segment was later confirmed to have an important role in capsaicin binding, but there were indications of multiple binding sites on the protein (Sutton et al., 2005). Later, the structures and precise interactions were determined for unliganded, agonist- and antagonist-bound TRPV1 in lipid nanodiscs, demonstrating the complex activation and the role of the S4-S5 linker (Gao et al., 2016).

TRPV1 receptor is activated by capsaicin

The central role of TRPV1 in the transduction of pain is well established. Its regulation is very complex (Szallasi and Blumberg, 2007). It plays an important role in nociception and therefore it is being thoroughly researched as a drug target. Upon activation by chemical (e.g. capsaicin) or physical (e.g. heat, low pH) stimuli, the ion channel opens and allows extracellular calcium cations to pass into the intracellular matrix, causing depolarization of the sensory neuron (Figure 17). This triggers a release of neuromodulatory peptides responsible for fighting inflammation and promoting healing, but exposure to capsaicin also increases pain sensitivity. After this pain sensitivity, comes a refractory period called desensitization, when reactivity to pain is relatively reduced, for days or even years depending on factors like dose and condition (Caterina and Julius, 2001). This desensitization to pain effect is a valuable property of capsaicin and the reason for the interest in developing capsaicin analogues as therapeutics. There are already products available containing capsaicin or analogous compounds, such as capsaicin patches and site-specific injections for treatment of osteoarthritis, post-herpetic neuralgia and diabetic polyneuropathy; and some which are still in clinical trials like resiferatoxin (more potent analogue), olvanil (non-pungent agonist) (Moran and Szallasi, 2018, Koivisto et al., 2022). There are significant side-effects with capsaicin treatment, like causing intense initial pain depending on the dose, burns or hyperthermia. Therefore, efforts are directed towards finding better analogues with less side effects (Koivisto et al., 2022).

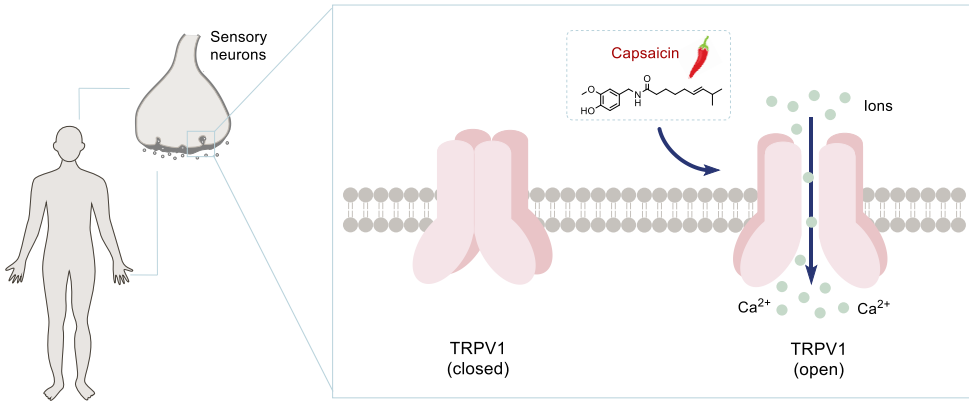


Figure 17. Capsaicin activates TRPV1 receptor present on sensory neurons. Figure adapted from (Muratovska et al., 2022).

Yeast as a biosensor

Could yeast be used as a biosensor to measure modulation of recombinant TRPV1 *in vivo*? This may be a valuable tool in the development of yeast cell factories for the biosynthesis of novel capsaicinoids with activity on the receptor. As a screening tool, it can be used to identify TRPV1 modulating compounds from strain libraries producing a variety of capsaicinoid derivatives. Ideally, measuring the change in intracellular calcium levels can be correlated to receptor activation in yeast overexpressing TRPV1 and selected cells can be isolated and re-cultivated.

Generally, biosensors contain a biological recognition (sensing) element and can deliver a response (signal) upon a change in the environment. The biological elements can be molecular (*in vitro*) using an enzyme, antibody, ion channel, nucleic acid, as well as cell-based, using live cells coupled with a reporter or transducer element as biosensors for detecting various changes in the environment (Pancrazio et al., 1999). Sensing of changes in the extracellular and intracellular environment, for example to avoid stressful conditions or detect beneficial environment for growth, as well as for screening purposes, is useful in developing synthetic biology applications.

Fluorescence-based techniques using fluorescent probes have been largely used for high throughput screening methods (HTS). Yeast-based biosensors have been developed as transcription dependant biosensors, using a reporter gene controlled by an inducible promotor. Transcription factor-based reporter systems have been developed utilizing green fluorescent protein (GFP) for example, to follow yeast growth and population heterogeneity (Carlquist et al., 2012), redox state (Knudsen et al., 2014, Zhang et al., 2016), to study response in sugar sensing (Brink et al., 2016) and other applications. Several other colours of fluorescent proteins have also

been investigated for their use to monitor cellular properties alone or even in combination for multicolour flow cytometry (Perruca-Foncillas et al., 2022, Torello Pianale et al., 2021).

More of interest here, are reporter systems for screening protein-ligand interactions for drug discovery (Vidan and Snyder, 2001). A protein ligand sensor that can detect binding of small molecules has been used in temperature sensitive *S. cerevisiae*, causing increase in growth upon binding (Tucker and Fields, 2001). Activation of mammalian G-protein coupled receptors (GPCR) has been also coupled to histidine prototrophy for library screening of peptide agonists in *S. cerevisiae* (Klein et al., 1998). GPCRs are important membrane proteins activated by many external stimuli (light, odours, hormones, growth factors) causing a conformational change triggering signalling pathways (Weis and Kobilka, 2014). They are one of the largest family of human membrane proteins, and similarly to TRPV1, are an important therapeutic target. Finding new GPCR targets is challenging mostly due to screening constrains and yeast can be a good screening platform for targets. However, issues with GPCR expression in yeast have been due to yeast membranes containing ergosterol instead of cholesterol, which benefits their expression. Therefore, more recently yeast has been engineered to produce cholesterol and express GPCRs linked to the yeast pheromone response pathway, as a potential biosensor for HTS of agonist binding and antagonist inhibition studies (Bean et al., 2022). In a similar manner of combining a cannabinoid receptor (GPCR) with yeast pheromone signalling pathway, yeast has been developed as a biosensor for HTS of cannabinoid modulators (Miettinen et al., 2022). Several fluorescence techniques have been used in studying GPCR receptors for their functionality: fluorescent ligands have been used to determine receptor-ligand binding; FRET (Fluorescence Resonance Energy Transfer) based sensors for conformational changes after activation (signal transduction); fluorescent antibodies, ligands, proteins and peptides for localization and visualization (Böhme and Beck-Sickinger, 2009).

Fluorescently labelled probes or dyes binding to various molecules are simple to use and do not require functional expression of fluorescent proteins. In this thesis, a calcium binding fluorescent dye Fluo-4 was used to evaluate the activity of recombinant TRPV1 in yeast (Paper IV). This dye is sensitive for quantifying within 100 nM-1 M Ca^{2+} range, is stable, easy to use and does not impair the cell's normal physiological signalling (Gee et al., 2000). Assessment of the intracellular calcium levels are based on excitation of the fluorescent dye-calcium complex, which emits a signal that can be measured by fluorimetry, flow cytometry or visualised using fluorescent microscopy. Assays for studying modulation of TRPV1 have been described using Ca^{2+} fluorescent indicators (Fluo-4 and Fura-2) in mammalian cells expressing the receptor (Moriello et al., 2023).

Fluorescence-based assay

In this work, attempts were made to develop a yeast-based assay for measuring TRPV1-dependant calcium ion influx in the cell in order to screen for effective receptor modulators. Earlier method to detect the modulation of ion channels was the patch-clamp electrophysiology which measures the channel activity via an ionic current (Myers et al., 2008, Koplas et al., 1997). The traditional patch-clamp method has a low sample throughput, requires a complex procedure and is labour intensive, so automated patch-clamp assays have been developed; however they are also limited in throughput and require high operator skills (Zicha et al., 2013). To construct efficient cell factories, HTS methods are needed, therefore, the aim of this project was to investigate the feasibility of a flow cytometry-based HTS method to qualify the modulation of TRPV1 (Figure 18). Since the TRPV1 activation causes a calcium influx in the cell, the theoretical background was to stain the cells with the fluorescence probe (Fluo-4), which forms complexes with intracellular calcium and emits a fluorescent signal that can be measured. In this context, upon TRPV1 activation by modulators, the channel changes conformation and a calcium influx leads to a higher calcium concentration in the cell, which should result in an increase of fluorescence intensity. The difference in intensity can be detected via flow cytometry and this method could eventually be used for screening of modulators, based on TRPV1 activation or de-activation.

Previously, mammalian (rat) TRPV has been functionally expressed in yeast in order to generate mutants of the receptor and identify which amino acid residues are important for the receptor's function (Myers et al., 2008). In this work, the same rat homologue of the TRPV1 protein was chromosomally integrated or overexpressed by a multicopy plasmid, with the aim of developing a yeast-based TRPV1 biosensor (Paper IV). The effect of TRPV1 overexpression on cell growth and intracellular calcium levels, evaluation of growth conditions for the assay, and combination of TRPV1 expression with *in vivo* synthesis of capsaicinoids in a single-cell, were few aspects addressed in this work.

TRPV1 overexpression negatively affected cell fitness and increased intracellular calcium levels without addition of modulators (Paper IV). The effect of cultivation conditions (pH, temperature and external calcium addition) on the modulation of the TRPV1 receptor indicated that physical conditions influence the intracellular calcium levels and consequently the TRPV1 activation. Additionally, the effect of added chemical modulators (capsaicin and capsazepine) was followed in the recombinant yeast by measuring the fluorescence correlated to the intracellular calcium levels; however, no significant effects was observed. Lastly, *in vivo* synthesis of nonivamide completely arrested growth in a strain overexpressing TRPV1. This research is a first step towards developing a yeast-based biosensor for screening of compounds produced by recombinant yeast cells which modulate the TRPV1 receptor (e.g. capsaicinoids). Future work should focus on improving

different aspects of the staining assay such as optimizing conditions for stable intracellular calcium levels prior modulation, dye concentration and incubation times. Primary treatment with a TRPV1 antagonist such as capsazepine or ruthenium red (Bevan et al., 1992, Myers et al., 2008) could be beneficial prior to the addition (or *in vivo* synthesis) of an agonist. This could decrease or prevent potential endogenous activation of the receptor and lower baseline fluorescence intensity.

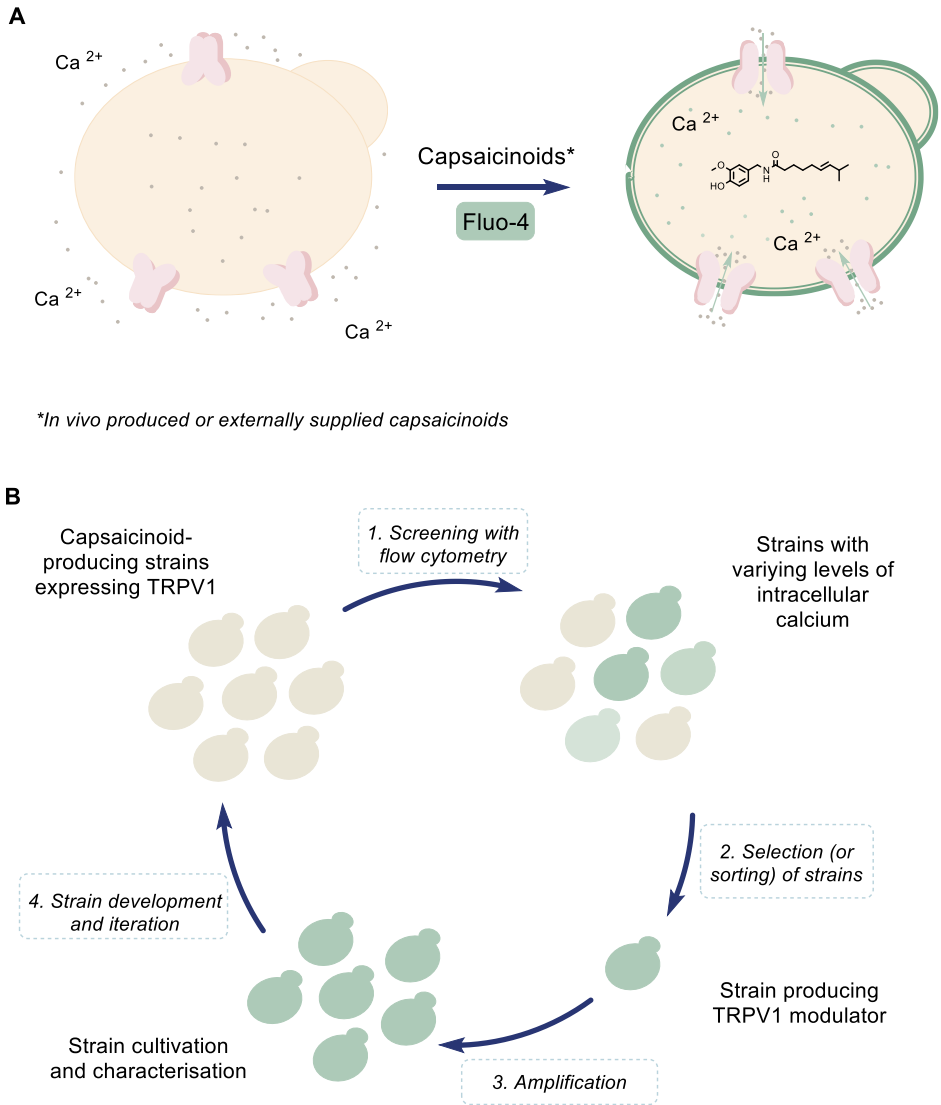


Figure 18. Schematic representation of the concept of yeast as a biosensor for screening of TRPV1 modulators. A. The principle of the assay is based on staining intracellular calcium with Fluo-4 upon calcium influx caused by TPRV1 activation by capsaicinoids. B. Yeast as a screening tool to identify TRPV1 receptor modulators and develop better cell factories.

Chapter 6
Summary and Outlook

The work described in this thesis addressed the production of capsaicinoids in recombinant *S. cerevisiae*. More specifically, the production of nonivamide from nonanoic acid and vanillin *in vivo* was established.

Reductive amination of vanillin to vanillylamine requires vanillin aminotransferase gene overexpression and an alanine dehydrogenase for amine donor recycling and removal of pyruvate, when alanine is used as amine donor. The alanine dehydrogenase expression also removed the need for amine donor supplementation. By-product levels can be decreased by deleting endogenous alcohol dehydrogenases and by using anaerobic conditions with ethanol as a co-substrate. Anaerobic conditions, however, may not be beneficial to the amidation step.

N-acyltransferase and CoA-ligase enzymes were identified with activity towards forming nonivamide, but the production yields can be further improved through protein engineering strategies or process optimisation. The best performing NAT enzyme was CaAT from *Capsicum annuum,* while the best performing CLs were the PhCL from *Petunia hybrida* and IpfF from *Sphingomonas sp. Ibu-2*. The ACS and CS from *Capsicum* gave the lowest amounts of product which could be due to their low expression in yeast.

S. cerevisiae was also engineered to express a TRPV1 receptor with the goal of developing an assay for evaluation of *in vivo* produced capsaicinoids as TRPV1 modulators. The expression of the receptor in *S. cerevisiae* negatively impacted the growth fitness and caused an increase in intracellular calcium levels, without the addition of chemical modulators.

Remaining challenges in the production of capsaicinoids

Cell tolerance to compounds

Nonivamide and capsaicin are inhibitory to yeast, likely due to association with lipophilic cell constituents like the cell membrane and disturbing its fluidity or integrity. Interaction of capsaicin with a synthetic lipid bilayer membrane has been previously observed (Swain and Kumar Mishra, 2015). Localisation and transport

of these compounds *in vivo* could be studied in future work. Additionally, and perhaps even more limiting is that the precursor to nonivamide - nonanoic acid, a nine carbon long fatty acid (FA), is inhibitory to yeast cell growth, like other medium-chain fatty acids (MCFA). Medium-chain FA were found to be more inhibitory to yeast than short-chain or long-chain FA (Paper I). The difference in inhibition between short- and medium-chain FA could be explained by the latter having higher lipophilicity and interaction with the plasma membrane (Liu et al., 2013). Long-chain FA, mostly C16-C18 are native in yeast as part of the phospholipid bilayer (Klug and Daum, 2014) and therefore likely not causing damage. To address this issue, cells can be adapted to tolerate higher concentrations of medium chain fatty acids through adaptive laboratory evolution or even engineered for their *de novo* synthesis (Zhu et al., 2020). Process engineering strategies can be applied; for example, continuous low level substrate feeding, product removal or supplementing the media with additives (e.g. oleic acid) that could alleviate toxicity (Liu et al., 2013).

Fatty acid synthesis in yeast

In order to reach complete synthesis of various capsaicinoids in *S. cerevisiae*, the fatty acid synthesis machinery needs to be engineered for production of specific and/or branched medium chain fatty acids, as well as address toxicity issues related to that production (Muratovska et al., 2022). Capsaicin, for example, has an acyl chain which is unsaturated and branched with a medium carbon length. Yeast naturally produces mainly 16 and 18 carbon straight chain fatty acids by the activity of the fatty acid synthase (FAS) system in the cytosol, consisting of a large multifunctional protein (Klug and Daum, 2014). It has been previously shown that FASI in combination with heterologous thioesterases could enable yeast to produce MCFA (Zhu et al., 2020). In a protein engineering approach, five active site mutations of the fungal FAS were performed and *S. cerevisiae* strain was engineered to produce short and medium chain FA (Gajewski et al., 2017). A yeast has also been engineered as a biosensor for screening of short and medium chain FA production, which could prove useful for high throughput screening of producer cells (Baumann et al., 2018). Engineering the fatty acid synthesis pathway is needed with regard to the mechanisms responsible for branching, degree of saturation and chain length in order to produce a range of capsaicinoids.

Promiscuous native enzymes

Constructing a production pathway in a cell comes with many advantages, such as stability of enzymes, availability of co-factors, ATP and potentially lower process costs; however, there are other components in the cell like proteins and metabolites that could affect the engineered metabolic pathway. Yeast activates free fatty acids which are produced or acquired from the media to acyl-CoA esters through the activity of six described acyl-CoA synthetases (Leber et al., 2015). In this work it was found that there are indeed endogenous CoA-ligase enzymes in yeast that could

interact with the added nonanoic acid and possibly other fatty acids. With only the AT3 (*Capsicum* acyltransferase) enzyme expressed, yeast could produce low levels of nonivamide (Paper I). This could be a valuable strategy if the goal would be to activate various fatty acids in order to create chemical variability, but would require a broad-spectrum acyltransferases or several. It is unlikely that there are yeast-native acyltransferase enzymes acting on vanillylamine and nonanoyl-CoA due to the insignificant difference in the nonivamide levels detected between a control strain and a strain only expressing the CL enzyme. This confirms the need for a NAT with high activity. Additionally, there were native enzymes acting on vanillin and impacting the transamination reaction. Deletion of *adh6* indeed lowered vanillin alcohol levels; however, depending on the reaction conditions, it didn't completely arrest the production of vanillyl alcohol (Paper II). Strategies in optimization of fermentation conditions in order to drive the metabolic flux towards the product can additionally improve production levels.

Heterologous enzyme performance

One of the main reasons for low production of nonivamide from vanillin and nonanoic acid is likely low activity of the amide-forming enzymes. This work has shown the possibility of production of nonivamide in a whole-cell as a proof of concept; however, the levels of product reached can be further improved. It is known that secondary metabolite enzymes have usually low catalytic rates and that their expression could be challenging (Romero-Suarez et al., 2022). On average, secondary metabolism enzymes have been reported to be 30-fold slower than ones from the central metabolism and the physicochemical properties of the substrates (e.g. hydrophobicity, CoA substitutions) also affect the kinetic parameters of the enzymes (Bar-Even et al., 2011). Some examples of synthetic biology strategies one can use to improve the production include engineering the gene expression levels of the enzyme in the cells, spatial engineering by directing enzymes in a cell compartment, protein engineering strategies (rational design or directed evolution) and/or activity-based screening for better enzymes and computational approaches (Jensen and Keasling, 2015, Cravens et al., 2019). Expression of genes coming from heterologous hosts can pose some issues with proper folding or localisation, if in the native organism they were active in specific compartments. Upon transferring of those enzymes in yeast or another heterologous host there would be a loss of the spatial regulatory strategies. Compartmentalization can also be achieved in yeast cells by directing enzymes in different cell regions and organelles, for example to better access a substrate or isolate a reaction from other reactions (Srinivasan and Smolke, 2020).

Screening strain libraries producing capsaicinoid derivatives

Yeast-based biosensors have been used for strain optimisation and screening purposes for detecting a large range of analytes, both through transcription-dependant and transcription-independent assays (Adeniran et al., 2015) Current

protocols for capsaicinoids extraction and chemical analysis limit the number of products from recombinant strains which could be analysed. Developing a yeast-based biosensor strain with the purpose of screening for capsaicinoids and their activity (e.g. TRPV1 modulation) could help with the bottleneck in the Test part of the Design-Build-Test-Learn cycle. The TRPV1 yeast-based biosensor has potential to be used for this purpose if made functional, for example by disabling possible cell-related (endogenous) receptor modulation or engineering the yeast-native calcium regulatory system. Alternatively, a transcription-factor based system where a capsaicinoid-inducible promotor is coupled to a GFP reporter molecule, could be developed, similarly to the biosensor developed for detection of short- and medium-chain FA (Baumann et al., 2018).

Acknowledgements

I am grateful for the amazing people I have had the pleasure of meeting and the moments we spent during this relatively short time. You have inspired me and taught me in more ways that I can describe. No one walks this journey completely alone, so thank you for walking alongside.

My honest gratitude to my supervisor Magnus Carlquist for all your guidance, knowledge and positivity, it has been a pleasure. Thank you for your constant support, and for inspiring and challenging me. Due to that, I have grown in many positive ways during these years at the division, for which I am grateful.

To my co-supervisors Carl Grey and Urban Johanson, thank you for all the fruitful discussions and support whenever it was needed. You have inspired me to look at things from a different perspective.

I would like to thank Marie Gorwa-Grauslund; your support during all the study plan meetings, staff appraisals and your valuable feedback overall during the years, has helped me improve in many ways.

I had the pleasure of meeting Bärbel Hahn-Hägerdal, Gunnar Lindahl and Peter Rådström during my time at the division, so thank you for the discussions, invaluable input and feedback on the project. Jenny Schelin, thank you for always being so kind and positive, and for your support when needed. Catherine Paul, your passion about water research is contagious, thank you for teaching me so much about it. Ed van Niel, thank you for the laughs, for sharing interesting information and your book suggestions. Johannes Hedman, thanks for engaging my curiosity about different aspects of PCR. Linda Jansson, thank you for your warmth and kindness. To Christer Larsson, thank you for your positivity and always being kind enough to fix anything. Anette Ahlberg, thank you for all the nice conversations and help with any administrative tasks.

Thank you to all of the Metabolic Engineering group members for creating a communal space to discuss research in an encouraging and supporting way.

Thank you to all my co-authors, especially Paulo César Silva and João Heitor Colombelli Manfrão-Netto, for your contributions and discussions.

To the students at the time, Robert Seute, Malte Marthin and Frederik Lund, thank you for working with me on this project.

Thank you to the former and current office mates, Kristjan Pullerits, Karen Ofuji Osiro, Thitiwut Vongkampang, Yasmine Akel, Fredrik Lund, Julia Södergren, Paulo César Silva, Lisa Lindahl, Bruna Bolzico. I will always cherish the time we spent in kindergarten, thank you for all the conversations, the selfless support, all the puzzles and jokes.

I am grateful to my former colleagues Lisa Wasserstrom, Sandy Chan, Maja Sidstedt, Eoin Byrne, Javier Garcia Hidalgo, Marie Baehr, Celina Borgström, for making me feel welcome at the department and your help.

To my current colleagues, thank you for all the coffee breaks and contributing to such a nice working environment. Daniel Brink, thank you for inspiring me. Starting from the 10-week project, to the master thesis project and then to the PhD, you encouraged me to continue with research. I am grateful for your support with all matters. I fondly remember working in the lab with you around. Your friendship has made every day more fun, so thanks for the memories. To Raquel Perruca Foncillas, thank you for being there since the first days I came to Lund. I appreciate your positivity, your constant support and voice of reason, and your contagious creativity of course. Thank you for going to pottery with me and for all the moments at work and outside. You are a great friend and colleague and I am grateful to know you. To Nikhil Rao, thank you for all the conversations, your help and support, and for your friendship during this journey - we got this. To Fredrik Lund, thanks for doing your first project at TMB with me, thank you for your friendship and office debates. To Tova Lindh, thank you for your friendship and for making me go back to the gym. Thanks for all the moments, whether making funny postcards, traveling, cooking dinner or you giving me plant advice. To Viktor Persson, thank you for your kindness, for all the cakes and bakes you brought at TMB, for organizing social activities and for making the most amazing chill pepper nail. To Krishnan Sreenivas, thank you for all the interesting conversations, your advice, debates, and your help and kindness throughout these years. To Tage Rosenquist, thanks for your cheerfulness and your jokes, I really appreciate your sense of humor. Thanks to Sofia Badiali, even though you were here for a short time, your positivity left an imprint. Thanks to Isabel Katharina Erb and Matilda Johansson, for your friendliness and enthusiasm, I wish you both lots of success in your work. To each and every person that I have come across during my PhD journey, but maybe didn't mention, thank you for the moments.

I am thankful for the unconditional love and support from my family and friends. Фамилијата ми е инспирација, мотивација и храброст. Нема зборови да опишат колку сум благодарна за се што сте ми овозможиле. Matej, hvala za vsakodnevno podporo, potrpezljivost in ljubezen. Hvala ti, da mi stojiš ob strani.

"One day in retrospect, the years of struggle will strike you as the most beautiful." ***- Sigmund Freud***

References

Abdelaziz, O. Y., Li, K., Tunå, P. & Hulteberg, C. P. 2017. Continuous catalytic depolymerisation and conversion of industrial kraft lignin into low-molecular-weight aromatics. *Biomass Conversion and Biorefinery,* 8**,** 455-470.

Abiraj, K. & Gowda, D. C. 2003. Zinc/ammonium formate: a new facile system for the rapid and selective reduction of oximes to amines. *Journal of Chemical Research,* 2003**,** 332-334.

Adeniran, A., Sherer, M. & Tyo, K. E. J. 2015. Yeast-based biosensors: design and applications. *FEMS Yeast Research,* 15**,** 1-15.

Aluru, M. R., Mazourek, M., Landry, L. G., Curry, J., Jahn, M. & O'Connell, M. A. 2003. Differential expression of fatty acid synthase genes, Acl, Fat and Kas, in *Capsicum* fruit. *J Exp Bot,* 54**,** 1655-64.

Anderson, M., Afewerki, S., Berglund, P. & Cordova, A. 2014. Total synthesis of capsaicin analogues from lignin-derived compounds by combined heterogeneous metal, organocatalytic and enzymatic cascades in one pot. *Advanced Synthesis & Catalysis,* 356**,** 2113-2118.

Andexer, J. N. & Richter, M. 2015. Emerging enzymes for ATP regeneration in biocatalytic processes. *Chembiochem,* 16**,** 380-6.

Antonio, A. S., Wiedemann, L. S. M. & Veiga Junior, V. F. 2018. The genus *Capsicum:* a phytochemical review of bioactive secondary metabolites. *RSC Advances,* 8**,** 25767-25784.

Appendino, G., Minassi, A., Morello, A. S., De Petrocellis, L. & Di Marzo, V. 2002. *N*-Acylvanillamides: development of an expeditious synthesis and discovery of new acyl templates for powerful activation of the vanilloid receptor. *Journal of Medicinal Chemistry,* 45**,** 3739-45.

Araújo, J. D. P., Grande, C. A. & Rodrigues, A. E. 2010. Vanillin production from lignin oxidation in a batch reactor. *Chemical Engineering Research and Design,* 88**,** 1024-1032.

Arce-Rodriguez, M. L. & Ochoa-Alejo, N. 2019. Biochemistry and molecular biology of capsaicinoid biosynthesis: recent advances and perspectives. *Plant Cell Reports,* 38**,** 1017-1030.

Atanasov, A. G., Waltenberger, B., Pferschy-Wenzig, E. M., Linder, T., Wawrosch, C., Uhrin, P., Temml, V., Wang, L., Schwaiger, S., Heiss, E. H., Rollinger, J. M., Schuster, D., Breuss, J. M., Bochkov, V., Mihovilovic, M. D., Kopp, B., Bauer, R., Dirsch, V. M. & Stuppner, H. 2015. Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnology Advances,* 33**,** 1582-1614.

Awan, A. R., Shaw, W. M. & Ellis, T. 2016. Biosynthesis of therapeutic natural products using synthetic biology. *Advanced Drug Delivery Reviews,* 105**,** 96-106.

Aza-Gonzalez, C., Nunez-Palenius, H. G. & Ochoa-Alejo, N. 2011. Molecular biology of capsaicinoid biosynthesis in chili pepper (*Capsicum* spp.). *Plant Cell Reports,* 30**,** 695-706.

Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., Jahurul, M. H. A., Ghafoor, K., Norulaini, N. A. N. & Omar, A. K. M. 2013. Techniques for extraction of bioactive compounds from plant materials: A review. *Journal of Food Engineering,* 117**,** 426-436.

Bar-Even, A., Noor, E., Savir, Y., Liebermeister, W., Davidi, D., Tawfik, D. S. & Milo, R. 2011. The moderately efficient enzyme: evolutionary and physicochemical trends shaping enzyme parameters. *Biochemistry,* 50**,** 4402-10.

Barbero, G. F., Molinillo, J. M., Varela, R. M., Palma, M., Macias, F. A. & Barroso, C. G. 2010. Application of Hansch's model to capsaicinoids and capsinoids: a study using the quantitative structure-activity relationship. A novel method for the synthesis of capsinoids. *Journal of Agricultural and Food Chemistry,* 58**,** 3342-9.

Baumann, L., Rajkumar, A. S., Morrissey, J. P., Boles, E. & Oreb, M. 2018. A yeast-based biosensor for screening of short- and medium-chain fatty acid production. *ACS synthetic biology,* 7**,** 2640-2646.

Bean, B. D. M., Mulvihill, C. J., Garge, R. K., Boutz, D. R., Rousseau, O., Floyd, B. M., Cheney, W., Gardner, E. C., Ellington, A. D., Marcotte, E. M., Gollihar, J. D., Whiteway, M. & Martin, V. J. J. 2022. Functional expression of opioid receptors and other human GPCRs in yeast engineered to produce human sterols. *Nature Communications,* 13**,** 2882.

Benitez-Mateos, A. I., Roura Padrosa, D. & Paradisi, F. 2022. Multistep enzyme cascades as a route towards green and sustainable pharmaceutical syntheses. *Nature Chemistry,* 14**,** 489-499.

Bennett, D. J. & Kirby, G. W. 1968. Constitution and biosynthesis of capsaicin. *Journal of the Chemical Society C-Organic***,** 442-&.

Bevan, S., Hothi, S., Hughes, G., James, I. F., Rang, H. P., Shah, K., Walpole, C. S. & Yeats, J. C. 1992. Capsazepine: a competitive antagonist of the sensory neurone excitant capsaicin. *British Journal of Pharmacology,* 107**,** 544-52.

Black, P. N. & DiRusso, C. C. 2007. Yeast acyl-CoA synthetases at the crossroads of fatty acid metabolism and regulation. *Biochimica et Biophysica Acta,* 1771**,** 286-98.

Blum, E., Liu, K., Mazourek, M., Yoo, E. Y., Jahn, M. & Paran, I. 2002. Molecular mapping of the C locus for presence of pungency in *Capsicum*. *Genome,* 45**,** 702-5.

Blum, E., Mazourek, M., O'Connell, M., Curry, J., Thorup, T., Liu, K., Jahn, M. & Paran, I. 2003. Molecular mapping of capsaicinoid biosynthesis genes and quantitative trait loci analysis for capsaicinoid content in *Capsicum*. *Theoretical and Applied Genetics,* 108**,** 79-86.

Böhme, I. & Beck-Sickinger, A. G. 2009. Illuminating the life of GPCRs. *Cell Communication and Signaling,* 7**,** 16.

Bornscheuer, U. T., Huisman, G. W., Kazlauskas, R. J., Lutz, S., Moore, J. C. & Robins, K. 2012. Engineering the third wave of biocatalysis. *Nature,* 485**,** 185-94.

Bouchez, P., Teixeira Benites, V., Baidoo, E. E. K., Mortimer, J. C., Sullivan, M. L., Scheller, H. V. & Eudes, A. 2019. Production of clovamide and its analogues in *Saccharomyces cerevisiae* and *Lactococcus lactis*. *Letters in Applied Microbiology,* 69**,** 181-189.

Breuer, M., Ditrich, K., Habicher, T., Hauer, B., Kesseler, M., Sturmer, R. & Zelinski, T. 2004. Industrial methods for the production of optically active intermediates. *Angewandte Chemie International Edition,* 43**,** 788-824.

Brink, D. P., Borgstrom, C., Tueros, F. G. & Gorwa-Grauslund, M. F. 2016. Real-time monitoring of the sugar sensing in *Saccharomyces cerevisiae* indicates endogenous mechanisms for xylose signaling. *Microbial Cell Factories,* 15**,** 183.

Brochado, A. R., Matos, C., Moller, B. L., Hansen, J., Mortensen, U. H. & Patil, K. R. 2010. Improved vanillin production in baker's yeast through *in silico* design. *Microbial Cell Factories,* 9**,** 84.

Bryan, M. C., Dunn, P. J., Entwistle, D., Gallou, F., Koenig, S. G., Hayler, J. D., Hickey, M. R., Hughes, S., Kopach, M. E., Moine, G., Richardson, P., Roschangar, F., Steven, A. & Weiberth, F. J. 2018. Key Green Chemistry research areas from a pharmaceutical manufacturers' perspective revisited. *Green Chemistry,* 20**,** 5082-5103.

Burgos-Valencia, E., Echevarria-Machado, I., Narvaez-Zapata, J. A. & Martinez-Estevez, M. 2020. Gene expression related to the capsaicinoids biosynthesis in the *Capsicum* genus: Molecular and transcriptomic studies. *Brazilian Journal of Botany,* 43**,** 201-212.

Carlquist, M., Fernandes, R. L., Helmark, S., Heins, A. L., Lundin, L., Sorensen, S. J., Gernaey, K. V. & Lantz, A. E. 2012. Physiological heterogeneities in microbial populations and implications for physical stress tolerance. *Microbial Cell Factories,* 11**,** 94.

Carrizo Garcia, C., Barfuss, M. H., Sehr, E. M., Barboza, G. E., Samuel, R., Moscone, E. A. & Ehrendorfer, F. 2016. Phylogenetic relationships, diversification and expansion of chili peppers (*Capsicum*, *Solanaceae*). *Annals of Botany,* 118**,** 35-51.

Castillo, E., Torres-Gavilan, A., Severiano, P., Arturo, N. & Lopez-Munguia, A. 2007. Lipase-catalyzed synthesis of pungent capsaicin analogues. *Food Chemistry,* 100**,** 1202-1208.

Caterina, M. J. & Julius, D. 2001. The vanilloid receptor: a molecular gateway to the pain pathway. *Annual Review of Neuroscience,* 24**,** 487-517.

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D. & Julius, D. 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature,* 389**,** 816-24.

Chatterjee, M., Ishizaka, T. & Kawanami, H. 2016. Reductive amination of furfural to furfurylamine using aqueous ammonia solution and molecular hydrogen: an environmentally friendly approach. *Green Chemistry,* 18**,** 487-496.

Choi, K.-H., Heath Richard, J. & Rock Charles, O. 2000. β-Ketoacyl-Acyl carrier protein synthase III (FabH) is a determining factor in branched-chain fatty acid biosynthesis. *Journal of Bacteriology,* 182**,** 365-370.

Claros Cuadrado, J. L., Pinillos, E. O., Tito, R., Mirones, C. S. & Gamarra Mendoza, N. N. 2019. Insecticidal properties of capsaicinoids and glucosinolates extracted from *Capsicum chinense* and *Tropaeolum tuberosum*. *Insects,* 10**,** 132.

Cosens, D. J. & Manning, A. 1969. Abnormal electroretinogram from a *Drosophila* mutant. *Nature,* 224**,** 285-7.

Cravens, A., Payne, J. & Smolke, C. D. 2019. Synthetic biology strategies for microbial biosynthesis of plant natural products. *Nature Communications,* 10**,** 2142.

Curry, J., Aluru, M., Mendoza, M., Nevarez, J., Melendrez, M. & O'Connell, M. A. 1999. Transcripts for possible capsaicinoid biosynthetic genes are differentially accumulated in pungent and non-pungent *Capsicum* spp. *Plant Science,* 148**,** 47-57.

D'Auria, J. C. 2006. Acyltransferases in plants: a good time to be BAHD. *Current Opinion in Plant Biology,* 9**,** 331-40.

Deng, X., Chen, L., Hei, M., Liu, T., Feng, Y. & Yang, G. Y. 2020. Structure-guided reshaping of the acyl binding pocket of 'TesA thioesterase enhances octanoic acid production in *E. coli*. *Metab Eng,* 61**,** 24-32.

Eichenberger, M., Hansson, A., Fischer, D., Durr, L. & Naesby, M. 2018. *De novo* biosynthesis of anthocyanins in *Saccharomyces cerevisiae*. *FEMS Yeast Research,* 18**,** foy046.

Eudes, A., Mouille, M., Robinson, D. S., Benites, V. T., Wang, G., Roux, L., Tsai, Y. L., Baidoo, E. E., Chiu, T. Y., Heazlewood, J. L., Scheller, H. V., Mukhopadhyay, A., Keasling, J. D., Deutsch, S. & Loque, D. 2016. Exploiting members of the BAHD acyltransferase family to synthesize multiple hydroxycinnamate and benzoate conjugates in yeast. *Microbial Cell Factories,* 15**,** 198.

Flagan, S. F. & Leadbetter, J. R. 2006. Utilization of capsaicin and vanillylamine as growth substrates by *Capsicum* (hot pepper)-associated bacteria. *Environmental Microbiology,* 8**,** 560-565.

Friedman, J. R., Nolan, N. A., Brown, K. C., Miles, S. L., Akers, A. T., Colclough, K. W., Seidler, J. M., Rimoldi, J. M., Valentovic, M. A. & Dasgupta, P. 2018. Anticancer activity of natural and synthetic capsaicin analogs. *Journal of Pharmacology and Experimental Therapeutics,* 364**,** 462-473.

Fu, B. X., Xiao, G. Z., Zhu, Y. L., Chen, Y. Y., Lai, Y. M. & Yuan, J. F. 2021. Renewable vanillylamine synthesis from lignin-derived feedstocks. *ACS Agricultural Science & Technology,* 1**,** 566-571.

Fuchs, M., Koszelewski, D., Tauber, K., Kroutil, W. & Faber, K. 2010. Chemoenzymatic asymmetric total synthesis of (*S*)-Rivastigmine using ω-transaminases. *Chemical Communications,* 46**,** 5500-2.

Fuchtbauer, S., Mousavi, S., Bereswill, S. & Heimesaat, M. M. 2021. Antibacterial properties of capsaicin and its derivatives and their potential to fight antibiotic resistance - A literature survey. *European Journal of Microbiology and Immunology,* 11**,** 10-17.

Gajewski, J., Pavlovic, R., Fischer, M., Boles, E. & Grininger, M. 2017. Engineering fungal *de novo* fatty acid synthesis for short chain fatty acid production. *Nature Communications,* 8**,** 14650.

Galanie, S., Thodey, K., Trenchard, I. J., Filsinger Interrante, M. & Smolke, C. D. 2015. Complete biosynthesis of opioids in yeast. *Science,* 349**,** 1095-100.

Gao, Y., Cao, E., Julius, D. & Cheng, Y. 2016. TRPV1 structures in nanodiscs reveal mechanisms of ligand and lipid action. *Nature,* 534**,** 347-51.

Gee, K. R., Brown, K. A., Chen, W. N., Bishop-Stewart, J., Gray, D. & Johnson, I. 2000. Chemical and physiological characterization of fluo-4 Ca(2+)-indicator dyes. *Cell Calcium,* 27**,** 97-106.

Gomm, A., Lewis, W., Green, A. P. & O'Reilly, E. 2016. A new generation of smart amine donors for transaminase-mediated biotransformations. *Chemistry,* 22**,** 12692-5.

Gottardi, M., Knudsen, J. D., Prado, L., Oreb, M., Branduardi, P. & Boles, E. 2017. *De novo* biosynthesis of *trans*-cinnamic acid derivatives in *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology,* 101**,** 4883-4893.

Gulick, A. M. 2009. Conformational dynamics in the Acyl-CoA synthetases, adenylation domains of non-ribosomal peptide synthetases, and firefly luciferase. *ACS Chemical Biology,* 4**,** 811-27.

Guo, F. & Berglund, P. 2017. Transaminase biocatalysis: optimization and application. *Green Chemistry,* 19**,** 333-360.

Han, K., Jeong, H. J., Sung, J., Keum, Y. S., Cho, M. C., Kim, J. H., Kwon, J. K., Kim, B. D. & Kang, B. C. 2013. Biosynthesis of capsinoid is controlled by the Pun1 locus in pepper. *Molecular Breeding,* 31**,** 537-548.

Hansen, E. H., Moller, B. L., Kock, G. R., Bunner, C. M., Kristensen, C., Jensen, O. R., Okkels, F. T., Olsen, C. E., Motawia, M. S. & Hansen, J. 2009. *De novo* biosynthesis of vanillin in fission yeast (*Schizosaccharomyces pombe*) and baker's yeast (*Saccharomyces cerevisiae*). *Applied Environmental Microbiology,* 75**,** 2765-74.

Hazelwood Lucie, A., Daran, J.-M., van Maris Antonius, J. A., Pronk Jack, T. & Dickinson, J. R. 2008. The Ehrlich pathway for fusel alcohol production: a century of research on *Saccharomyces cerevisiae* metabolism. *Applied and Environmental Microbiology,* 74**,** 2259-2266.

Hernandez Lozada, N. J., Lai, R. Y., Simmons, T. R., Thomas, K. A., Chowdhury, R., Maranas, C. D. & Pfleger, B. F. 2018. Highly active C(8)-Acyl-ACP thioesterase variant isolated by a synthetic selection strategy. *ACS synthetic biology,* 7**,** 2205-2215.

Hetzler, B. E., Trauner, D. & Lawrence, A. L. 2022. Natural product anticipation through synthesis. *Nature Reviews Chemistry,* 6**,** 170-181.

Hohne, M., Kuhl, S., Robins, K. & Bornscheuer, U. T. 2008. Efficient asymmetric synthesis of chiral amines by combining transaminase and pyruvate decarboxylase. *Chembiochem,* 9**,** 363-5.

Jamil, O. K., Cravens, A., Payne, J. T., Kim, C. Y. & Smolke, C. D. 2022. Biosynthesis of tetrahydropapaverine and semisynthesis of papaverine in yeast. *Proceedings of the National Academy of Sciences,* 119**,** e2205848119.

Jankowski, M. D., Henry, C. S., Broadbelt, L. J. & Hatzimanikatis, V. 2008. Group contribution method for thermodynamic analysis of complex metabolic networks. *Biophysical Journal,* 95**,** 1487-99.

Jensen, M. K. & Keasling, J. D. 2015. Recent applications of synthetic biology tools for yeast metabolic engineering. *FEMS Yeast Research,* 15**,** 1-10.

Jordt, S. E. & Julius, D. 2002. Molecular basis for species-specific sensitivity to "hot" chili peppers. *Cell,* 108**,** 421-30.

Kabita, K. C., Sanatombi, K. & Sharma, S. K. 2020. Efficient enhancement of capsaicinoids biosynthesis in cell suspension cultures of *Capsicum chinense* Jacq. cv. 'Umorok' by elicitors and differential gene expression analysis of elicited cultures. *Plant Cell Tissue and Organ Culture,* 141**,** 145-154.

Kaga, H., Goto, K., Takahashi, T., Hino, M., Tokuhashi, T. & Orito, K. 1996. A general and stereoselective synthesis of the capsaicinoids via the orthoester Claisen rearrangement. *Tetrahedron,* 52**,** 8451-8470.

Kaneda, T. & Smith, E. J. 1980. Relationship of primer specificity of fatty acid *de novo* synthetase to fatty acid composition in 10 species of bacteria and yeasts. *Canadian Journal of Microbiology,* 26**,** 893-8.

Kang, S., Kang, K., Chung, G. C., Choi, D., Ishihara, A., Lee, D. S. & Back, K. 2006. Functional analysis of the amine substrate specificity domain of pepper tyramine and serotonin *N*-hydroxycinnamoyltransferases. *Plant Physiology,* 140**,** 704-15.

Kaulmann, U., Smithies, K., Smith, M. E. B., Hailes, H. C. & Ward, J. M. 2007. Substrate spectrum of ω-transaminase from *Chromobacterium violaceum* DSM30191 and its potential for biocatalysis. *Enzyme and Microbial Technology,* 41**,** 628-637.

Keasling, J. D. 2010. Manufacturing molecules through metabolic engineering. *Science,* 330**,** 1355-8.

Kehie, M., Kumaria, S., Tandon, P. & Ramchiary, N. 2015. Biotechnological advances on *in vitro* capsaicinoids biosynthesis in capsicum: a review. *Phytochemistry Reviews,* 14**,** 189-201.

Kelly, S. A., Pohle, S., Wharry, S., Mix, S., Allen, C. C. R., Moody, T. S. & Gilmore, B. F. 2018. Application of ω-transaminases in the Pharmaceutical Industry. *Chemical Reviews,* 118**,** 349-367.

Klein, C., Paul, J. I., Sauvé, K., Schmidt, M. M., Arcangeli, L., Ransom, J., Trueheart, J., Manfredi, J. P., Broach, J. R. & Murphy, A. J. 1998. Identification of surrogate agonists for the human FPRL-1 receptor by autocrine selection in yeast. *Nature Biotechnology,* 16**,** 1334-1337.

Klempien, A., Kaminaga, Y., Qualley, A., Nagegowda, D. A., Widhalm, J. R., Orlova, I., Shasany, A. K., Taguchi, G., Kish, C. M., Cooper, B. R., D'Auria, J. C., Rhodes, D., Pichersky, E. & Dudareva, N. 2012. Contribution of CoA ligases to benzenoid biosynthesis in petunia flowers. *Plant Cell,* 24**,** 2015-30.

Klug, L. & Daum, G. 2014. Yeast lipid metabolism at a glance. *FEMS Yeast Research,* 14**,** 369-88.

Knudsen, J. D., Carlquist, M. & Gorwa-Grauslund, M. 2014. NADH-dependent biosensor in *Saccharomyces cerevisiae*: principle and validation at the single cell level. *Amb Express,* 4**,** 81.

Kobata, K., Sugawara, M., Mimura, M., Yazawa, S. & Watanabe, T. 2013a. Potent production of capsaicinoids and capsinoids by *Capsicum* Peppers. *Journal of Agricultural and Food Chemistry,* 61**,** 11127-11132.

Kobata, K., Sugawara, M., Mimura, M., Yazawa, S. & Watanabe, T. 2013b. Potent production of capsaicinoids and capsinoids by *Capsicum* peppers. *Journal of Agricultural and Food Chemistry,* 61**,** 11127-32.

Koivisto, A. P., Belvisi, M. G., Gaudet, R. & Szallasi, A. 2022. Advances in TRP channel drug discovery: from target validation to clinical studies. *Nature Reviews Drug Discovery,* 21**,** 41-59.

Koplas, P. A., Rosenberg, R. L. & Oxford, G. S. 1997. The role of calcium in the desensitization of capsaicin responses in rat dorsal root ganglion neurons. *Journal of Neuroscience,* 17**,** 3525-37.

Koszelewski, D., Göritzer, M., Clay, D., Seisser, B. & Kroutil, W. 2010a. Synthesis of optically active amines employing recombinant ω-transaminases in *E. coli* cells. *ChemCatChem,* 2**,** 73-77.

Koszelewski, D., Tauber, K., Faber, K. & Kroutil, W. 2010b. ω-Transaminases for the synthesis of non-racemic α-chiral primary amines. *Trends in Biotechnology,* 28**,** 324-32.

Larroy, C., Fernandez, M. R., Gonzalez, E., Pares, X. & Biosca, J. A. 2002. Characterization of the *Saccharomyces cerevisiae* YMR318C (ADH6) gene product as a broad specificity NADPH-dependent alcohol dehydrogenase: relevance in aldehyde reduction. *Biochemical Journal,* 361**,** 163-72.

Leber, C., Polson, B., Fernandez-Moya, R. & Da Silva, N. A. 2015. Overproduction and secretion of free fatty acids through disrupted neutral lipid recycle in *Saccharomyces cerevisiae*. *Metab Eng,* 28**,** 54-62.

Lee, W.-H., Seo, S.-O., Bae, Y.-H., Nan, H., Jin, Y.-S. & Seo, J.-H. 2012. Isobutanol production in engineered *Saccharomyces cerevisiae* by overexpression of 2-ketoisovalerate decarboxylase and valine biosynthetic enzymes. *Bioprocess and Biosystems Engineering,* 35**,** 1467-1475.

Leete, E. & Louden, M. C. 1968. Biosynthesis of capsaicin and dihydrocapsaicin in *Capsicum frutescens*. *Journal of the American Chemical Society,* 90**,** 6837-41.

Lefebvre, T., Destandau, E. & Lesellier, E. 2021. Selective extraction of bioactive compounds from plants using recent extraction techniques: A review. *Journal of Chromatography A,* 1635**,** 461770.

Li, Y., Li, S., Thodey, K., Trenchard, I., Cravens, A. & Smolke, C. D. 2018. Complete biosynthesis of noscapine and halogenated alkaloids in yeast. *Proceedings of the National Academy of Sciences,* 115**,** E3922-E3931.

Liao, M., Cao, E., Julius, D. & Cheng, Y. 2013. Structure of the TRPV1 ion channel determined by electron cryo-microscopy. *Nature,* 504**,** 107-12.

Liu, P., Chernyshov, A., Najdi, T., Fu, Y., Dickerson, J., Sandmeyer, S. & Jarboe, L. 2013. Membrane stress caused by octanoic acid in *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology,* 97**,** 3239-51.

Luengo, J. M. & Olivera, E. R. 2020. Catabolism of biogenic amines in *Pseudomonas species*. *Environmental Microbiology,* 22**,** 1174-1192.

Luo, X., Reiter, M. A., d'Espaux, L., Wong, J., Denby, C. M., Lechner, A., Zhang, Y., Grzybowski, A. T., Harth, S., Lin, W., Lee, H., Yu, C., Shin, J., Deng, K., Benites, V. T., Wang, G., Baidoo, E. E. K., Chen, Y., Dev, I., Petzold, C. J. & Keasling, J. D. 2019. Complete biosynthesis of cannabinoids and their unnatural analogues in yeast. *Nature,* 567**,** 123-126.

Lv, H., Li, J., Wu, Y., Garyali, S. & Wang, Y. 2016. Transporter and its engineering for secondary metabolites. *Applied Microbiology and Biotechnology,* 100**,** 6119-6130.

Ma, X., Koepke, J., Panjikar, S., Fritzsch, G. & Stockigt, J. 2005. Crystal structure of vinorine synthase, the first representative of the BAHD superfamily. *Journal of Biological Chemistry,* 280**,** 13576-83.

Manfrao-Netto, J. H. C., Lund, F., Muratovska, N., Larsson, E. M., Parachin, N. S. & Carlquist, M. 2021. Metabolic engineering of *Pseudomonas putida* for production of vanillylamine from lignin-derived substrates. *Microbial Biotechnology,* 14**,** 2448-2462.

Marini, E., Magi, G., Mingoia, M., Pugnaloni, A. & Facinelli, B. 2015. Antimicrobial and anti-virulence activity of capsaicin against erythromycin-resistant, cell-Invasive group A streptococci. *Frontiers in microbiology,* 6**,** 1281.

Materska, M. & Perucka, I. 2005. Antioxidant activity of the main phenolic compounds isolated from hot pepper fruit (*Capsicum annuum* L). *Journal of Agricultural and Food Chemistry,* 53**,** 1750-6.

Mazourek, M., Pujar, A., Borovsky, Y., Paran, I., Mueller, L. & Jahn, M. M. 2009. A dynamic interface for capsaicinoid systems biology. *Plant Physiology,* 150**,** 1806-21.

Miettinen, K., Leelahakorn, N., Almeida, A., Zhao, Y., Hansen, L. R., Nikolajsen, I. E., Andersen, J. B., Givskov, M., Staerk, D., Bak, S. & Kampranis, S. C. 2022. A GPCR-based yeast biosensor for biomedical, biotechnological, and point-of-use cannabinoid determination. *Nature Communications,* 13**,** 3664.

Minwoo, K., Shinje, K., Soohyun, K. & Byung-Dong, K. 2001. Isolation of cDNA clones differentially accumulated in the placenta of pungent pepper by suppression subtractive hybridization. *Molecules and Cells,* 11**,** 213-219.

Mohr, J. T., Krout, M. R. & Stoltz, B. M. 2008. Natural products as inspiration for the development of asymmetric catalysis. *Nature,* 455**,** 323-32.

Moran, M. M. & Szallasi, A. 2018. Targeting nociceptive transient receptor potential channels to treat chronic pain: current state of the field. *British Journal of Pharmacology,* 175**,** 2185-2203.

Moriello, A. S., De Petrocellis, L. & Vitale, R. M. 2023. Fluorescence-based assay for TRPV1 channels. *Methods in Molecular Biology,* 2576**,** 119-131.

Muratovska, N., Silva, P., Pozdniakova, T., Pereira, H., Grey, C., Johansson, B. & Carlquist, M. 2022. Towards engineered yeast as production platform for capsaicinoids. *Biotechnology Advances,* 59**,** 107989.

Murdoch, R. W. & Hay, A. G. 2013. Genetic and chemical characterization of ibuprofen degradation by *Sphingomonas* Ibu-2. *Microbiology (Reading),* 159**,** 621-632.

Myers, B. R., Bohlen, C. J. & Julius, D. 2008. A yeast genetic screen reveals a critical role for the pore helix domain in TRP channel gating. *Neuron,* 58**,** 362-73.

Nelson, R. K. 1919. Vanillyl-acyl amides. *Journal of the American Chemical Society,* 41**,** 2121-2130.

Newman, D. J. & Cragg, G. M. 2020. Natural products as sources of new drugs over the nearly four decades from 01/1981 to 09/2019. *Journal of Natural Products,* 83**,** 770-803.

Nicolaou, K. C. & Rigol, S. 2018. A brief history of antibiotics and select advances in their synthesis. *J Antibiot (Tokyo),* 71**,** 153-184.

Ogawa, K., Murota, K., Shimura, H., Furuya, M., Togawa, Y., Matsumura, T. & Masuta, C. 2015. Evidence of capsaicin synthase activity of the Pun1-encoded protein and its role as a determinant of capsaicinoid accumulation in pepper. *Bmc Plant Biology,* 15**,** 93.

Pancrazio, J. J., Whelan, J. P., Borkholder, D. A., Ma, W. & Stenger, D. A. 1999. Development and application of cell-based biosensors. *Annals of Biomedical Engineering,* 27**,** 697-711.

Payne, J. T., Valentic, T. R. & Smolke, C. D. 2021. Complete biosynthesis of the bisbenzylisoquinoline alkaloids guattegaumerine and berbamunine in yeast. *Proceedings of the National Academy of Sciences,* 118**,** e2112520118.

Pelckmans, M., Renders, T., Van de Vyver, S. & Sels, B. F. 2017. Bio-based amines through sustainable heterogeneous catalysis. *Green Chemistry,* 19**,** 5303-5331.

Peppin, J. F. & Pappagallo, M. 2014. Capsaicinoids in the treatment of neuropathic pain: a review. *Therapeutic Advances in Neurological Disorders,* 7**,** 22-32.

Perruca-Foncillas, R., Davidsson, J., Carlquist, M. & Gorwa-Grauslund, M. F. 2022. Assessment of fluorescent protein candidates for multi-color flow cytometry analysis of *Saccharomyces cerevisiae*. *Biotechnology Reports,* 34**,** e00735.

Philpott, H. K., Thomas, P. J., Tew, D., Fuerst, D. E. & Lovelock, S. L. 2018. A versatile biosynthetic approach to amide bond formation. *Green Chemistry,* 20**,** 3426-3431.

Ro, D. K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C., Withers, S. T., Shiba, Y., Sarpong, R. & Keasling, J. D. 2006. Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature,* 440**,** 940-3.

Rodriguez, A., Strucko, T., Stahlhut, S. G., Kristensen, M., Svenssen, D. K., Forster, J., Nielsen, J. & Borodina, I. 2017. Metabolic engineering of yeast for fermentative production of flavonoids. *Bioresource Technology,* 245**,** 1645-1654.

Romero-Suarez, D., Keasling, J. D. & Jensen, M. K. 2022. Supplying plant natural products by yeast cell factories. *Current Opinion in Green and Sustainable Chemistry,* 33**,** 100567.

Roughley, S. D. & Jordan, A. M. 2011. The medicinal chemist's toolbox: an analysis of reactions used in the pursuit of drug candidates. *Journal of Medicinal Chemistry,* 54**,** 3451-79.

Sánchez-Moreno, I., Oroz-Guinea, I., Iturrate, L. & García-Junceda, E. 2012. 7.20 Multi-Enzyme Reactions. *In:* CARREIRA, E. M. & YAMAMOTO, H. (eds.) *Comprehensive Chirality.* Amsterdam: Elsevier.

Savile, C. K., Janey, J. M., Mundorff, E. C., Moore, J. C., Tam, S., Jarvis, W. R., Colbeck, J. C., Krebber, A., Fleitz, F. J., Brands, J., Devine, P. N., Huisman, G. W. & Hughes, G. J. 2010. Biocatalytic asymmetric synthesis of chiral amines from ketones applied to sitagliptin manufacture. *Science,* 329**,** 305-9.

Schmidt, G. W., Jirschitzka, J., Porta, T., Reichelt, M., Luck, K., Torre, J. C., Dolke, F., Varesio, E., Hopfgartner, G., Gershenzon, J. & D'Auria, J. C. 2015. The last step in cocaine biosynthesis is catalyzed by a BAHD acyltransferase. *Plant Physiology,* 167**,** 89-101.

Shin, J. S. & Kim, B. G. 1999. Asymmetric synthesis of chiral amines with ω-transaminase. *Biotechnology and Bioengineering,* 65**,** 206-11.

Sorokina, M. & Steinbeck, C. 2020. Review on natural products databases: where to find data in 2020. *Journal of Cheminformatics,* 12**,** 20.

Srinivasan, P. & Smolke, C. D. 2019. Engineering a microbial biosynthesis platform for *de novo* production of tropane alkaloids. *Nature Communications,* 10**,** 3634.

Srinivasan, P. & Smolke, C. D. 2020. Biosynthesis of medicinal tropane alkaloids in yeast. *Nature,* 585**,** 614-619.

Starai, V. J., Celic, I., Cole, R. N., Boeke, J. D. & Escalante-Semerena, J. C. 2002. Sir2-dependent activation of acetyl-CoA synthetase by deacetylation of active lysine. *Science,* 298**,** 2390-2.

Stewart, C., Jr., Kang, B. C., Liu, K., Mazourek, M., Moore, S. L., Yoo, E. Y., Kim, B. D., Paran, I. & Jahn, M. M. 2005. The Pun1 gene for pungency in pepper encodes a putative acyltransferase. *The Plant Journal,* 42**,** 675-88.

Stewart, C., Jr., Mazourek, M., Stellari, G. M., O'Connell, M. & Jahn, M. 2007. Genetic control of pungency in *C. chinense* via the Pun1 locus. *J Exp Bot,* 58**,** 979-91.

Stovicek, V., Holkenbrink, C. & Borodina, I. 2017. CRISPR/Cas system for yeast genome engineering: advances and applications. *FEMS Yeast Research,* 17**,** fox030.

Strucko, T., Magdenoska, O. & Mortensen, U. H. 2015. Benchmarking two commonly used *Saccharomyces cerevisiae* strains for heterologous vanillin-β-glucoside production. *Metabolic Engineering Communications,* 2**,** 99-108.

Sutton, K. G., Garrett, E. M., Rutter, A. R., Bonnert, T. P., Jarolimek, W. & Seabrook, G. R. 2005. Functional characterisation of the S512Y mutant vanilloid human TRPV1 receptor. *British Journal of Pharmacology,* 146**,** 702-11.

Suzuki, T. & Iwai, K. 1984. Chapter 4 Constituents of red pepper species: chemistry, biochemistry, pharmacology, and food science of the pungent principle of *Capsicum* Species. *In:* BROSSI, A. (ed.) *The Alkaloids: Chemistry and Pharmacology.* Academic Press.

Swain, J. & Kumar Mishra, A. 2015. Location, partitioning behavior, and interaction of capsaicin with lipid bilayer membrane: study using its intrinsic fluorescence. *The Journal of Physical Chemistry B,* 119**,** 12086-93.

Szallasi, A. & Blumberg, P. M. 2007. Complex regulation of TRPV1 by vanilloids. BTI - TRP ion channel function in sensory transduction and cellular signaling cascades. CRC Press/Taylor & Francis CTI - Frontiers in Neuroscience.

Takpho, N., Watanabe, D. & Takagi, H. 2018. Valine biosynthesis in *Saccharomyces cerevisiae* is regulated by the mitochondrial branched-chain amino acid aminotransferase Bat1. *Microbial Cell,* 5**,** 293-299.

Tehlivets, O., Scheuringer, K. & Kohlwein, S. D. 2007. Fatty acid synthesis and elongation in yeast. *Biochimica et Biophysica Acta,* 1771**,** 255-70.

Tewksbury, J. J., Reagan, K. M., Machnicki, N. J., Carlo, T. A., Haak, D. C., Penaloza, A. L. & Levey, D. J. 2008. Evolutionary ecology of pungency in wild chilies. *Proceedings of the National Academy of Sciences,* 105**,** 11808-11.

Torello Pianale, L., Rugbjerg, P. & Olsson, L. 2021. Real-time monitoring of the yeast intracellular state during bioprocesses with a toolbox of biosensors. *Frontiers in microbiology,* 12**,** 802169.

Truppo, M. D., Rozzell, J. D., Moore, J. C. & Turner, N. J. 2009. Rapid screening and scale-up of transaminase catalysed reactions. *Organic & Biomolecular Chemistry,* 7**,** 395-8.

Tucker, C. L. & Fields, S. 2001. A yeast sensor of ligand binding. *Nature Biotechnology,* 19**,** 1042-1046.

Tufvesson, P., Lima-Ramos, J., Jensen, J. S., Al-Haque, N., Neto, W. & Woodley, J. M. 2011. Process considerations for the asymmetric synthesis of chiral amines using transaminases. *Biotechnology and Bioengineering,* 108**,** 1479-93.

Uarrota, V. G., Maraschin, M., de Bairros, A. F. M. & Pedreschi, R. 2021. Factors affecting the capsaicinoid profile of hot peppers and biological activity of their non-pungent analogs (*Capsinoids*) present in sweet peppers. *Critical Reviews in Food Science and Nutrition,* 61**,** 649-665.

Vanable, E. P., Habgood, L. G. & Patrone, J. D. 2022. Current progress in the chemoenzymatic synthesis of natural products. *Molecules,* 27**,** 6373.

Vidan, S. & Snyder, M. 2001. Making drug addicts out of yeast. *Nature Biotechnology,* 19**,** 1022-1023.

Von Roepenack-Lahaye, E., Newman, M. A., Schornack, S., Hammond-Kosack, K. E., Lahaye, T., Jones, J. D., Daniels, M. J. & Dow, J. M. 2003. *p*-Coumaroylnoradrenaline, a novel plant metabolite implicated in tomato defense against pathogens. *Journal of Biological Chemistry,* 278**,** 43373-83.

Walton, N. J., Mayer, M. J. & Narbad, A. 2003. Vanillin. *Phytochemistry,* 63**,** 505-15.

Wang, X., Liang, Z., Hou, J., Bao, X. & Shen, Y. 2016. Identification and functional evaluation of the reductases and dehydrogenases from *Saccharomyces cerevisiae* involved in vanillin resistance. *BMC Biotechnology,* 16**,** 31.

Wawrosch, C. & Zotchev, S. B. 2021. Production of bioactive plant secondary metabolites through *in vitro* technologies-status and outlook. *Applied Microbiology and Biotechnology,* 105**,** 6649-6668.

Weber, N., Gorwa-Grauslund, M. & Carlquist, M. 2014a. Exploiting cell metabolism for biocatalytic whole-cell transamination by recombinant *Saccharomyces cerevisiae*. *Applied Microbiology and Biotechnology,* 98**,** 4615-24.

Weber, N., Gorwa-Grauslund, M. & Carlquist, M. 2017. Improvement of whole-cell transamination with *Saccharomyces cerevisiae* using metabolic engineering and cell pre-adaptation. *Microbial Cell Factories,* 16**,** 3.

Weber, N., Ismail, A., Gorwa-Grauslund, M. & Carlquist, M. 2014b. Biocatalytic potential of vanillin aminotransferase from *Capsicum chinense*. *BMC Biotechnology,* 14**,** 25.

Weis, W. I. & Kobilka, B. K. 2014. The molecular basis of G Protein–Coupled Receptor activation. *Annual Review of Biochemistry,* 87**,** 897-919.

Willey, J. M., Sherwood, L., Woolverton, C. J. & Prescott, L. M. 2017. *Prescott's microbiology,* New York: McGraw-Hill, McGraw-Hill Education.

Williams, D. C., McGarvey, D. J., Katahira, E. J. & Croteau, R. 1998. Truncation of limonene synthase preprotein provides a fully active 'pseudomature' form of this monoterpene cyclase and reveals the function of the amino-terminal arginine pair. *Biochemistry,* 37**,** 12213-20.

Wong, G. Y. & Gavva, N. R. 2009. Therapeutic potential of vanilloid receptor TRPV1 agonists and antagonists as analgesics: Recent advances and setbacks. *Brain Research Reviews,* 60**,** 267-77.

Zhang, J., Hansen, L. G., Gudich, O., Viehrig, K., Lassen, L. M. M., Schrubbers, L., Adhikari, K. B., Rubaszka, P., Carrasquer-Alvarez, E., Chen, L., D'Ambrosio, V., Lehka, B., Haidar, A. K., Nallapareddy, S., Giannakou, K., Laloux, M., Arsovska, D., Jorgensen, M. A. K., Chan, L. J. G., Kristensen, M., Christensen, H. B., Sudarsan, S., Stander, E. A., Baidoo, E., Petzold, C. J., Wulff, T., O'Connor, S. E., Courdavault, V., Jensen, M. K. & Keasling, J. D. 2022. A microbial supply chain for production of the anti-cancer drug vinblastine. *Nature,* 609**,** 341-347.

Zhang, J., Sonnenschein, N., Pihl, T. P., Pedersen, K. R., Jensen, M. K. & Keasling, J. D. 2016. Engineering an NADPH/NADP(+) redox biosensor in yeast. *ACS synthetic biology,* 5**,** 1546-1556.

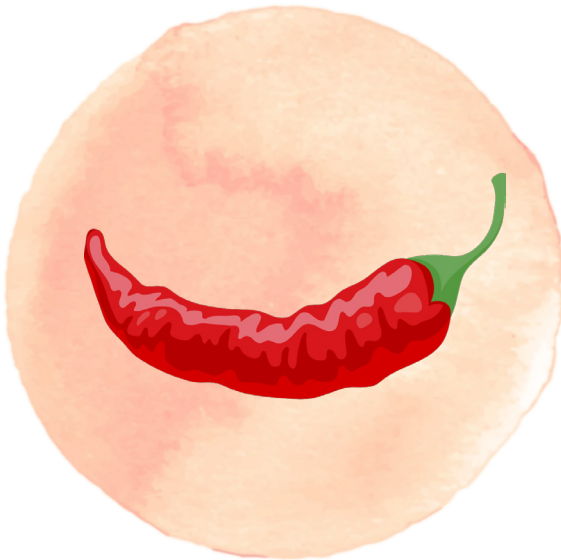
Zheng, Y., Li, L., Liu, Q., Qin, W., Yang, J., Cao, Y., Jiang, X., Zhao, G. & Xian, M. 2012. Boosting the free fatty acid synthesis of *Escherichia coli* by expression of a cytosolic *Acinetobacter baylyi* thioesterase. *Biotechnology Biofuels,* 5**,** 76.

Zhu, Z., Zhou, Y. J., Krivoruchko, A., Grininger, M., Zhao, Z. K. & Nielsen, J. 2017. Expanding the product portfolio of fungal type I fatty acid synthases. *Nature Chemical Biology,* 13**,** 360-362.

Zhu, Z. W., Hu, Y. T., Teixeira, P. G., Pereira, R., Chen, Y., Siewers, V. & Nielsen, J. 2020. Multidimensional engineering of *Saccharomyces cerevisiae* for efficient synthesis of medium-chain fatty acids. *Nature Catalysis,* 3**,** 64-74.

Zicha, S., Radresa, O., Laplante, P., Morton, M., Jones, K., Main, M., Trivedi, S., Julien, R. P., Griffin, A., Labrecque, J., Ahmad, S. & Brown, W. 2013. Novel methodology to identify TRPV1 antagonists independent of capsaicin activation. *Journal of Biomolecular Screening,* 18**,** 544-55.

Printed by Media-Tryck, Lund 2023 NORDIC SWAN ECOLABEL 3041 0903



ISBN: 978-91-7422-928-8

Applied Microbiology
Department of Chemistry
Faculty of Engineering
Lund University