Xanthophyllomyces dendrorhous as a platform organism for the production of terpenes

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CHAPTER 1

INTRODUCTION AND SCOPE OF THE THESIS

Microbial fermentation has been a mean for humans to convert raw materials into refined food for thousands of years; bread, wine and beer are the most well-known examples. In recent years, with the development of genetic engineering, microorganisms have been modified for the production of non-native substances with the aim of reducing costs and increasing the availability of compounds such as human insulin, plant derived drugs or biofuel. Genetic engineering has then become the basis of two main biological fields: synthetic biology and metabolic engineering.

While synthetic biology aims at the complete engineering of entire cells via, among other tools, the creation of artificial genomes, metabolic engineering modifies the microbial native pathways to improve production of specific compounds.

When synthetic biology and metabolic engineering meet, the result is a microorganism whose pathways have been tuned towards the production of one or more valuable heterologous substances. Before such a proof of principle organism can become a proper "platform organism" or "cell factory", it has to meet industrial standards of yield, titer and productivity.

The process to obtain a strain that is optimized for the production of a single compound or for a class of compounds in general requires a development time between three and six years with a minimum of one hundred people working on this objective every year. In order to reduce the time invested in research, companies have been focusing on just a few already well-established industrial microorganisms. Depending on the structure of the compounds, different microorganisms have been chosen. For example, *Corynebacterium* species have been used extensively for the production of amino acids, while different strains of *Saccharomyces cerevisiae* have been engineered to maximize the yields of biofuels, food ingredients or pharmaceuticals. The advantages of using model organisms for industrial purposes include available genome databases, the existence of single or multiple gene mutants, extensively studied metabolic pathways and molecular engineering tools optimized for fast genetic transformation and functional screening. Nevertheless, it is important to consider that the biodiversity in nature can provide numerous organisms which have not been fully characterized, yet, but that might provide further advantages over the common laboratory and industrial strains.

A microorganism that is already capable of producing compounds similar to the molecular targets or has a high flux of the precursors involved in the synthesis of

a specific chemical would be a good candidate when searching for alternatives to the already well known microbes.

In order to identify a potential platform organism for the production of terpenoids and, in particular sesquiterpenes, alternative to *S. cerevisiae*, the potential of the basidiomycetous yeast *Xanthophyllomyces dendrorhous* was elucidated in this thesis.

The importance of terpenes, their functions, their secondary metabolic pathways and research on the metabolic engineering of microorganism for the production of expensive terpene based drugs with limited availability are reviewed in **Chapter 2**. In this chapter, *X. dendrorhous* is introduced and an update on the ongoing research on this yeast is provided.

Chapter 3 demonstrates the production of the first non-native terpene, pentalenene, in *X. dendrorhous*. The transformation of the yeast is obtained by the concurrent inactivation by insertion of two genes involved in the carotenoid pathway toward the production of astaxanthin.

In **Chapter 4**, the synthase gene from *Coprinus cinereus*, *Cop6*, is transferred to *S. cerevisiae* and *X. dendrorhous* for a comparison in α-cuprenene production between the two organisms. The putative germacrene synthase gene from *C. cinereus*, *Cop4*, is used to demonstrate the differences in terpene production between the two yeasts. Furthermore, the transformation of *X. dendrorhous* with a vector containing *Cop6* and the two cytochromes *COX1* and *COX2* results in a mutant strain able to express the three genes and produce the compound lagopodin A, as explained in **Chapter 5**.

The possibility of expressing the terpene cyclase amorphadiene synthase (*ADS*) in different cell compartments is expanded in **Chapter 6**. The targeting sequences for the mitochondria and for the peroxisomes in *S. cerevisiae* are fused to the ADS and to the green fluorescent protein (GFP) to demonstrate the transferring of the proteins to the right compartments and to compare the amorphadiene production in the different strains.

CHAPTER 2

METABOLIC ENGINEERING OF MICROORGANISMS FOR THE PRODUCTION OF TERPENOIDS

Remco Muntendam*, Elena Melillo*, Annamargareta Ryden, Oliver Kayser

(*Authors contributed equally)

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Terpenoids and their properties

With more than 25,000 identified structures from plants, microorganisms, insects and marine organisms, terpenoids are one of the largest class of natural compounds [1]. Terpenoids serve many, very diverse functions in the natural world ; their roles span from membrane fluidity and hormone signaling (sterols) [2], photosynthesis in plants and antioxidant agents in fungi (carotenoids) [3, 4], to electron transport or antimicrobial compounds (quinones) [5, 6] and protein glycosylation (dolichol) [7].

Food and pharmaceutical industries have already commercialized several compounds with terpene skeletons possessing interesting properties. Some good examples are menthol (**Fig. 1A**) as flavoring agent [8], artemisinin (**Fig. 1B**) as antimalarial drug [9] and paclitaxel (Taxol) (**Fig. 1C**) showing antineoplastic properties [10].

Furthermore, enzymes in the two biosynthetic routes, the mevalonate (MVA) and the 2*C*-methyl-D-erythrotol-4-phosphate (MEP) pathway, used by several organisms to produce terpenes have been targeted for a wide range of medicinal purposes. For example, statins, the cholesterol lowering drugs, act by inhibiting the first enzyme in the MVA pathway, hydroxy methylglutaryl CoA reductase (HMGR) and thus depleting cells of downstream terpenoids, including farnesyl diphosphate (FPP). FPP is a key intermediate in the synthesis of cholesterol and its reduced production results in a decreased *de novo* cholesterol synthesis [11]. While statins inhibit the HMGR from the MVA pathway, the second enzyme of the MEP pathway, 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR), which converts 1-deoxy-D-xylulose-5-phosphate (DXP) into the branched compound MEP [12], is the molecular target for fosmidomycin. The MEP pathway enzymes are highly conserved but show no homology to mammalian proteins [13]. This implies that the use of such a specific inhibitor would result in novel antimicrobial drugs with broad spectrum activity and little toxicity to humans.

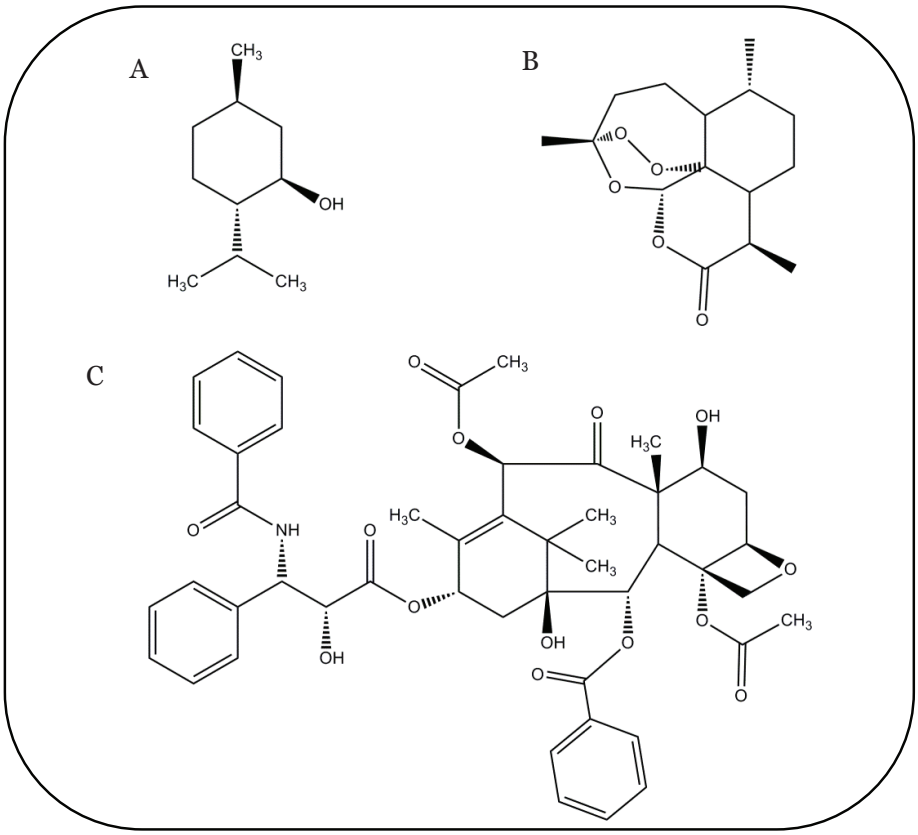

Figure 1. (**A**) menthol; (**B**) artemisinin and (**C**) paclitaxel

Terpene compounds like limonene, menthol and carotenoids are available in discrete quantities in nature and can be produced and purified simply. But terpenoids like artemisinin and paclitaxel are rare and have low abundance, causing supply problems. Metabolic engineering is a new concept in molecular biology and considered as new avenue for the production of rare and high-cost natural products. In the artemisinin and paclitaxel biosynthesis pathways most enzymes are known, allowing the introduction of the complete primary and secondary pathway in heterologous hosts possible. The main idea is the complete transfer of a plant terpenoid pathway to a microorganism to optimize the biosynthetic production rate by fermentation of the genetically engineered microorganisms. However, the targeted bacterial and fungal pathways are regulated through complex networks. Furthermore, the regulation of the terpenoid pathway is far from simple and control

mechanisms are unknown. In this chapter the challenges and promises of recent developments in terpenoid pathway engineering will be discussed.

Biosynthesis of terpenoids in several organisms

Nature has developed two biosynthetic pathways for the production of the terpenoid backbones, namely MVA and MEP. The MVA pathway (**Fig. 2**) has been described by the Nobel Prize winners Bloch and Lynen, who elucidated the cholesterol pathway [14]. For almost 40 years it was thought that all isoprenes descended from the MVA pathway, although studies indicated that not all of the produced terpenoid structures could be explained solely by the MVA pathway. In 1993, an alternative pathway for isopentenyl diphosphate (IPP) biosynthesis (Fig. 3) was reported in bacteria, through intensive analysis of ^{13}C labeling in hopanoid production by the microorganisms studied [15]. Soon after the publications describing the alternative pathway, others reported the same pathway in algae, diatoms, and protozoa [16, 17]. The nomenclature for the alternative pathway was confusing as various names had been used in literature, like DOXP-, DXP-, MEP-, Rohmer- or non-mevalonate pathway. Only recently the pathway was officially named the "MEP pathway", according to the first official precursor in the biosynthetic route in *Escherichia coli* [18].

Both pathways produce IPP; however, delivery of precursors depends on alternative metabolic routes. The MVA pathway is fed by the precursor acetyl-CoA obtained in the citrate cycle. The MEP pathway is, instead, mainly fed by D-glyceraldehyde 3-phosphate, produced via the pentose phosphate pathway, and pyruvate, present in several metabolic routes, including the citrate cycle and glycolysis [19]. However, in photosynthetic organisms and in anaerobic conditions, the MEP pathway is mainly fed through production of the same precursors by the Calvin cycle [20, 21].

Higher plants possess both pathways, while prokaryotes, with some exceptions [22], have the MEP pathway, and other eukaryotes may use either the MEP or MVA pathway. In addition, higher plants have separated the two pathways in different compartments. The MVA pathway is localized in the cytosol, while the MEP pathway is separated in plastids. The coexistence of both pathways in higher plants and the intracellular trafficking of precursors makes higher plants unique compared to prokaryotes and the eukaryotes that have only one of two biosynthetic routes.

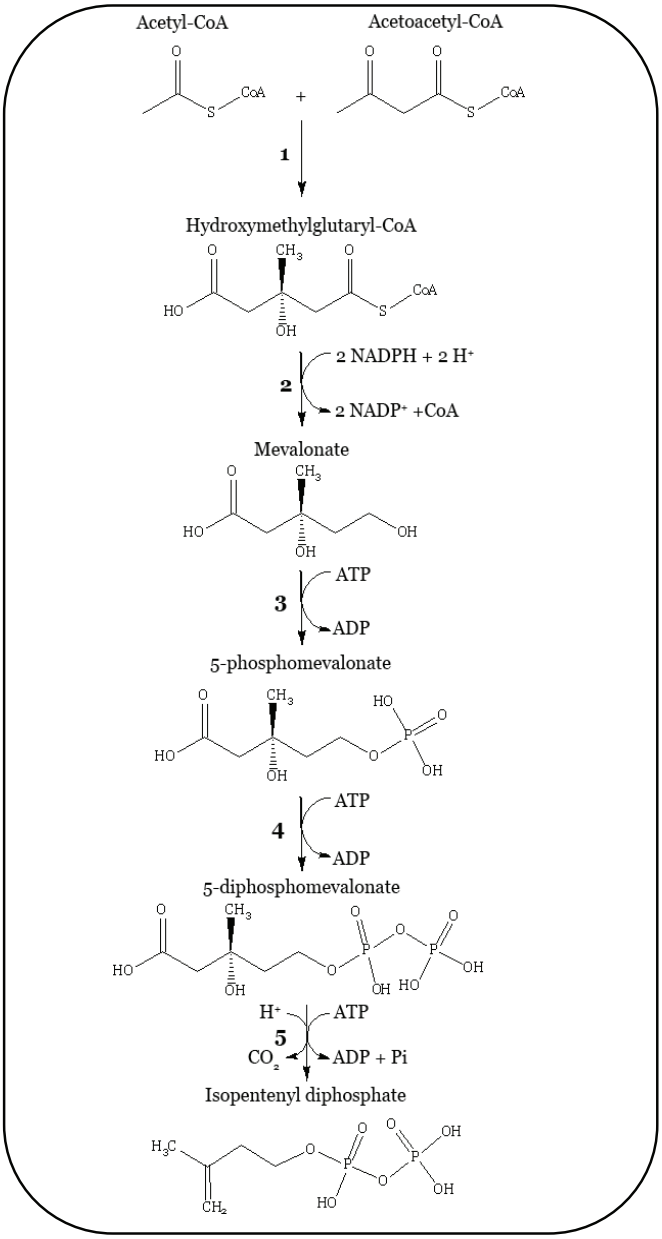

Figure 2. The MVA pathway.
1. HMG-CoA synthase (HMGS);
2. HMG-CoA reductase (HMGR);
3. mevalonate kinase;
4. phosphomevalonate kinase;
5. diphosphomevalonate decarboxylase

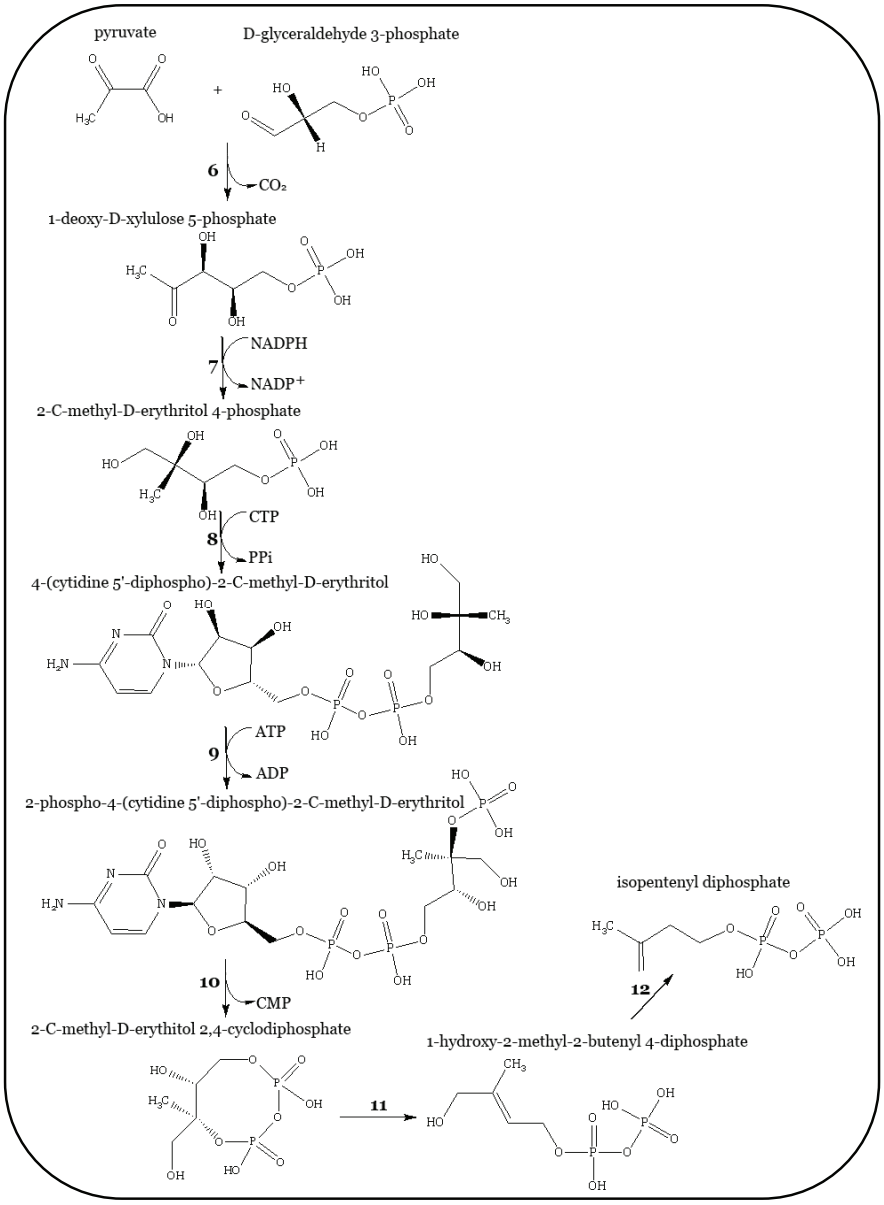

Figure 3. The MEP pathway. **6**. 1-deoxy-D-xylulose-5-phosphate synthase; **7.** 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR); **8.** 4-diphosphocytidyl-2-*C*-methyl-D-erythritol synthase; **9.** 4-diphosphocytidyl-2-*C*-methyl-D-erythritol kinase; **10.** 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase; **11.** 2-C-methyl-D-erythritol-2,4-cyclodiphosphate reductase; **12.** 1-hydroxy-2-methyl-2-butenyl-4-diphosphate reductase (HDR).

The interchange of metabolites, named “cross talking,” occurs in varying degrees between species and depends on physiological conditions [23-27]. The

regulatory system controlling this cross talking is not yet fully understood [28] and elucidating this system remains a major challenge in biochemistry and metabolic engineering.

Various terpene synthases are known to convert the terpenoid backbone into pathway-specific structures; however, some are less specific and can produce more than one structure [29]. During the enzymatic modification of common terpenoid backbones (**Fig. 4**), like geranyl diphosphate (GPP), FPP or geranyl-geranyl diphosphate (GGPP) a divalent metal, generally magnesium, is used to stabilize the diphosphate group. After this transformation, the pyrophosphate is lost and more species-specific enzymes (e.g., cytochromes) are used by the organisms to create the known broad diversity of secondary relevant terpenoids [30, 31].

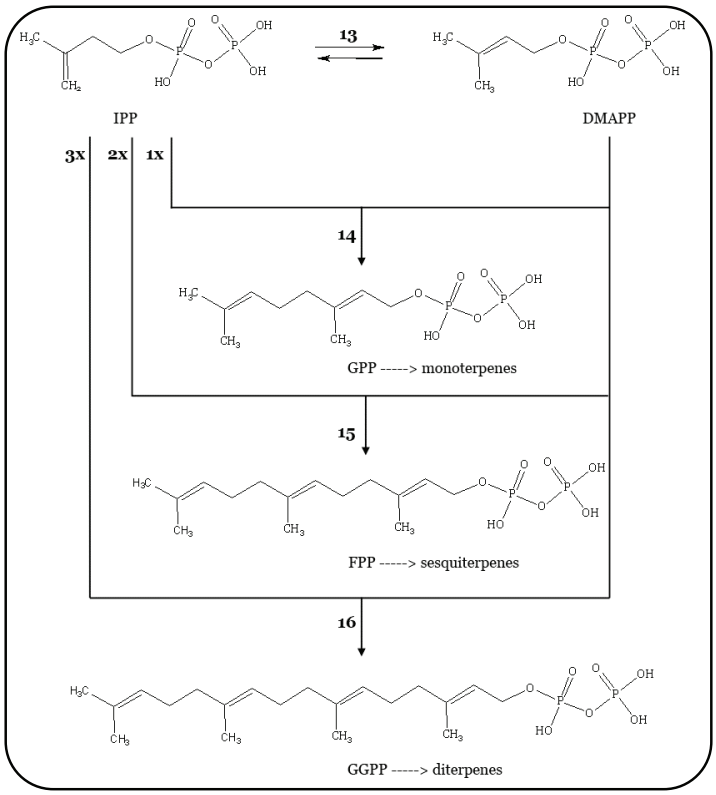

Figure 4. Fusion of IPP and dimethylallyl diphosphate (DMAPP) to GPP, FPP and GGPP.
13. Isopentenyl diphosphate isomerase;
14. GPP synthase (GPPS);
15. FPP synthase (FPPS);
16. GGPP synthase (GGPPS)

Sesquiterpenoids and diterpenoids often possess macrocyclic rings as exemplified in artemisinin, paclitaxel, and ginkgolide. The enzymes responsible for the ring closing step are the terpenoid cyclases. A comparison between terpenoid cyclases shows that, despite the lack of sequence identity, structural similarity is high

[32, 33]. The major difference between terpenoid synthases derived from plants and those produced by microorganisms is the presence of an N-terminal sequence in plant enzymes. This N-terminal sequence is similar to a glucosyl hydrolase [33], and is crucial for the enzyme activity [34], although the specific function remains unknown. The diversity of the secondary terpenoids is caused by promiscuity, mutations, and various enzymes acting downstream on the terpene skeleton and may explain chemotypes within a family or species as it is known for *Mentha x piperita* or *Thymus vulgaris*. This variation can be used for the selection of highly producing species for commercial medicinal plant cultivation, commercial microbiological fermentation and more sophisticated strategies in metabolic engineering.

Metabolic fluxes can differ among families, species and even within a multicellular organism. An example is the production of astaxanthin by various hosts. Several studies were performed on the production capacities of yeasts, plants, and microalgae. These studies show that wild-type production levels of astaxanthin differ dramatically between hosts. *Chlorella zofingiensis* (a microalga) produces about 0.1% astaxanthin of the dry weight, *Nicotiana tabacum* around 0.24% [35], *Xanthophyllomyces dendrorhous* between 0.4% and 2.5% [36], and the highest production in a wild-type organism of asthaxanthin is obtained by the microalga *Haematococcus pluvialis* with production levels of around 3% of dry weight [37, 38]. Next to accumulation levels, the produced enantiomer is also different in various species. For instance, in *X. dendrorhous* the all-trans isomer is mainly produced [39], where *H. pluvialis* produces mainly the all-cis isomer [40].

Metabolic engineering of the terpenoid pathway

Metabolic engineering is considered as one of the major concepts in biotechnology. At the moment, genetic engineering allows the transfer of a biosynthetic pathway to any selected host. The isoprenoid pathway is an example of how biosynthetic-relevant genes can be reassembled from biological sources (e.g., plants, bacteria and fungi) into a heterologous microorganism. Engineering of plant terpenoids into microbial hosts has focused mainly on isoprenoid-derived compounds such as carotenoids [41], artemisinin [42], and paclitaxel [43]. The production of carotenoids and artemisinin demonstrates that complex natural products can be produced by microbial fermentation with yields approaching

commercial viability. Development of a microbial production platform for the biosynthesis of complex terpenoids offers large-scale and cost-effective industrial production via fermentation, which is independent from climate (too dry, too wet) and cultivation risks (inefficient pest control, weeds competing for food, bad soil conditions). Well-characterized and genetically easy to manipulate heterologous hosts like *E. coli* and *S. cerevisiae* allow specific engineering of biosynthetic pathways for increased yields and the generation of novel compounds. Although extensive engineering is frequently required to successfully reconstitute biosynthetic pathways in these hosts, the potential to easily manipulate the biosynthesis is a significant advantage when compared to engineering in poorly characterized host strains or non-microbial systems.

Engineering the primary metabolism

The first enzyme committed to the MEP pathway (**6**) is 1-deoxy-D-xylulose-5-phosphate synthase (DXS). This enzyme is documented to be involved in the rate-limiting step in the MEP pathway [44] and, as a consequence, it is often subject to overexpression with the goal to increase production of GPP and, in extension, monoterpenes. A recent example of *DXS* overexpression in *Lavandula latifolia* resulted in a 4.6-fold increase of essential oils in leaves and a 1.74-fold increase in flowers compared with the control plant [45]. However, Botella-Pavia and Besumbes proved that in transgenic *Arabidopsis thaliana* grown under light conditions hydroxymethylbutenyl diphosphate reductase (HDR) (**12**), a late enzyme in the MEP pathway that simultaneously synthesizes IPP and DMAPP, functions as the rate-determining enzyme, rather than DXS. In their experimental setup with recombinant *A. thaliana* expressing taxadiene synthase (*TXS*), *DXS* was overexpressed, and taxadiene production increased six times compared to the plant line solely expressing the taxadiene synthase. Furthermore, constitutive coexpression of *TXS* and *HDR* resulted in a 13 times higher production of taxadiene (TXD) [46, 47]. These studies clearly indicate that more knowledge on pathway regulation is essential for a better understanding of the metabolic engineering and flux improvement.

A well-known regulation mechanism in the MVA biosynthetic pathway acts on the enzyme HMGR (**2**). This enzyme converts the substrate HMG-CoA to mevalonate and represents the rate-determining step in the MVA pathway [48].

Several groups have successfully overexpressed this gene to increase the production of downstream isoprenoids. The enzyme is inhibited by phosphorylation as a control mechanism and is stimulated by accumulation of downstream sterols. Accumulation of phytosterols in seeds was observed in transgenic tobacco plants expressing a mutated form of HMGR from *Arabidopsis thaliana*. The serine in the recognition site for the HMGR-kinase was replaced by an alanine, causing insensitivity to the phosphorylation and thus reduced degradation. Although accumulation of phytosterols was only observed in the seeds and not in the leaves, the authors did not describe the presence of other isoprene derivatives, whose concentration could be elevated by the increased presence of mevalonic acid [49]. Another example of HMGR modification is the truncation of the N-terminal membrane anchor domain and exclusive expression of the catalytic C-terminal domain of the protein. The truncated form of HMGR (tHMGR) increased the flux through the MVA pathway as it is unresponsive to negative feedback by FPP [50, 51].

Strategies to maximize IPP production are more complex than single gene expression or inclusion of various endogenous enzymes from the MVA or MEP pathway. Currently employed strategies include the expression of multiple heterologous genes or even of complete heterologous pathways [52-54]. One approach to introduce the MVA pathway from *S. cerevisiae* into *E. coli*, led to an elevated production (112.2 mg/l) of amorphadiene (AD). This is a 6.5 fold increase to when only amorphadiene synthase (*ADS*) was expressed in *E. coli* [55]. While expressing heterologous genes or pathways for overproduction of isoprenoids is highly desirable, a serious risk is host-specific down-regulation of the heterologous biosynthetic genes. For maximal production the engineered pathway was required to be tuned suboptimal in several cases. Pitera and colleagues described a good example in which the heterologous MVA pathway engineered in *E. coli*, which normally harbors exclusively the MEP pathway (**Fig. 3**), leads to accumulation of enzymatic products that induce growth inhibition or become toxic to the host. Only by fine-tuning of the gene expression these bottlenecks of isoprenoid production became acceptable and relieved the growth inhibition. The tuning was accomplished by monitoring the specific metabolic profiles of well-growing and growth-limited mutants. By titration of gene expression and analysis of metabolite production, HMG-CoA accumulation proved to be one of the reasons of growth inhibition. After introduction of tHMGR, the rate of biosynthesis was increased, and growth was restored by approaching the growth rate of the wild type [56].

Bottlenecks within pathways have an important role, as they serve as control points within a native organism to regulate resource utilization and production of metabolites. Metabolic engineering strives to elevate these bottlenecks by adapting expression levels of native or heterologous proteins and inherently increase production rates and levels of metabolites. A way to empirically identify the optimal tuning of genes and, hence, eliminate bottlenecks in a pathway involves the creation of a library from the intergenic regions containing various mRNA secondary structures, RNase cleavage sites, and ribosomal binding site sequestering sequences [57]. Another parameter that has to be considered is the promoter, which can have a significant influence on product yield, as described in a case of chromosomal replacement of endogenous promoter with the exogenous strong bacteriophage T5 promoter in *E. coli* [58].

Parallel usage of transcriptomics, proteomics and metabolomics becomes more often the chosen strategy to elucidate the circuit before engineering [59-61]. After engineering a pathway, it is desirable to analyze the metabolic profile to be able to compare before–after situations and detect effects on the pathway originating from more distant networks. The ultimate goal is to be able to predict which steps need to be engineered to maximize the metabolic flux in a desired pathway. This can be done by comprehensive ^{13}C-glucose labeling studies in which the distribution of the heavy atom among the metabolites is calculated based on the known biochemistry of the participating pathways and, at the same time, taking into account general cofactors such as ATP, NADH, and NADPH [62]. Biological sensors represent a new technique that is used in addition to standard analytical methods such as NMR, GCMS and HPLC, to detect higher carbon flux through a pathway. An example of a biosensor system is based on a strain where the MEP pathway has been permanently disrupted by deleting an early gene in the pathway. Gene disruption makes the cell auxotrophic for isoprenoids due to lack of production of IPP and DMAPP. As a consequence, this strain can produce isoprenoid building blocks exclusively via an engineered partial MVA pathway containing the enzymes downstream of HMGR or when MVA is supplemented to the media. This strain was further engineered to express green fluorescent protein (GFP) constitutively, for real-time growth rate detection. Furthermore, the auxotrophic strain was co-cultivated with a second strain producing mevalonate allowing survival of the first strain. The growing bacteria gave a fluorescent signal, which was correlated to the levels of mevalonic acid produced by the second strain [63].

The use of auxotrophic strains also helps in the struggle for plasmid stabilization in microorganisms like *E. coli*. An example is the cloning of a heterologous MVA pathway in an *E. coli* knockout where the last step responsible for IPP production had been deleted from the genome and rescued by a similar gene on the plasmid. By using an auxotrophic strain and the plasmid to rescue the IPP production, higher plasmid stability was established [64].

Predictions on metabolic fluxes have successfully been performed to identify target genes to be knocked out for an increased lycopene production in *E. coli*. This research has led to the construction of a knockout variant that produces 40% more lycopene compared to the parental strain [65]. A recent approach to obtain maximum production levels did not only rely on a single knockout or overexpression of single genes, but instead on a multidimensional target search combining knockout and overexpression targets [66]. A multidimensional search was performed combining several one-dimensional search programs. Hereby, it became possible to screen *in silico* combinations of knockouts and overexpression patterns that would not have been detected using a single one-dimensional search. Based on initial selection of mutant candidates from *in silico* predictions, 40 mutants were constructed and screened for optimized carotenoids production. Grown in a minimal medium with glucose, an identified superior single strain produced 16 mg of carotenoids per gram of cells in 24 hours. Recombinant carotenoids production is a comparatively simple system for the validation of *in silico* prediction models as the products can be detected easily by spectroscopy due to their typical yellow to red color [67].

Out of simplicity reasons, most engineering efforts have focused on the primary pathway to optimize carotenoid production and have neglected parallel or distant pathways competing for precursors and cofactors. System biology opens the possibility to handle the interactions between biological networks at transcriptomics and at metabolomics levels. An example is the work of Papon and colleagues which looked outside the MVA and MEP biosynthetic pathways for non-direct regulation of IPP production. Here, supplementation of cytokinin and ethylene had a positive effect on the expression level of three genes from the MEP pathway in *Catharanthus roseus* cell suspensions [68]. In yeast, redirection of the carbon flux from competing pathways, such as sterols, into the desired pathway for the production of terpenoids has proven to be successful. By downregulating *ERG9*, a squalene synthase in *S. cerevisiae*, the production of the sesquiterpenoid AD was increased 5-fold when compared to a strain in which only *ADS* was expressed [69].

A novel strategy increases the pathway competition, rather than reducing it. The main idea is to overexpress an enzyme situated at a branching point in the biosynthetic network. One interesting branching enzyme in yeast is the acetaldehyde dehydrogenase competing for cytosolic acetaldehyde, which compound if otherwise transported and used in the mitochondria. With additional overexpression of a heterologous cytosolic acetyl-CoA synthase and the *ADS* a metabolic sink was created to drive the carbon flux into the MVA pathway and produce AD [70].

High yields of carotenoids in cauliflower (*Brassica oleracea* L. var. botrytis) were accomplished when a metabolic sink was combined with overexpression of carotenoid biosynthetic enzymes [71]. In plants, carotenoids are synthesized in the membranes of the plastids and accumulated in the chromoplasts that can store carotenoids with tremendous efficacy and act as a metabolic sink preventing possible negative feedback in the biosynthetic pathway. By overexpression in *B. oleracea* of *Or*, a gene involved in differentiation of plastids into chromoplasts, a 5 times increase in concentration of β-carotene was obtained in a heterozygous mutant and an 8 times increase in a homozygous mutant. A remarkable result was that *Or* did not affect the expression levels of carotenoid or MEP pathway biosynthetic genes [72, 73]. However, when making use of organelles as production sites, it is important to consider the adverse effect of depletion of common metabolites that cannot be transported across membranes. As an example, depletion of acetyl-CoA in tobacco mitochondria, caused by the expression of an acetyl-CoA hydrolase, resulted in a growth-affected phenotype [74].

Engineering the secondary metabolism: artemisinin, paclitaxel and astaxanthin

In contrast to the primary metabolism, no common biological components exist which would be present in all different secondary pathways. Secondary metabolite routes are per definition species specific, and therefore a high diversity in molecular structures between organisms in the terpenoid biosynthesis is to be expected [75]. Artemisinin is an excellent example since *Artemisia annua* produces low amounts of artemisinin, while its relatives *Artemisia absinthium* [76] and *Artemisia afra* [77] produce no artemisinin at all. The properties of many terpenoids are interesting for recombinant production, although many pathways are yet too

complex or lack an identification of enzymes; for this reason, three case studies will be discussed: artemisinin, paclitaxel, and astaxanthin.

Artemisinin (**Fig. 1A**) is a sesquiterpenoid lactone endoperoxide extracted from *A. annua* L. (Asteraceae; known also as "sweet wormwood"). The activity of artemisinin against malaria parasite *P. falciparum* has been well established. Artemisinin has been proven to act by selective inhibition of the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA). The same study shows that artemisinin induces a change in the membranous structures in the cytoplasm of parasites inside erythrocytes [9]. Examples of derivatives of artemisinin include artemether and artemisone, which are on the market or currently under development [78].

The low production yield of artemisinin from *A. annua* (0.01–1% of dry weight) has led to difficulties in managing the demand while offering an acceptable price for most patients [79, 80]. The wish to improve the overall supply of artemisinin at reduced market price has encouraged interest in molecular biology and biochemistry of artemisinin biosynthesis (**Fig. 5**) [31, 81-85]. A recent study demonstrates that both the cytosolic MVA pathway and the plastidic MEP pathway might be involved in the production of IPP for artemisinin biosynthesis [86]. The biosynthesis starts with the cyclization of FPP to AD by ADS (**17**) [87]. Subsequent enzymatic steps can be executed in different orders. The primary conversion scheme *in planta* for production of artemisinin includes the oxidation of AD to artemisinic alcohol followed by a further oxidation at C12 from alcohol to aldehyde by the cytochrome P450 CYP71AV1 (**18**). In the subsequent step, the carbon double bond at C11, 13 is reduced giving dihydroartemisinic aldehyde by artemisinic aldehyde reductase Dbr2 (**19**), then dihydroartemisinic aldehyde is transformed into dihydroartemisinic acid by the CYP71AV1. The formation of the 1,2,4-trioxane moiety is poorly understood at the moment and is suggested to occur in a non-enzymatic manner (Ro et al. 2006; Covello et al. 2007; Rydén and Kayser 2007). Identification of these last unknown enzymes is essential for the production of artemisinin in heterologous systems. The applicability of heterologous expression was proven for the first time by the expression of the genes ADS and cytochrome P450 CYP71AV1 in yeast. The transgenic *S. cerevisiae* strain produces more artemisinic acid in contrast to relative plant biomass (4.5% dry weight in yeast compared to 1.9% artemisinic acid, and 0.16% artemisinin in *A. annua*) and in a shorter time (4–5 days for yeast

versus several months for *A. annua* [84]. When these two enzymes were expressed in yeast together with *Dbr2*, dihydroartemisinic acid was produced at a level of 15.7 (±1.4)-mg/l culture and the related artemisinic acid was produced at levels of 11.8 mg/l [88]. The production of artemisinic acid was reduced compared to a mutant in which the artemisinic aldehyde reductase was not present (29.4 mg/l). In the latter mutant instead dihydroartemisinic acid was produced. The synthesis of dihydroartemisinic acid is preferred because it is converted towards artemisinin straightforward and with a reduced cost. Westfall and colleagues further modified *S. cerevisiae* to yield amorphadiene at a concentration of 40 g/L. In spite of the major increase in amorphadiene production the production of artemisinic acid remained similar at 1.8 g/L [89].

Today, artemisinin production has also been improved by means of genetic transformation of *A. annua* plants [80]. Cotton FPP synthase was transferred to *A. annua*, and the transgenic plant showed an artemisinin production of 8–10 mg/g dry weight, which is 2 or 3 times higher than the wild type plants [90]. In addition, the overexpression of the IPP transferase gene in *A. annua* increased artemisinin content by 30–70% in contrast to the native control plant [91].

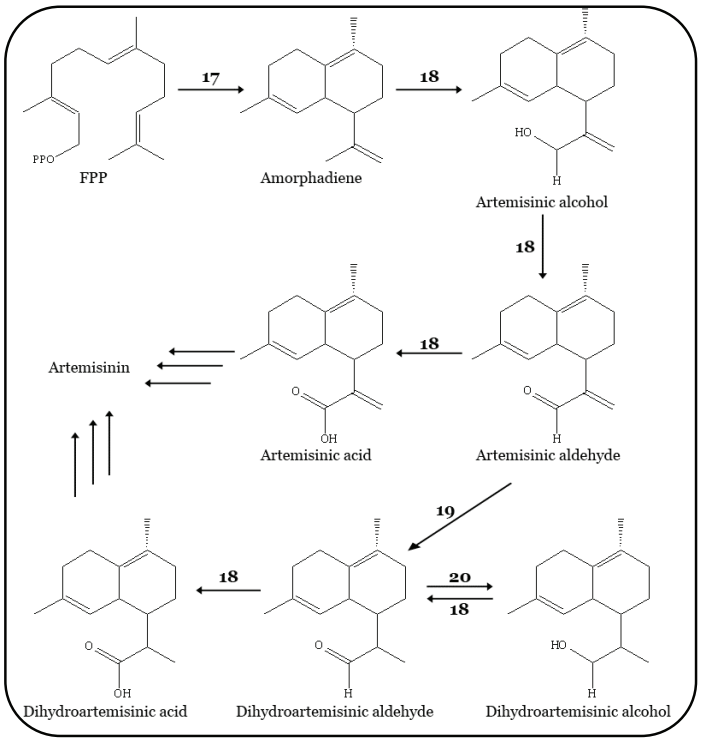

Figure 5.
Artemisinin biosynthetic pathway.
17. ADS;
18. CYP71AV;
19. Dbr2;
20. Reductase 1 [85]

Paclitaxel is a well-known example of a clinically important natural product with dramatic problems of supply. Paclitaxel is isolated from the bark of the Pacific yew (*Taxus brevifolia*), which is a rare and slowly growing tree. In addition, the amount of the active constituent produced in the tree is about 1 mg per 750 kg of bark [92], and its harvest is destructive for the tree itself. An alternative production system is the Taxus cell culture, which allows increased paclitaxel production after methyl jasmonate induction [93]. Furthermore, fungi including endophytes produce taxanes, including paclitaxel, in amounts of 500 mg/l media [94].

The biosynthesis (**Fig. 6**) of paclitaxel has been studied intensively, and some of the enzymes involved in the pathway, like TXS (**21**) [95], taxadiene 5α-hydroxylase (**22**), cytochrome P450 reductase (**23**), taxane 2α-*O*-benzoyltransferase (TBT) (**24**) [96], 10-deacetylbaccatin III-10-*O*-acetyltransferase (DBAT) (**25**) [97] and baccatin III-3-amino-3-phenylpropanoyltransferase (BAPT) (**26**) [98], have been elucidated. Other enzymes (taxadien-5α-ol-O-acetyl transferase, taxoid 10β-hydroxylase, taxoid 2α-hydroxylase, taxoid 9α-hydroxylase, taxane 13α-hydroxylase, taxoid 7β-hydroxylase, and 3′-N-debenzoyl-2′-deoxytaxol N-benzyoyltransferase) have been identified and/or characterized but their order of action in the pathway has not been elucidated, yet [99]. More enzymes, including taxoid 1β-hydroxylase, taxoid 9-keto-oxidase, β-phenylalanoyl-CoA ligase, taxoid 2′-hydroxylase, and those involved with oxetane ring formation, still need to be identified.

A functional expression of the TXS was achieved in *A. thaliana* seedlings and leaves [47]. In the transgenic plant leaves, expressing the gene for TXS under an inducible promoter, the production of TXD reached 600 ng/gram of dry weight. These experiments prove that significant increases in the production of TXD can be accomplished by selecting temporal and spatial coordinates of TXS expression, establishing a platform technology for future metabolic engineering of taxanes in crop plants. The identification of alternative enzymes from other organisms can represent an additional advantage in the production of paclitaxel precursors. When Pacific yew extracts were treated with three enzymes originating from *Nocardioides albus*, *Nocardioides luteus* and *Moraxella* spp., a C-13 taxolase, a C-10 deacetylase, and a xylosidase respectively, the mix of taxanes was converted primarily to 10-deacetylbaccatin III, and the amount of this precursor was increased by 4 to 24 times [100].

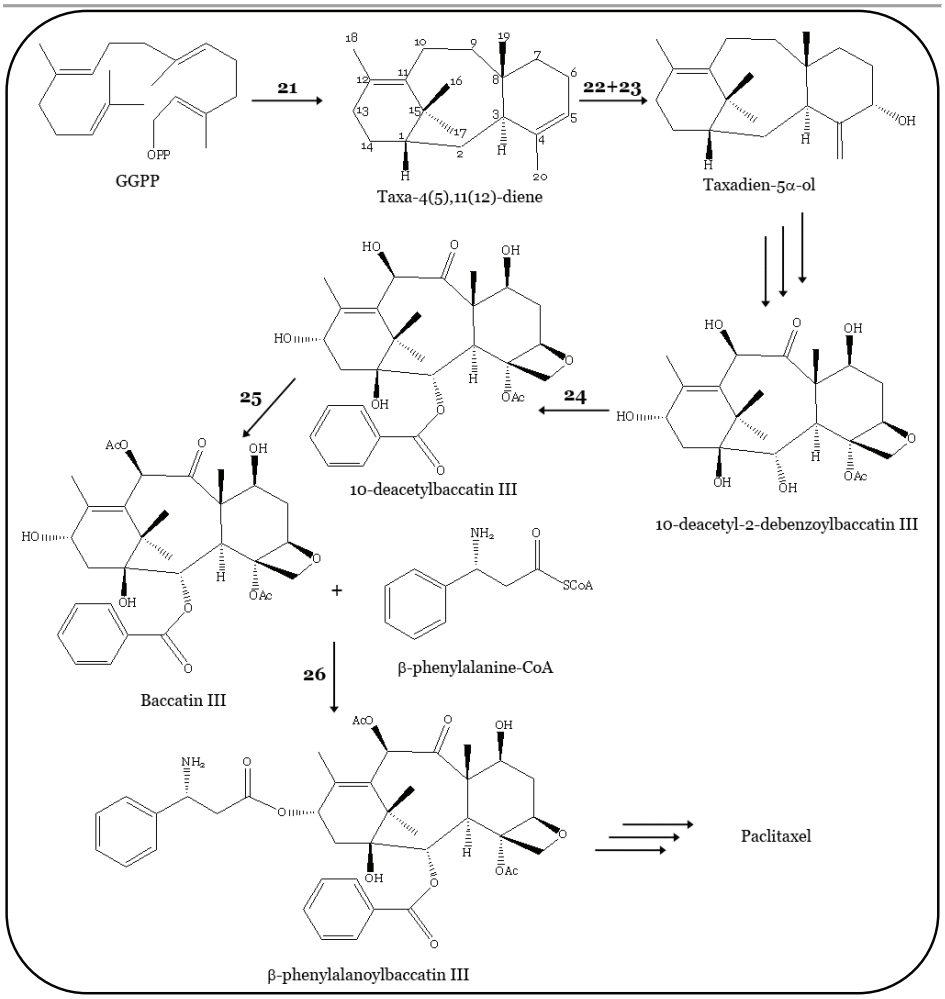

Figure 6. Paclitaxel biosynthetic pathway. **21.** TXS; **22.** taxadiene 5α-hydroxylase; **23.** Cytochrome P450 reductase; **24.** TBT; **25.** DBAT; **26.** BAPT.

The first expression of the partial paclitaxel pathway up to TXD was conducted by Huang and colleagues who realized that the limiting activity of the cytochrome P450 in *E. coli* was a major drawback [92]. In 2006 the first 8 known genes from paclitaxel pathway (GGPPS included) were expressed in *S. cerevisiae*. The production of the early precursor of paclitaxel, baccatin III, reached 0.7 mg/l up to 1 mg/l of medium. The downstream products could not be measured, indicating that the bottleneck was encountered at the 5α-hydroxylase level [101]. The production of TXD was further increased to 8.7 mg/L in *S. cerevisiae* by introducing a GGPPS from *Sulfolobus acidocalidarius*.

The most recent experiments study *E. coli* via a multivariate-modular approach to metabolic-pathway engineering. The pathway for the production of TXD was divided into an upstream native and a downstream heterologous pathway. The promoters driving the genes expression and the replicating origins of the plasmids were fine-tuned to produce the maximum amount of TXD while minimalizing the negative feedback by the metabolite indole. The final strain could produce approximately one gram of TXD per liter of culture in a fed-batch bioreactor. When the strain was further transformed with a chimeric construct consisting of a truncated taxadiene 5α-hydroxylase and a *Taxus* cytochrome P450 reductase, the bacteria produced taxadiene-5α-ol up to 60 mg/L. The lower taxadiene-5α-ol concentration compared to taxadiene is partially explained by the concurrent production of the hydroxylation byproduct 5(12)-oxa-3(11)-cyclotaxane [43]. Although, six more hydroxylation steps are required to obtain baccatin III, some of which are still unknown, this study confirms that the potential of the MEP pathway in *E. coli* can be unlocked and exploited for the marketing of microbially derived terpenoids both for pharmaceutical and energy purposes.

Carotenoids are a class of tetraterpenoid pigments that are often associated with flowers and fruits and are known to act as potent antioxidants [102]. All chlorophyll-containing photosynthetic organisms produce carotenoids, as do red yeasts and microalgae. These compounds are used in industry as coloring agents for agriculture, food and cosmetics, as precursors of vitamin A in humans [103] and animals [104], and as feed supplements for poultry and fish [105]. Carotenoids are also applied in prevention of macular degeneration [106, 107] and are suggested to be useful as anticancer agents [108]. Within the plant, carotenoids are synthesized inside the plastids. Some examples of carotenoid compounds are β-carotene, lutein, lycopene, α-carotene, β-cryptoxanthin and astaxanthin [109].

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is an oxygenated carotenoid which has valuable industrial applications. It is commonly used as a food additive in aquaculture to obtain the desired degree of pigmentation of flesh from salmon and trout allowing successful marketing. The European Commission considers astaxanthin as a food dye and the US Food and Drug Administration approved it as food for animals in 2009. Furthermore, several studies on medical properties of astaxanthin reported anti-inflammatory, antineoplastic, antibacterial, photoprotective, neuroprotective and immunomodulatory effects. Currently, the

organisms with the highest identified production of astaxanthin are the alga *H. pluvialis* and the yeast *X. dendrorhous*. While the astaxanthin produced in the esterified form by the microalga has a high stability, the carotenoid coming from the yeast has a better bioavailability for trout and salmon since is mainly composed of the free, non-esterified stereoisomer. In spite of the availability of astaxanthin in living organisms, 97% of the market is covered by the chemically synthesized compound. The chemical synthesis of astaxanthin is the cheapest process to obtain the carotenoid. It has been calculated that, in order to industrially compete with the synthetic approach, an organism would have to produce a minimum of four milligrams of astaxanthin per gram of cell dry weight and should be suitable for growth in industrial scale bioreactors [110]. The possible reduction of generated waste during production of the carotenoid and the possibility to increase the market price, by "naturally" isolating the carotenoid from microorganisms, has propelled the research around native and heterologous astaxanthin producers.

Microalgae are among the most common producers of astaxanthin, and *H. pluvialis* is the dominant species for the production of this carotenoid giving yields ranging between 1.5% and 3% of dry weight [37]. Recently, a protocol was developed to genetically transform *H. pluvialis* and improve the astaxanthin synthesis capacity [111].

The *Adonis* species are among the few plants to produce astaxanthin and the breeding of *Adonis palaestina* increased the carotenoid content from 7.9 to 17.9 mg/g of dried petals [112]. Furthermore, Tobacco, *Tagetes*, carrots and potatoes are a small selection of hosts that were genetically modified to obtain astaxanthin [35, 113-115].

Among yeasts, *X. dendrorhous*, grown in different media and under diverse conditions, has been the subject of several studies on the production and isolation of astaxanthin. The biosynthetic pathway for astaxanthin has been elucidated in this yeast (**Fig 7**). The first committed step fuses three molecules of IPP to one molecule of DMAPP giving GGPP (**16**) and is catalyzed by CrtE, a GGPP synthase. Both IPP and DMAPP used in this step are produced via the MEV pathway; in the second step two GGPP molecules are combined by the phytoene synthase CrtYB (**27**). Phytoene subsequently undergoes a desaturation step by the phytoene desaturase CrtI (**28**) to obtain lycopene. The gene *crtYB* also codes for a lycopene synthase which can catalyze the ring closure at either or both ends of the lycopene molecule. When just a single ring closure occurs, the resulting molecule is γ-carotene which, by action of the

same desaturase CrtI, is transformed into torulene and eventually into 3-hydroxy-3',4'-didehydro-β,-ψ-caroten-4-one (HDCO). β-carotene is made when both ends of the lycopene are closed to give a 6 membered ring. The order of the subsequent reactions is unknown, but it has been suggested to involve a series of hydroxylations and ketolyzations most likely catalyzed by CrtS, also known as astaxanthin synthetase, and CrtR, a cytochrome P450 reductase [116].

The production of astaxanthin in the wild type strain of *X. dendrorhous* ranges between 200 and 400 μg per gram of yeast dry weight, which is not enough to be commercially competitive with the chemically synthesized compound. Further chemical or physical mutagenesis led to isolation of mutants with a higher astaxanthin concentration, reaching a maximum concentration of one milligram of compound per gram of dry yeast weight [36, 117].

Since the biosynthetic pathway for astaxanthin is known and a transformation protocol for *X. dendrorhous* has been established, genetic engineering has played a major role in the enhancement of production of the tetraterpene [118]. *X. dendrorhous* is the organism studied in this thesis and a more comprehensive overview of the state of the art on this yeast will be provided in the next section.

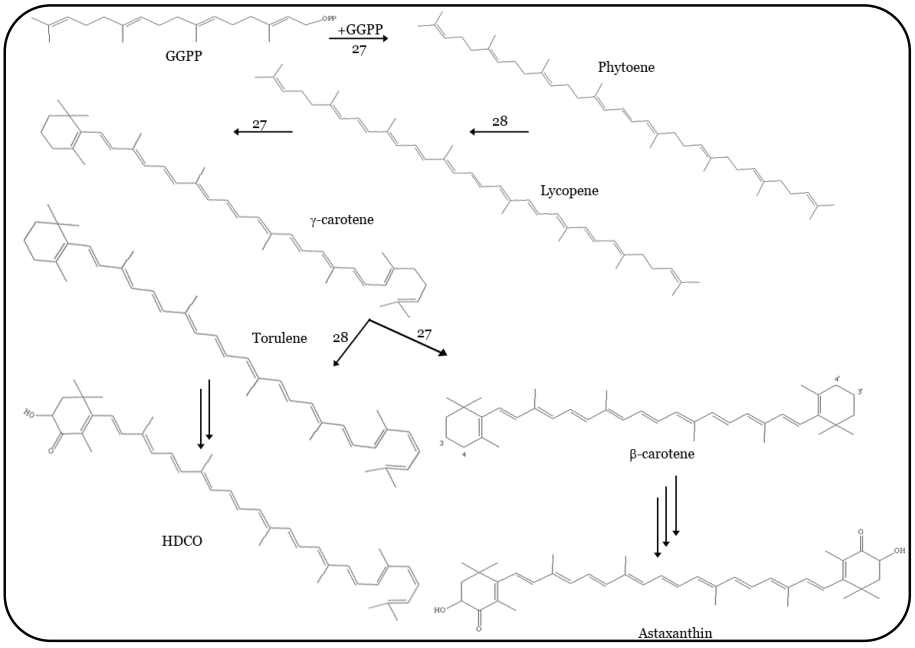

Figure 7. Astaxanthin biosynthetic pathway in *X. dendrorhous*. **27.** CrtYB; **28.** CrtI.

Selecting microorganisms to engineer the terpenoid pathways: X. dendrorhous *as a potential platform organism*

Commonly, the hosts *E. coli* and *S. cerevisiae* are favored in metabolic engineering, but other hosts can be used as well. Both laboratory domestic organisms have been studied extensively over decades, and it is advantageous that elucidation of their genome, transcriptome and metabolome is nearly complete; however, *in silico* models will yet need to be provided [119]. In theory, the possibility exists to adapt every host for the production of natural compounds, but, as various organisms exhibit different genetic backgrounds, they also react differently to genetic manipulations and the introduced recombinant genes.

Although nearly all organisms are able to produce terpenoid structures [11, 120], their production strategy and structural diversity varies dramatically between species. Synthetic biology currently provides the tools to explore the rich variety of enzymes by which nature is capable of creating multiple structures. These tools can be used by metabolic engineers: by selecting the right host for recombinant expression, it is possible to increase production levels by minimal adaption of the genetic system. The prerequisite for metabolic engineering of a plant or microorganism is a profound understanding of the isoprenoid biosynthesis at a molecular level for the production of the different classes of terpenoids. Although, the primary MVA and MEP pathways leading to the biosynthesis of GPP, FPP, and GGPP are well characterized, the knowledge about related secondary natural product biosynthesis is still insufficient. Artemisinin is an excellent example of a compound for which early biosynthetic steps have been elucidated, but enzymes involved in the late-stage transformation to construct the trioxan ring skeleton remain yet unknown.

When selecting a host other than *E. coli* or *S. cerevisiae*, it is wise to choose microorganisms with a natural high precursor flux. For instance, the yeast *X. dendrorhous* is industrially known for carotenoid accumulation [121-123]. Since this yeast is specialized to produce long-chain terpenoids, it is easier to use this ability for the production of terpenoid structures.

In 1972, *X. dendrorhous*, also known as *Phaffia rhodozyma*, was isolated in Japan and Alaska from birch trees by Herman J. Phaff for the first time. Ten strains were isolated from the trees and all of them produced red pigments, reproduced by budding and were able to ferment different sugars. The isolates were found to be all

species of a novel genus that was given the name of its discoverer, *Phaffia*. *X. dendrorhous* is defined as the sexual form of *P. rhodozyma* and has been taxonomically categorized as a basydiomycota based on the cell wall structure, the budding process, the pigments, the urease activity and the Q-10 ubiquinone system [124]. It is considered to be a psychrophilic yeast with a growth temperature ranging between 0°C and 27°C, with an optimum, depending on the strain, between 18°C and 22°. The cell wall polysaccharides include mainly β-(1,3) and β-(1,6) glucan and α-(1,3) glucan but little or no chitin is present. The cells are additionally surrounded by an acidic polysaccharides-based capsule. The sexual activity includes both sporulation and mating between two cells under starvation conditions and individual spores from genetically marked strains revealed evidence of karyogamy, meiosis and recombination.

The genome of *X. dendrorhous* has not been sequenced and very little is known about its structural and functional organization; the ploidity level is also still uncertain since some strains seem to be diploid and others aneuploid. The number of chromosomes found in the nucleus varies from 9 to 17; furthermore, several linear plasmids and cytoplasmic dsRNA viruses have been found in mitochondria and in the cytoplasm of the yeast cells, respectively [125].

Astaxanthin production is the main driving force for the research on X. dendrorhous. Not surprisingly, most of the genes isolated and sequenced from the yeast genome are involved in either the MEV or the carotenoid pathway. **Table 1** provides a list of all the known genes from *X. dendrorhous* published in literature and in patents.

The presence of introns characterizes all the sequences isolated, including several rDNA ORFs (which have not been included in the list, since these lack a complete characterization).

The concentration of astaxanthin in the wild type strains of the red yeast (between 200 and 400 μg per gram of yeast dry weight) is not sufficient for the industrialization of this organism. In order to find a superior astaxanthin producer, several groups initiated research on the mutagenesis of the wild type strains or tried to identify a natural high-producer [36, 117]. In 1996, the first gene (*act1*) was isolated from *X. dendrorhous* [126], but already in 1995 a transformation protocol with a plasmid used for *S. cerevisiae* had been established. In spite of the positive results, the transformation efficiency was extremely low and the plasmid was lost easily when the selection pressure was removed [127]. To improve the transformation

efficiency, a complete new vector was designed to allow the integration of the new sequence in the genomic rDNA. The mutants screening was based on resistance against the antibiotic geneticin, since the used vector included a resistance cassette with the kanamycin resistance gene (Km^r) under regulation of the promoter of the *act1* gene [128]. The same study described a new transformation protocol that increased the efficiency of the process. Although, cells containing the selection marker for the resistance against geneticin could be isolated, the gene expression driven by the promoter from the actin gene was still low. To overcome the problem of an insufficient gene expression, Verdoes and colleagues identified and cloned the native gene for the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) [129]. The promoter of this gene in other organisms is considered as a very strong promoter and has been used in several vectors to control gene expression in *S. cerevisiae* and *Pichia pastoris* [130, 131].

Table 1. Sequenced and characterized genes from *X. dendrorhous*

Gene (Genbank ID)	Gene full name	Ref.
act1 (X89898)	Actin	[126]
idi (AB019035)	IPP isomerase	[132]
gpd (Y08366)	Glyceraldehyde-3-phosphate dehydrogenase	[129]
crtE	GGPP synthase	[133]
eph1 (AF166258)	Epoxide hydrolase	[134]
crtYB (AJ133646)	Phytoene synthase/lycopene cyclase	[135]
crtI (Y150071)	Phytoene desaturase	[136]
Sqs	Squalene synthase	[137]
Hmc	HMG-CoA synthase	[138]
Hmg	HMG-CoA reductase	[138]
Mvk	Mevalonate kinase	[138]
Mpd	Mevalonate diphosphate decarboxylase	[138]
Fps	FPP synthase	[138]
crtS (ABA43719)	β-carotene oxygenase (Astaxanthin synth., Cy P450)	[116]
crtR (ACI43098)	Cytochrome P450 reductase	[139]
gdhA (EU7914561)	Glutamate dehydrogenase	[140]
Xd-INV (ACL79833)	β-fructofuranosidase	[141]
TEF-1α	Translation elongation factor 1-α	[142]
pdc (HQ 694558)	Pyruvate decarboxylase	[143]

With the identification of a strong promoter and the optimization of the transformation protocol, the identification, isolation and overexpression of the genes involved in the astaxanthin pathway became easier and faster. Sequences were obtained and overexpressed in other organisms, like *S. cerevisiae* [144, 145] or used in knock-out experiments to identify the different phenotypes caused by the absence of particular genes [121]. Some of these studies highlighted the possibility of alternative splicing of some genes, producing different proteins with different functions. A good example is given by the *crtYB* gene coding for two proteins involved in the astaxanthin pathway, a phytoene synthase (CrtY) and a β-carotene oxygenase (CrtB) [146].

In addition to isolation of natural or induced high astaxanthin producers and the genetic engineering of the wild type strains of *X. dendrorhous*, several studies have focused on growth conditions and media that would increase the carotenoids production in the red yeast. For example, Marcoleta and colleagues indicated ethanol as a strong inducer and glucose as a repressor of astaxanthin production [143]. Citrate and glutamate, precursors of the carotenogenesis, were also found to increase astaxanthin content when added to the cultures [147, 148]. Rodríguez-Sáiz and colleagues, together with the review published by Schmidt and colleagues, have summarized the major accomplishments in the industrial production of astaxanthin and provided an extensive overview of the studies performed on *X. dendrorhous* [149, 150].

In addition to the research on astaxanthin production in *X. dendrorhous*, a few studies have focused on proteins outside of the carotenoid pathway, like the extracellular β-fructofuranosidase, involved in the hydrolyzation of sucrose-like sugars, or the pyruvate decarboxylase induced by glucose feeding [141].

One aspect that has not been investigated is exploitation of the terpene precursors pool present in *X. dendrorhous*. As seen in the literature, Phaffia's potential to produce terpenoid structures is still far from being fully exploited. The presence of the MEV pathway, the knowledge about the carotenogenesis and the availability of transformation vectors and protocols provide us with the necessary techniques to investigate *X. dendrorhous* as a platform organism for the production of terpenes. By expressing different terpene synthases in the red yeast, knocking out the carotenoid pathway at different stages, comparing the production of the same compound in different microorganisms and examining the possibility of producing a

complete terpene-based drug we show that *Phaffia* is indeed a suitable host for the production of terpenoids.

CHAPTER 3

HETEROLOGOUS EXPRESSION OF PENTALENENE SYNTHASE (*PSS*) FROM *STREPTOMYCES UC5319* IN *XANTHOPHYLLOMYCES DENDRORHOUS*

Elena Melillo, Remco Muntendam, Wim J. Quax, Oliver Kayser

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For the first time, the pentalenene synthase (PSS) gene from Streptomyces UC5319 was expressed in *Xanthophyllomyces dendrorhous,* a native producer of astaxanthin. For the expression of the gene and the concurrent knock out of the native *crtE* or *crtYB* genes, two new vectors were engineered and used for the transformation of the wild-type strain of *X. dendrorhous*. The transformations resulted in white colonies, showing a complete shutdown of the carotenoid production. Furthermore, an additional vector was constructed for the insertion of the *PSS* gene in the rDNA of the yeast. All the mutant strains produce the sesquiterpene pentalenene and show no difference in growth when compared to the wild-type strain. In this report, we demonstrate that *X. dendrorhous* is a suitable host for the expression of heterologous terpene cyclases and for the production of foreign terpene compounds.

Introduction

Xanthophyllomyces dendrorhous belongs to the phylum of the basidiomycota and shows a characteristic red pigmentation [125] given by the carotenoid astaxanthin, produced by the yeast in response to oxidative stress [4]. Since the market size of astaxanthin in 2007 reached nearly 220 million US dollars [150], the improvement of the production of the tetraterpene in *X. dendrorhous* has been the main aim of the research performed on this yeast. Screening of mutants [151], genetic engineering [121] and optimization of medium and growth conditions [110, 152] represented the most common approaches chosen to increase the yield of astaxanthin from *X. dendrorhous*. Integrative plasmids and a transformation protocol have been developed for the study of the carotenoid pathway. The biosynthetic pathway for the production of carotenoids in *X. dendrorhous* has been elucidated [118] and some of the genes have been transferred to *Saccharomyces cerevisiae* [144]. The genes *crtE*, *crtI*, *crtYB, crtR* and *crtS* were identified, overexpressed and/or deleted in *X. dendrorhous*, yielding different phenotypes caused by the modified concentrations and ratios of the carotenoids in the yeast [121, 139, 153, 154].

The production of carotenoids by *X. dendrorhous* implies the presence of an upstream mevalonate (MEV) pathway that can provide the precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), for the formation of terpenes [155]. IPP and DMAPP constitute the building blocks for several different compounds, like volatile scented monoterpenes [156] sesquiterpenes with antimalarial or antibacterial properties [87, 157], sterols for membrane fluidity [2] and diterpenes with antineoplastic characteristics [43] While some of the terpenes are widely available in nature, e.g. limonene and menthol, some others are produced by the native host in low amounts or the extraction from the natural source is too inefficient. Thus, the need arises for alternative methods of production for such precious terpenoid drugs.

One of the potential strategies is genetic engineering of heterologous hosts for the production of molecules of interest or precursors that can be then easily chemically modified [158-160]. The metabolic engineering approach has been extensively applied, mostly to *Escherichia coli* and *S. cerevisiae*. While both models share several advantages, like the availability of the complete genome sequence and

advanced genetic engineering tools, they may show a low natural terpene flux [155]. A faster and less invasive solution might come from genetically engineering organisms that already have a higher level of terpene precursors, for example, *X. dendrorhous*.

We decided to use the pentalenene synthase (*PSS*) gene in our new expression vector as a proof that *X. dendrorhous* can functionally express a heterologous terpene cyclase. *PSS* is a prokaryotic sesquiterpene cyclase that catalyzes the formation of pentalenene using farnesyl diphosphate (FPP) as substrate [161] (**Fig. 1**). The gene is relatively short (~1 kb) and it was isolated from *Streptomyces UC5319*.

For the expression of the cyclase, two different approaches were chosen. In the first strategy, the gene was inserted in one of the two new vectors engineered using pPR2TN as a template [135] allowing the insertion of the expression cassette into the genes *crtE* or *crtYB*. As second approach, a modified pPRcDNA1crtE vector [153] was used for the integration of the PSS gene in the rDNA sequence.

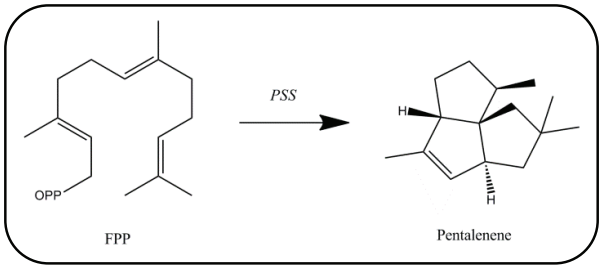

Figure 1. Conversion of FPP towards pentalenene catalyzed by pentalenene synthase, PSS.

Materials and methods

Strains and culture conditions

E. coli DH5α strain was used for the cloning steps; bacteria were grown in LB (1% Trypton, 0.5% Yeast Extract, 1% NaCl) with 100 mg/ml ampicillin in a shaking incubator at 37°C, 250 rpm.

The wild type strain *X. dendrorhous* CBS 6938 (ATCC 96594) was used as control and for all transformation procedures. YPD (1% Yeast Extract, 2% Peptone and 2% Glucose) medium with and without 40 µg/ml geneticin (G-418 Sulfate,

Gibco) was used for the yeast growth in a shaking incubator at 21°C, 180 rpm. The ratio between medium and air space in the flasks was always kept at 1:10.

Engineering of recombination vectors and transformation of X. dendrorhous

pGEMT vector was used as a backbone for the engineering of all the vectors in this study. The cassette for the resistance against geneticin was amplified from the plasmid pPR2TN [135].

The expression cassette was created by fusing the *GPD* promoter to the *GPD* terminator sequence from pPR2TN. The product presented a small multiple cloning site between the promoter and the terminator sequences with the unique restriction sites for *NheI*, *HpaI*, *AvaI* and *XhoI*, and was flanked by restriction sites for *SpeI* and *BamHI*. The complete cassette was ligated into pGEMT; the construct was then digested with *NdeI* and *SpeI* and ligated to the resistance cassette that had been cut with the same restriction enzymes, resulting in one complete vector, pMCS.

The genes used as recombination sequences for the vectors were amplified from genomic DNA extracted from *X. dendrorhous*. The *crtE* gene, flanked by the sequence recognized by *EcoRI*, was amplified and ligated into pGEMT (pCrtE). Part of the *crtYB* gene was amplified and a *BamHI* site was inserted in the middle of the fragment by overlapping PCR; the gene was, then ligated into pGEMT (pCrtYB).

The *PSS* gene (GenBank ID: U05213.1) was a kind gift of Prof. Claudia Schmidt-Dannert from University of Minnesota. After amplification and ligation in pGEMT, the gene was excised with *NheI* and *XhoI* and ligated into the multiple cloning site of the pMCS, which had been previously digested with the same enzymes, resulting in the new construct pPSS. The plasmid was digested with *BamHI* and ligated in pCrtE and pCrtYB, yielding the two final constructs pCrtE-PSS and pCrtYB-PSS, respectively (**Fig. 2 A, B**).

The pPRcDNA1crtE plasmid [153] was a kind gift of Prof. Sandmann, Goethe Universität, Frankfurt. The *crtE* gene was excised from the plasmid using *EcoRI* and *SacI* and replaced with the restriction sites for *NheI* and *XhoI* that were then used to clone the *PSS* gene giving the new vector pPR-PSS. pPR-PSS was linearized with *SwaI* (**Fig. 2 C**), purified and concentrated.

Transformation of *X. dendrorhous* was performed according to the protocol of Wery and colleagues [128]. After the transformations with pCrtE-PSS and pCrtYB-PSS, only white colonies were chosen and grown again on selective medium with geneticin.

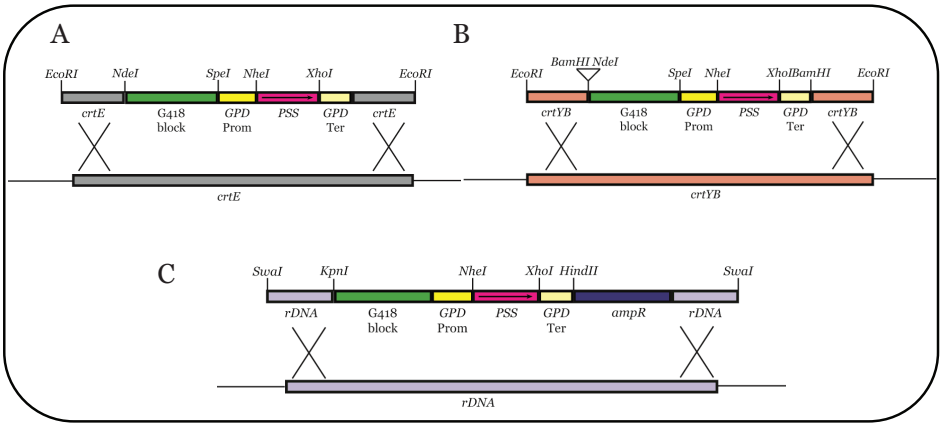

Figure 2. Scheme of the *EcoRI* fragment of pCrtE-PSS (**A**) and pCrtYB-PSS (**B**) vectors and of the linearized pPR-PSS vector (**C**) used for the transformation of *X. dendrorhous*. The crosses indicate the double crossing over events with the genomic DNA that will have to occur in order to obtain the stable mutants.

GC-MS analysis of organic layer

Dodecane was added to 10 ml of cultures in the concentration of 5% and hexadecane had a final concentration of 37.7 μg/ml of medium. Cultures taken at different time points were pelleted for 10 minutes at 3000 xg; the upper dodecane layer was isolated and diluted 1:10 in ethyl acetate. The diluted dodecane extract was analyzed in total ion scan using a ZB-1ms dimethylpolysiloxane column (Phenomenex 0.25 mm inner diameter, 0.25 μm thickness, 15 m length) on a Shimadzu GCMS-QP5000. Two microliters of solution were injected splitless in the GC using helium as carrier gas. The injector temperature was 250°C; the oven initial temperature was 50°C with an increment of 5°C/min up to 105°C and then up to 200°C with an increase of 30°C/ min. The solvent cut-off was of 10 minutes and 30 seconds due to high dodecane content.

Results

Mutants in the carotenoid pathway

The transformations of *Xanthophyllomyces dendrorhous* resulted in two different mutants in the terpenoid pathway. The geranyl-geranyl diphosphate synthase (GGPPS) gene *crtE* was the target for the creation of the first mutant, Δ*crtE*, while the second mutated strain (Δ*crtYB*) was characterized by the insertion of the resistance cassette in the gene *crtYB*, coding for the protein acting both as phytoene synthase and as lycopene cyclase. Both Δ*crtE* and Δ*crtYB* showed a white colony phenotype in contrast to the pink color of the wild type, as can be seen in **Figure 3**. This clear lack of carotenoids, in addition to the ability to grow on selective medium, was a strong proof that the integration of foreign DNA had occurred. The presence of the resistance cassette in the genomic DNA was confirmed by PCR (data not shown).

In order to establish whether the lack of carotenoids in the mutants was affecting the growth of the yeast, a time course experiment was performed (**Fig. 4**). No significant changes in the growth rate could be detected.

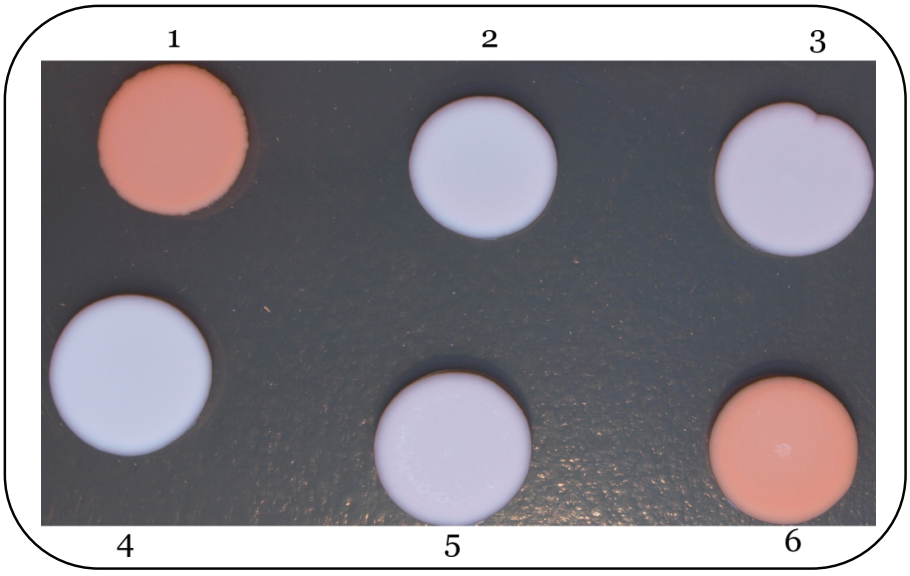

Figure 3. Phenotype of wild type and mutant colonies. Strains: ***1***, WT; ***2***, *ΔcrtYB*; ***3***, *ΔcrtE*; ***4***, *ΔcrtYB-PSS*; ***5***, *ΔcrtE-PSS*; ***6***, *pPR-PSS*. YPD medium was used to resuspend colonies grown on plates; approximately 10 µl of the solutions were spotted on a new YPD plate and let grow at 21°C for 3 days

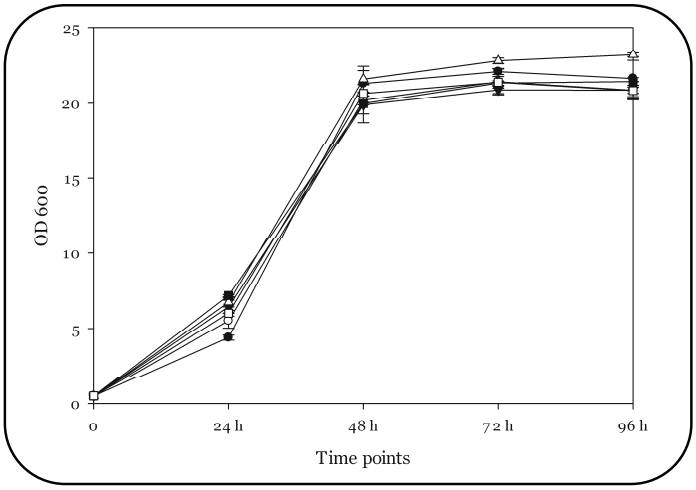

Figure 4. Growth curves of wild type (*closed circles*), *ΔcrtYB* (*open circles*), *ΔcrtE* (*closed triangles*) *ΔcrtYB-PSS* (*open triangles*), *ΔcrtE-PSS* (*closed squares*) and *pPR-PSS* (*open squares*) strains grown in YPD medium, with geneticin where necessary. Overnight cultures were diluted in 10ml of medium to an OD of 0.05 for each culture and each time point. Experiments were performed in duplo.

X. dendrorhous *strains transformed with* PSS *gene*

The vectors for the expression of the pentalenene synthase (PSS) gene in *X. dendrorhous* were engineered starting from the constructs used to create the mutants *ΔcrtE* and *ΔcrtYB*. The new expression cassette was introduced in the vector, flanking the antibiotic resistance region (**Fig. 2 A, B**). To be able to transform *X. dendrorhous*, both pCrtE-PSS and pCrtYB-PSS were digested with *EcoRI* and the fragment was purified and concentrated. Two mutants lacking carotenoids (white) but containing the *PSS* gene were isolated after transformation of the wild-type strain and were named *ΔcrtE-PSS* and *ΔcrtYB-PSS*. The new strains showed the same color phenotype (**Fig. 3**) and the same growth curves (**Fig. 4**) as the respective mutants without the *PSS* gene.

In order to evaluate the effect of the production of carotenoids on the pentalenene concentration, we constructed the vector pPR-PSS (**Fig. 2 C**) and used it for the transformation of the wild type *X. dendrorhous* resulting in *PSS* expression without interference with the carotenoid pathway. As shown in **Figure 3**, this mutant (*pPR-PSS*) exhibits similar pigmentation as the wild type strain, and the growth curve is comparable to the one obtained with the non-transformed yeast (**Fig. 4**).

Production of pentalenene in ΔcrtE-PSS, ΔcrtYB-PSS *and* pPR-PSS

The dodecane layer, expected to harbor the pentalenene, was isolated from the cultures after 24, 48, 72 and 96 hours cultivation. The chromatograms of the diluted dodecane from *ΔcrtE-PSS*, *ΔcrtYB-PSS* and *pPR-PSS* showed a peak at about 11.5 minutes that was absent in the wild type strain, *ΔcrtE* and *ΔcrtYB* (**Fig. 5**). The fragmentation pattern of the peak (**Fig. 6**) matched the pentalenene pattern as reported in the literature (**Fig. 7**, adapted from [162]) and presented two characteristic peaks for sesquiterpenes (m/z 204 and m/z 189) in the percentage of 68% and 47.6%, respectively, when considering the base peak (m/z 105.1) as 100%. Other major peaks could be found at m/z 175.1 (13.1%), 161.2 (41.1%), 147.2 (73.7%), 119.2 (57.2%) and 91.1 (91.3%). In order to be able to compare the production among the different strains, hexadecane was added at known concentration to the cultures as internal standard together with the dodecane. The concentration of pentalenene in the cultures was increasing with the optical density of the cultures as shown in **Figure 8**, which is in concert with the constitutive regulation reported for the *GPD* promoter we have here used.

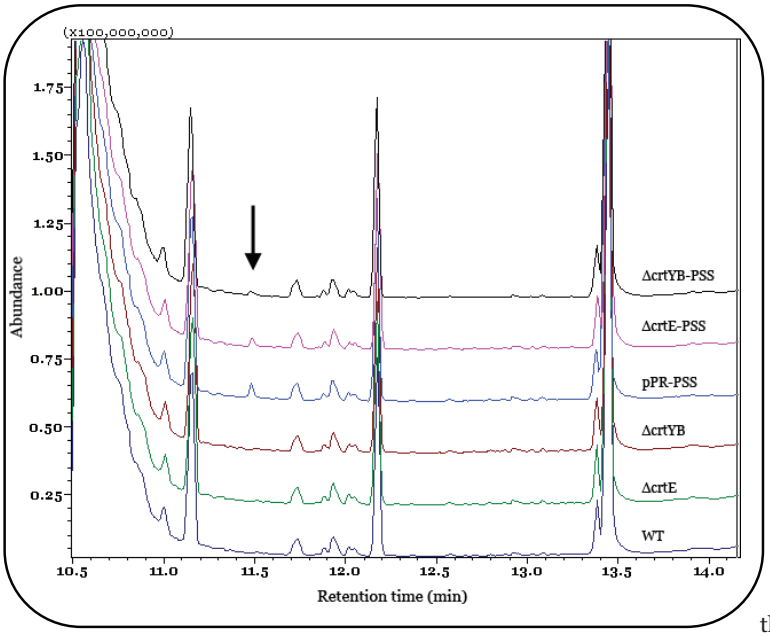

Figure 5. Production of pentalenene in *ΔcrtE-PSS*, *ΔcrtYB-PSS* and *pPR-PSS*. The pentalenene peak (indicated by the arrow) is found at 11.5 minutes from injection time. The peak at about 13.5 minutes is the hexadecane used for standardization of the analysis. The other peaks are impurities in the dodecane.

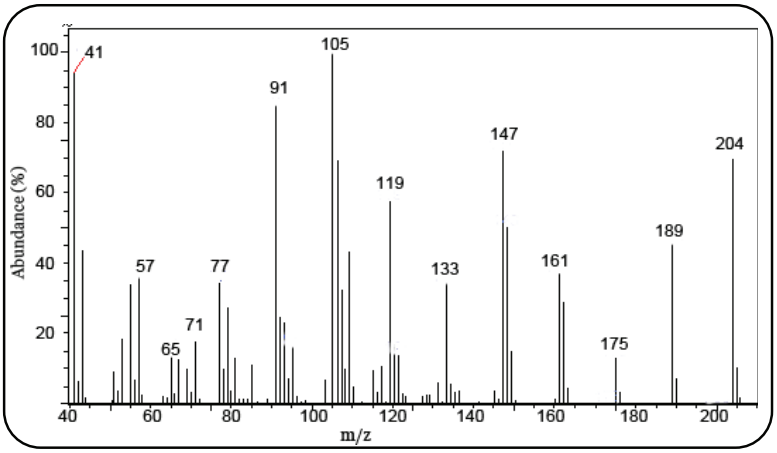

Figure 6. Fragmentation pattern obtained for the peak corresponding to pentalenene at 11.5 minutes.

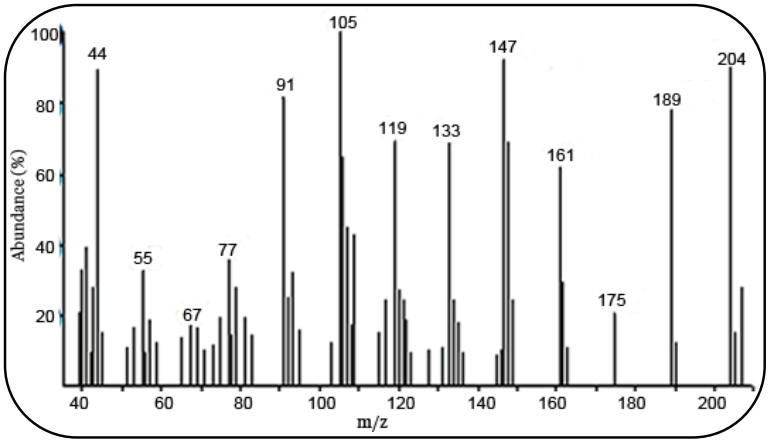

Figure 7. Published pentalenene fragmentation pattern (modified from [162]).

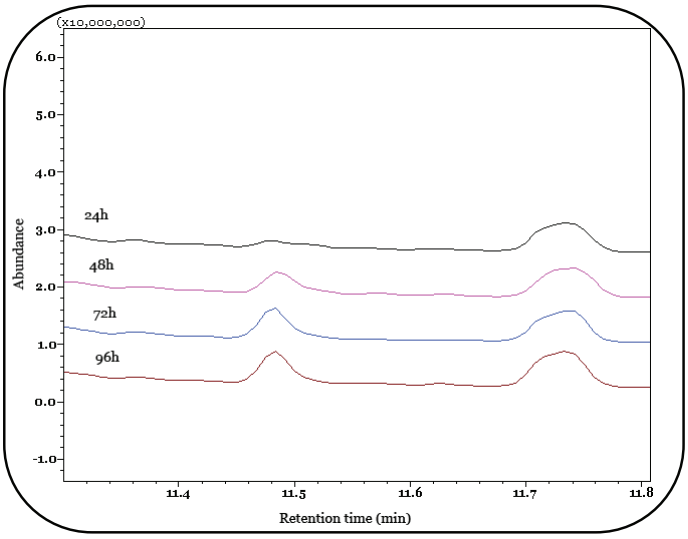

Figure 8. Pentalenene production by *pPR-PSS* after 24, 48, 72 and 96 hours from inoculation point. The retention time of the pentalenene is 11.48 minutes.

Discussion

X. dendrorhous has been the target of studies focusing on the biosynthesis and production optimization of the dye astaxanthin. Experiments involving genetic engineering of this yeast have converged towards the identification of the genes involved in the carotenoid pathway and the overexpression or deletion of those genes to analyze the differences in carotenoids production. In spite of the presence of tools for the transformation of the yeast, no effort has been put into expressing heterologous genes in *X. dendrorhous*, except for the sequences conferring resistance against geneticin and hygromycin [128, 139].

Here we report for the first time the production of a heterologous terpene cyclase in *X. dendrorhous*. We designed and engineered two new vectors for the functional expression of the heterologous *PSS* and the concurrent knocking out of one of the host's genes. The mutants obtained after selection on antibiotic showed a clear change in phenotype, which is due to the insertional inactivation of *crtE* or *crtYB*. The strains switched from the wild-type orange pigmentation to a completely white color, indicating a complete lack of astaxanthin and of any other coloring carotenoid. Carotenoids, like astaxanthin, are known to be produced by the yeast in response to oxygen reactive species produced under high oxygen conditions or during cell aging [4]; we demonstrated that the absence of carotenoids did not seem to affect the growth of the mutants since they followed the same growth curve and reached the same final OD as the wild type strain (**Fig. 4**).

The production of pentalenene could easily be revealed by culturing the yeast in presence of dodecane, which trapped the volatile compounds produced by the yeast. Detection by GC-MS did not show other terpenoids as by products, therefore we assume that pentalenene was the only biosynthesized terpenoid produced by the yeast that could be captured in the dodecane layer or at least detected.

The yield of pentalenene in the mutant strains ranged from 0.25 to 0.68 mg/L when compared with hexadecane as internal standard. The *pPR-PSS* strain showed a higher production compared to the other two mutant strains. The reason for this difference in production may be the integration of multiple copies of the gene in the genomic rDNA sequences. To support this theory, preliminary results in our lab show that low production of pentalenene in integration strains is probably caused by a low gene expression. When *pPR-PSS* mutants were selected on higher

concentrations of geneticin only colonies with a higher copy number of the genes could grow, and a screening of those colonies showed that the production of the sesquiterpene could reach 4.8 mg/L, which corresponded to 0.37 milligrams of pentalenene per gram of dried yeast.

The expression of a terpene cyclase in *X. dendrorhous* opens a new path toward the utilization of this yeast as a potential platform organism for the production of terpene based drugs. The possibility to knock out the carotenoid pathway and, thus, to channel the upstream precursors towards the production of new metabolites provides an additional advantage over the organisms that have been exploited until now for the terpene production. Breitenbach and colleagues have calculated that the wild type strain of *X. dendrorhous* grown in a poor medium can produce an average of 694 micrograms of total carotenoids per gram of yeast dry weight [153]. Furthermore, concentrations of astaxanthin of over 6.6 milligrams per gram of dry yeast weight could be obtained growing *X. dendrorhous* with different media, light and oxygen conditions [110].

These results confirm that the production of foreign terpenes in *X. dendrorhous* is not limited by the concentration of precursors, but rather by the gene expression driven by a weak regulating sequence. With a stronger promoter and with a codon optimized gene, the pentalenene production could reach even higher yields.

The maximum concentration of pentalenene we obtained in *X. dendrorhous* is still 20 times less than the maximum concentration of astaxanthin isolated from the wild-type strain, showing that the potential of this yeast is still far from being completely exploited.

Acknowledgements

We thank Prof. Claudia Schmidt-Dannert and Grayson Wawrzyn from the University of Minnesota for providing us with the *PSS* gene and for the expertise on terpene cyclases. We also thank Prof. Sandmann from the Goethe Universität in Frankfurt for sending us the pPRcDNA1crtE plasmid. E.M. was supported by an Ubbo Emmius fellowship.

CHAPTER 4

PRODUCTION OF α-CUPRENENE IN *XANTHOPHYLLOMYCES DENDRORHOUS*: A STEP CLOSER TO A POTENT TERPENE BIOFACTORY

Elena Melillo, Rita Setroikromo, Wim J. Quax, Oliver Kayser

Submitted

Background: The red yeast *Xanthophyllomyces dendrorhous* is a natural producer of the carotenoid astaxanthin. Because of its high flux, the native terpene pathway leading to the production of the tetraterpene is of particular interest as it can be redirected toward the production of other terpene compounds. The genetic tools for the transformation of the yeast with the concurrent knock-out of genes involved in the astaxanthin biosynthesis are made available and here we show that the production of the sesquiterpene α-cuprenene is possible in mutant strains of *X. dendrorhous* transformed with the *Cop6* gene originating from the fungus *Coprinus cinereus*. For the evaluation of the production levels, we chose to express the same gene and analyze the accumulation of α-cuprenene in *Escherichia coli* and *Saccharomyces cerevisiae*, as well. Here we propose that *X. dendrorhous* is a candidate in the search for the potential platform organism for the production of terpenes.

Results: All three *X. dendrorhous* mutants functionally express the *Cop6* gene and accumulate α-cuprenene. The production of α-cuprenene in the red yeast reached 80 mg/L, which represents a far higher concentration compared to the levels obtained in the *E. coli* and *S. cerevisiae* mutants. At this expression levels the pool of terpene precursors has not become a limiting factor in the *X. dendrorhous* mutants since the expression of the *Cop6* gene in the genomic rDNA of the yeast allows production of both α-cuprenene and astaxanthin without affecting the growth or the accumulation levels of both compounds.

Conclusions: We have shown that *X. dendrorhous* can produce α-cuprenene, and the results here presented, next to the capability of accumulating at least two more non-native sesquiterpenes, demonstrates the high potential of this yeast to become an interesting terpene-based drugs producer.

Background

Since ancient times, microorganisms have been used to produce bread, wine and dairy products in order to improve the quality of food and its nutrients. Today, microbes are utilized for the manufacture of a wide variety of fine or bulk chemicals including antibiotics [163, 164], vitamins [165], biofuels [166, 167], biodegradable and biocompatible plastics from waste [168] and terpene-based drugs [43, 89]. Originally, the choice for a production host was dictated by the ability of the organism to produce the desired compounds but in most cases the concentration of the chemicals of interest was not sufficient to cover the market demand.

With the development of genetic engineering, new tools became available to overcome these obstacles. The possibility of transferring single genes or even complete pathways to other microorganisms led to improved yields or easier bioprocessing conditions. Furthermore, metabolic engineering allowed the enhancement of the yields in the native hosts by finely tuning the metabolic networks of the cells towards the optimized production of the specific compound. More recently, the combination of synthetic biology and metabolic engineering has resulted in the creation of hosts capable of producing a non-native compound with high efficiency obtainable only by optimizing the native pathways of the cells [169].

The majority of the yeast or bacterial strains used by industry have been selected for the several advantages they deliver: they are easy to cultivate, they can grow on cheap media, they are generally regarded as safe (GRAS status) and their metabolic pathways are easy to modify via genetic engineering.

Xanthophyllomyces dendrorhous, a red basidiomycetous yeast, represents one of the microbial strains already used in industry and shares all the aforementioned advantages [150]. Today, *X. dendrorhous* is grown at industrial scale for its native capability to produce the valuable carotenoid astaxanthin.

Carotenoids, together with several other pharmaceutically important compounds, like artemisinin and paclitaxel, belong to the natural compounds class of the terpenes [155]. Several efforts have been put in the engineering of a platform organism for the production of industrially important terpenes [43, 89].

We hypothesize that, since *X. dendrorhous* can produce high levels of astaxanthin, which shares the same precursors with all other terpenes, it can also utilize those same precursors for the production of any other terpenoid compound.

The red yeast was already shown to be able to functionally express the pentalenene synthase from a *Streptomyces* strain involved in the biosynthesis of the antibiotic pentalenolactone [170]. In order to further evaluate the potential of the red yeast as a platform organism for terpenes, we expressed the sesquiterpene cyclase *Cop6* in *X. dendrorhous* mutant strains. The protein Cop6, originating from the fungus *Coprinus cinereus*, produces the cyclized sesquiterpene α-cuprenene, which is the basic structure for the formation of lagopodin A, an antimicrobial sesquiterpene quinone [162]. We also compared the accumulation levels of α-cuprenene with two of the most industrially utilized microbial strains, *E. coli* and *S. cerevisiae*.

Results

Production of cuprenene in E. coli

Isolation of volatile terpenoids in *E. coli* via addition of a dodecane organic phase to the liquid cultures has been shown to be extremely efficient [42]. We have decided to adopt the same strategy to capture the α-cuprenene produced by the *E. coli* strains transformed with the *Cop6* gene. In order to be able to compare the levels of α-cuprenene at the different time points and from different organisms we added hexadecane in known concentrations, as an internal standard, to the dodecane.

Twenty hours after induction of the expression, cells reached the highest density and they started dying after 30 hours. After 48 hours the concentration of cuprenene, based on the internal standard, reached approximately 0.25 mg/L of culture, a slight increase compared to the concentration at 20 hours (**Fig. 1**).

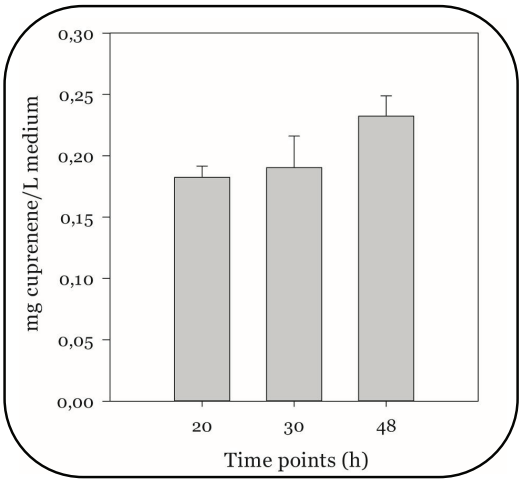

Figure 1. Cuprenene production during time course with *E. coli* pHis8Cop6

Growth curves and α-cuprenene production from S. cerevisiae and X. dendrorhous in rich medium

After the separate transformations of *X. dendrorhous* wild-type strain with the vectors pCrtE-Cop6, pCrtYB-Cop6 and pPR-Cop6, one colony from each transformation plate was chosen to be grown and analyzed. As expected, since the astaxanthin pathway was disrupted (**Fig. 2**), on the plates used to select *ΔE-Cop6* and *ΔYB-Cop6*, the mutant colonies presented a white phenotype. In contrast, *XdCop6* colonies, transformed with pPR-Cop6, in which the carotenoid pathway was not modified (**Fig. 2**), shared an orange pigmentation with the wild type strain.

The *S. cerevisiae* mutant, *ScCop6*, was isolated after transformation of the wild type strain of *S. cerevisiae* with the plasmid p426GPD-Cop6, which allows constitutive expression of the *Cop6* gene.

A time course analysis was performed on the *ScCop6* and on the three *X. dendrorhous* mutant strains, *XdCop6*, *ΔE-Cop6* and *ΔYB-Cop6*. In order to obtain a high biomass, all the strains were grown in the rich YPD medium. The time course consisted of four sampling times after the inoculation in fresh medium; we chose 24h, 48h, 72h and 96h to be able to observe all the steps of the growth curve. **Figure 3** compares the growth curves obtained with the time course analysis.

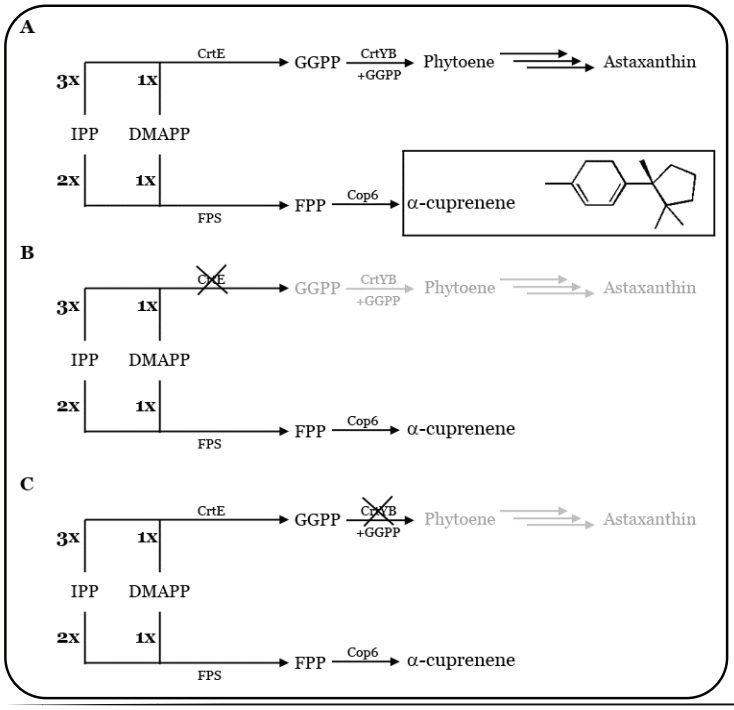

Figure 2. Schematic representation of *X. dendrorhous* mutant strains**.** (**A**) In the mutant *XdCop6*, the native astaxanthin pathway has not been modified but the gene *Cop6* has been integrated in the rDNA of the yeast allowing the mutant to produce both astaxanthin and α-cuprenene. (**B**) In the strain *ΔE-Cop6* the *Cop6* gene has been inserted in the *crtE* gene causing the disruption of the carotenoid production at the

GGPP synthesis level. (**C**) When *Cop6* is inserted in the *crtYB* gene, the *ΔYB-Cop6* strain is created. While there is still expression of the GGPPS, phytoene cannot be produced anymore, blocking the production of astaxanthin one step downstream of the *ΔE-Cop6*.

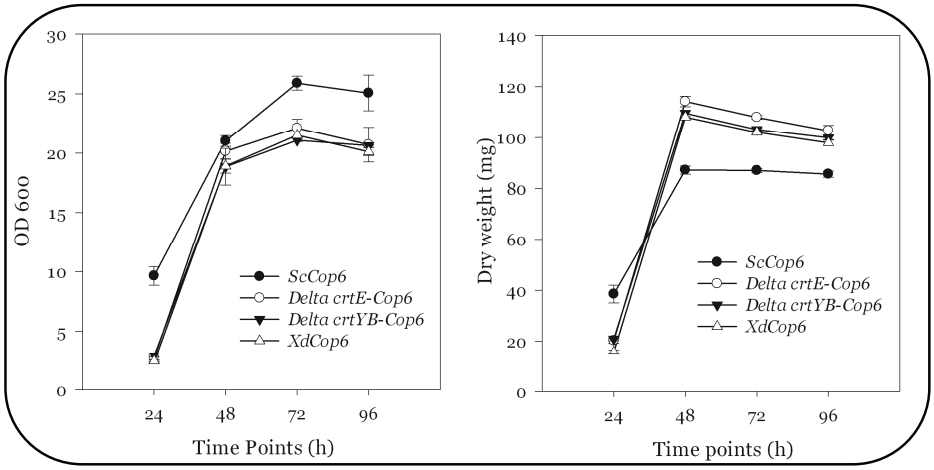

Figure 3. OD_{600} and cell dry weight of *ScCop6*, *ΔE-Cop6*, *ΔYB-Cop6* and *XdCop6* in 10 ml of YPD medium.

The three *X. dendrorhous* strains exhibit similar curves with respect to the OD_{600} and to the cell dry weight. In contrast, the *S. cerevisiae* mutant reaches a higher optical density but accumulates a lower biomass compared to the three *X. dendrorhous* mutants.

The diluted dodecane solutions from the transformed *S. cerevisiae* and from the wild type and mutant strains of *X. dendrorhous* were analyzed by GCMS, and a single peak appeared in the chromatograms from the mutants at 12.8 minutes (**Fig 4**). The fragmentation pattern of the peak was compared to the pattern corresponding to α-cuprenene produced in *S. cerevisiae* (**Fig. 5**). The mass and the relative ratio of the fragment peaks matched between the two patterns, allowing us to confirm that the only sesquiterpene produced by Cop6 in *X. dendrorhous* is α-cuprenene.

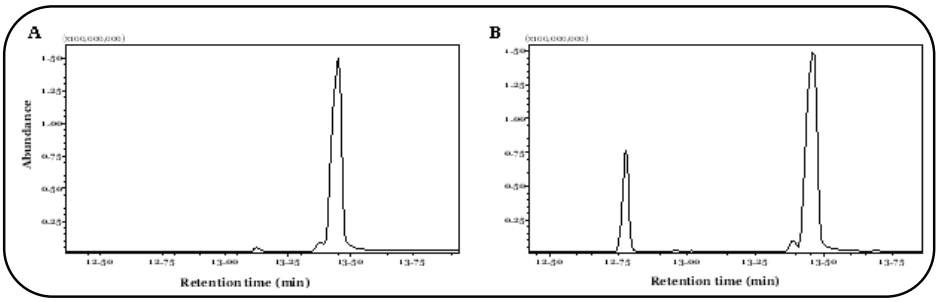

Figure 4. Chromatograms of the diluted dodecane from (**A**) *X. dendrorhous* wild type strain and (**B**) *ΔE-Cop6*. The peak at 13.5 minutes is the hexadecane used as internal standard. The α-cuprenene has a retention time of approximately 12.8 minutes.

The time-course production of α-cuprenene in the four strains is represented in **Figure 6**. The level of the sesquiterpene in *S. cerevisiae* sharply increased after 24 hours to reach a maximum of 6.6 mg/ L on the second day of culturing and then appeared to decrease during the following two days.

The α-cuprenene production in the three *X. dendrorhous* strains showed ten times higher levels with a major increase between 24 and 48 hours, as well. In contrast to *S. cerevisiae*, *XdCop6*, *ΔE-Cop6* and *ΔYB-Cop6* constantly produced α-cuprenene during the complete time course. The accumulation of the sesquiterpene appeared to be consistent in all the three *X. dendrorhous* strains and was directly proportional to the cell mass, in particular in the first three days after inoculation. The highest yield was obtained after 96 hours with the *XdCop6* strain and corresponded to almost 80 mg/L of culturing medium. *ΔE-Cop6* could produce up to 70 mg of α-cuprenene per liter of medium and *ΔYB-Cop6* 74 mg/L, both after growing for 96 hours.

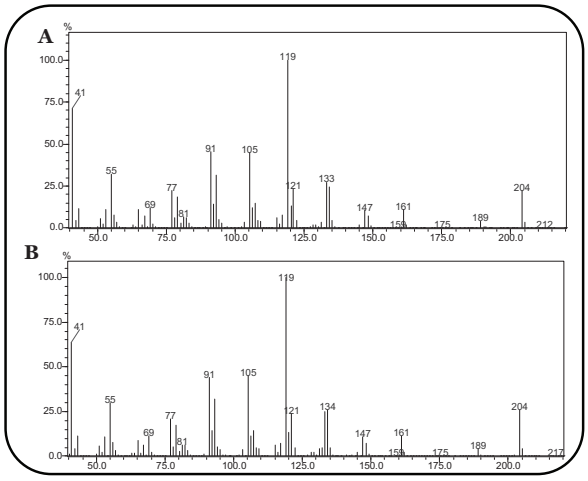

Figure 5. Fragmentation patterns. (**A**) α-cuprenene from *ScCop6*; (**B**) peak at 12.8 minutes from *XdCop6*.

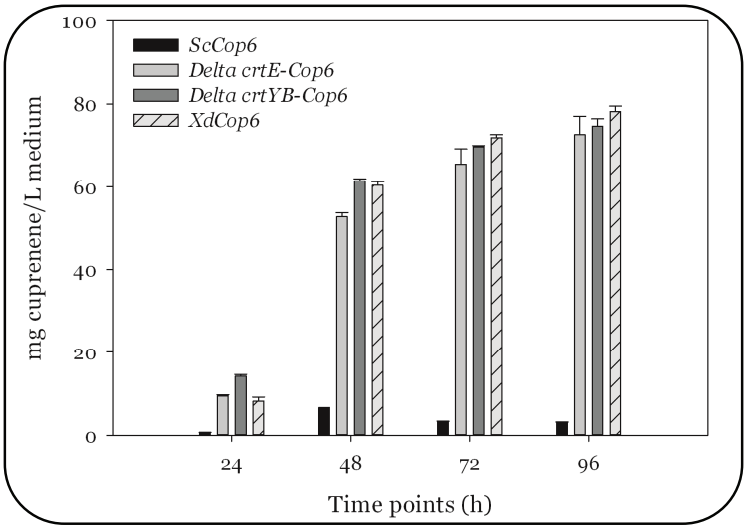

Figure 6. Production of α-cuprenene in rich YPD medium

Growth curves and α-cuprenene production from S. cerevisiae *and* X. dendrorhous *in minimal medium*

The *ScCop6* mutant was selected on medium lacking uracil in order to allow only the colonies containing the plasmid to grow. The rich YPD medium contains all the necessary nucleotides, thus the selective pressure on the *S. cerevisiae* mutant strain grown in this medium was inexistent. For this reason, we decided to grow all the strains in a minimal medium lacking uracil which would allow a more accurate comparison between the *ScCop6* and the three *X. dendrorhous* mutants. The same settings chosen for the time course in rich medium were applied for the growth and α-cuprenene production analysis in minimal medium.

An overall lower cell mass and OD_{600} compared to rich medium was observed for all the strains. *S. cerevisiae* started growing already after 24 hours while *XdCop6*, *ΔE-Cop6* and *ΔYB-Cop6* showed a lag phase between 0 and 24 hours and a log growth between 24 and 48 hours (**Fig. 7**). In minimal medium *ΔE-Cop6* exhibited a slightly reduced cell growth compared to *XdCop6* and *ΔYB-Cop6*

The accumulation of α-cuprenene in the three *X. dendrorhous* strains was significantly lower than the concentration obtained in YPD reaching a maximum of 20 mg/l (**Fig. 8**) for *ΔYB-Cop6*. When *ScCop6* was grown in minimal selective medium, the sesquiterpene production increased nearly to 12 mg of α-cuprenene per liter of medium.

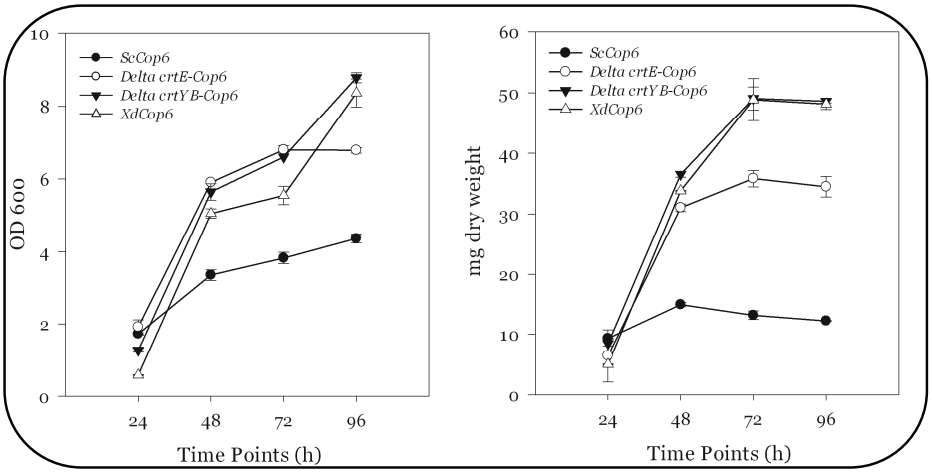

Figure 7. OD_{600} and cell dry weight of *ScCop6*, *ΔE-Cop6*, *ΔYB-Cop6* and *XdCop6* in 10 ml of minimal medium

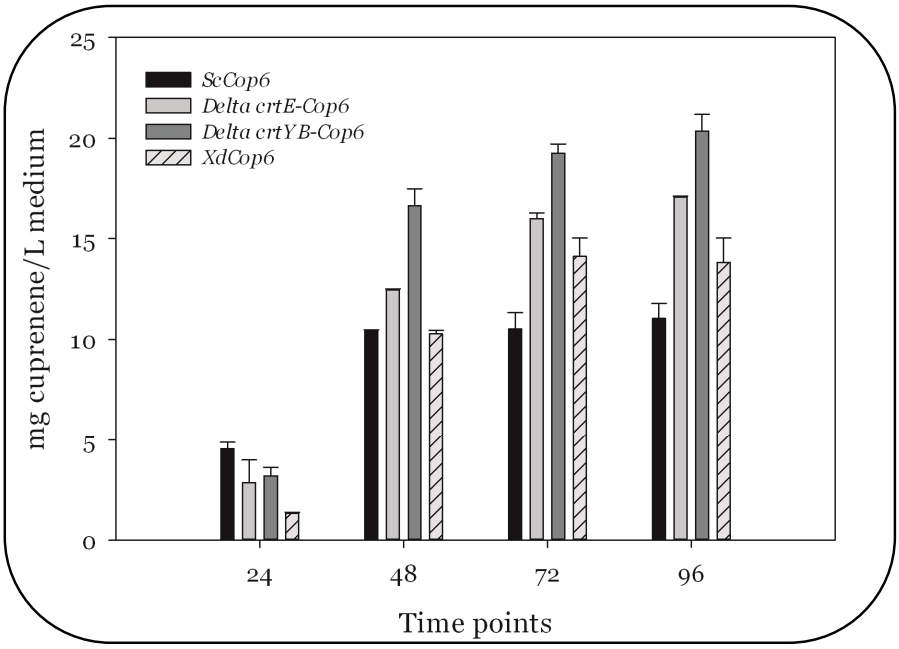

Figure 8. Production of α-cuprenene in minimal medium

Microscopy analysis of ScCop6 *and* XdCop6

Figures 3 and **7** indicate that *S. cerevisiae* and *X. dendrorhous* are characterized by a different growth rate and optical density. For a better understanding of the differences between the two yeasts, we performed a microscopy analysis on the strains *ScCop6* and *XdCop6*.

A Burke chamber was used to count the yeast cells. In spite of the macroscopic red pigmentation of *XdCop6*, the cells appeared white under the microscope white light. After counting samples in duplo from *ScCop6* and *XdCop6* and calculating the cell numbers, we observed that one OD_{600} unit in *S. cerevisiae* corresponded to 3 x 10^7 cells per milliliter, consistent with the data available in literature [171]. In contrast, the number of *X. dendrorhous* cells counted in one milliliter of culture with the same optical density was 4.8 x 10^6.

To further ascertain the morphological differences between *ScCop6* and *XdCop6*, we compared the average cells sizes. **Figure 9** compares the pictures of the two strains after four days of growth; the same magnification was used to visualize the cells. Cells from *XdCop6* are round shaped and have a granular appearance, while *ScCop6* cells, although showing a similar round morphology, show a more homogeneous cytosol. On average, the size of *X. dendrorhous* cells was 10 μm in diameter; *S. cerevisiae* cells were smaller with a size ranging between 7 and 8 μm. Not only *ScCop6* cells were on average smaller than *X. dendrorhous*, but they also never had a diameter bigger than 10 μm, in contrast with the red yeast cells.

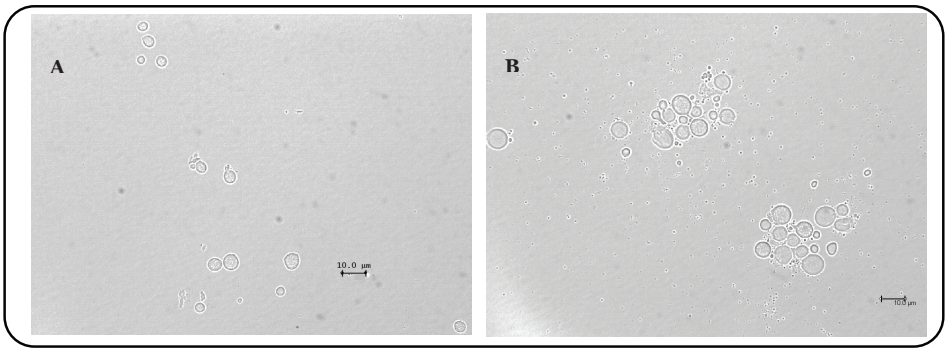

Figure 9. Microscopy photographs. (**A**) *ScCop6* and (**B**) *XdCop6*.

Discussion

E. coli and *S. cerevisiae* are two model organisms that also serve as industrial cell factories for the production of a wide variety of compounds ranging from pharmaceutically active substances to food ingredients and biofuels.

In spite of the fact that *X. dendrorhous* has not been studied as extensively as *E. coli* or *S. cerevisiae*, it shows great potential to become a platform organism for terpene production [170]. In order to assess the value of the red yeast as a cell factory, we expressed the *Cop6* gene in the three *X. dendrorhous* mutants and compared the production of α-cuprenene with *E. coli* and *S. cerevisiae* strains expressing the same gene.

The cDNA from the *Cop6* gene was expressed by all *X. dendrorhous* mutants, whereas the genomic version of the gene, when transferred to the red yeast, did not result in α-cuprenene accumulation (data not shown) indicating that *X. dendrorhous* cannot correctly splice the gene from *C. cinereus*.

When grown in rich medium, the *X. dendrorhous* mutant strains showed very similar growth rates: they reached OD_{600} values of 20 and produced a maximum of 12 grams of cell dry weight per liter of culture. Similarly, when looking at the α-cuprenene production, the three strains *XdCop6*, *ΔE-Cop6* and *ΔYB-Cop6* did not show big differences among each other, with levels of the sesquiterpene ranging from 70 to 80 mg of compound per liter of medium.

Comparing figures 3 and 7, it is clear that, differently from the experiments in the rich medium, the three *X. dendrorhous* strains shared an altered growth behavior in the minimal medium. While *XdCop6* and *ΔYB-Cop6* reached a maximum cell dry mass of nearly 5 g/L, *ΔE-Cop6* could not produce more than 3.5 g of dry cells per liter of medium. Concomitantly, the concentration of α-cuprenene in the dodecane was reduced to values ranging from 15 to 21 mg/L of culture. The decrease in sesquiterpene accumulation can partly be explained by the reduced cell mass and partly by the lower concentration of nutrients in the minimal medium which would induce the cells to minimize the energy consumption by shutting down unnecessary pathways.

When comparing cell mass accumulation and α-cuprenene production in all *X. dendrorhous*, *E. coli* and *S. cerevisiae* strains, the prokaryote showed the lowest values. The low biomass in the bacterium is most likely to be ascribed to a lack of

glucose in its growth medium, while the limited sesquiterpene production is due to the lower terpene flux in *E. coli* compared to the two eukaryotes.

The differences in growth curves and dry weight between the *X. dendrorhous* and the *S. cerevisiae* strains seem to have morphological reasons. *X. dendrorhous* cells are on average bigger than *S. cerevisiae* ones [172] and at the same optical density *S. cerevisiae* cell counts are almost 10 times higher than in *X. dendrorhous* cultures, meaning that the same OD_{600} value corresponds to more *S. cerevisiae* cells than it does for *X. dendrorhous*. Since, at the beginning of the time course experiments, the initial OD_{600} for all the strains was set at 0.05, the number of cells initially transferred to the fresh medium was higher in *ScCop6* than in all the *X. dendrorhous* mutants. This would explain the delay in growth we observed for *XdCop6*, *ΔE-Cop6* and *ΔYB-Cop6* compared to *ScCop6*. Additionally, the higher cell mass accumulation observed in the *X. dendrorhous* strains compared to *S. cerevisiae* may be due to the red yeast's bigger sized cells rather than to a higher number of cells.

The highest α-cuprenene production levels were obtained with the *X. dendrorhous* strains both in the rich and in the minimal medium experiments. Remarkably, in the YPD medium the gap in sesquiterpene accumulation between the red yeast and the *S. cerevisiae* strain was far more pronounced. We assume that, since the complete medium does not allow selective pressure on *ScCop6*, which was isolated by its ability to grow on minimal medium lacking uracil, the strain might have undergone a reduction in plasmid copy number.

While *ScCop6* mutants contain an average of 20 copies of *Cop6*, the *X. dendrorhous* white mutants possess just one copy of the gene since the recombination of the constructs can occur only once in the single *crtE* or *crtYB* genes. In order to obtain a mutant with a higher number of integrations of the gene in the genomic rDNA, we transformed the *X. dendrorhous* wild type strain with a higher concentration of the DNA fragment from the pPR-Cop6 vector and selected the transformants on YPD medium containing a concentration of geneticin 5 times higher than normal, hoping for gene amplification. Unfortunately, no colony grew after this transformation and we could not evaluate the effect of more gene copies on the α-cuprenene accumulation.

Nevertheless, we can safely assume that the concentration of the precursors is not a limiting factor in the sesquiterpene production in *X. dendrorhous*, since the

strain *XdCop6* can easily sustain the production of both α-cuprenene and astaxanthin, especially when grown in the YPD rich medium. The production of both terpene compounds in *XdCop6* confirms the hypothesis that a higher gene copy number would positively influence the α-cuprenene production in *X. dendrorhous*.

In conclusion, *X. dendrorhous* shows great promise since it has the GRAS status, it grows at room temperature in minimal media, and it has already been used by industry for the production of astaxanthin. We discovered that it can produce at least three non-native sesquiterpenes, pentalenene [170], α-cuprenene and cubebol (**S1-7**). Furthermore, *X. dendrorhous* is the best microorganism, among the ones we have analyzed, to be used for the production of α-cuprenene. A better understanding of the molecular biology of this yeast will prove useful for the identification of stronger promoters for a higher gene expression.

In light of the aforementioned advantages and of the provided results, *X. dendrorhous* is an interesting candidate for being used as a cell factory for the production of terpenes.

Methods

Strains and culture conditions

The *E. coli* strain DH5α was used for the cloning processes, while the strain BL21 (DE3) was transformed and cultured for the time course experiments. *S. cerevisiae* MRG 5 #502 (*MATa*, *ura*3-52, *leu*2-Δ1, *trp*1-Δ36, *his*3-Δ200, D*ade*2) was used for the transformation and time course analysis. *X. dendrorhous* wild type strain (CBS 6938) was used for the transformations and as negative control for all experiments. Both *E. coli* strains were grown in LB (10 g/L Trypton, 5 g/L Yeast Extract, 10 g/L NaCl) with 30 mg/ml kanamycin. The rich medium for *S. cerevisiae* and *X. dendrorhous* was YPD (10 g/L Yeast Extract, 20 g/L Peptone and 20 g/L Dextrose) with additional 40 mg/ml geneticin (G-418 Sulfate, Gibco) only for the selection and growth of the *X. dendrorhous* mutants. The minimal medium for *S. cerevisiae* consisted of 13.4 g/l Yeast Nitrogen Base without amino acids, 20 g/L Dextrose, 100 mg/L leucine, 40 mg/L histidine, 40 mg/L tryptophan and 40 mg/L of uracil. The same concentrations of Yeast Nitrogen Base and Dextrose were kept for

the minimal medium for *X. dendrorhous* and geneticin was added to the medium for the culturing of the mutants.

Construction of the E. coli *strain* EcCop6 *and time course analysis*

The plasmid pHis8Cop6 was a kind gift of Prof. Claudia Schmidt-Dannert from University of Minnesota and it contains the cDNA sequence of the *Cop6* gene from *Coprinus cinereus* under the control of the T7 promoter. *E. coli* BL21 (DE3) colonies containing pHis8Cop6 were selected on LB plates with kanamycin.

One transformed colony, *EcCop6*, was chosen for the time course analysis and a seed culture was started over night in LB plus kanamycin. The fresh cultures were inoculated and grown to OD_{600} 0.5 and then 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added for the induction of expression of the *Cop6* gene. The time course was performed in duplo and consisted of six 100-ml flasks with 10 ml of LB medium plus kanamycin and 500 µl of dodecane mixed with the internal standard, hexadecane (68 µg/ml of medium), grown in a shaking incubator at 250 rpm at 37°C for 48 hours. Two flasks were removed from the incubator at each sampling point (20, 30 and 48 hours after IPTG induction); the cultures were centrifuged at 4000 rpm for 10 minutes, the upper dodecane layer was isolated from the medium and used for the GCMS analysis. The cell pellet was washed once with water and freeze dried to determine the cell dry weight. The same procedure was applied to *S. cerevisiae* and *X. dendrorhous*, as well.

Construction of the S. cerevisiae *strain* ScCop6 *and time course analysis*

pHis8Cop6 was used as a template for the amplification of the gene to be cloned in the p426GPD episomal vector for the expression of *Cop6* in *S. cerevisiae*. The gene was amplified with primers flanked with the restriction sites for *EcoRI* and *SpeI* and was mutated in positions 497 and 558 to eliminate the native *EcoRI* sites. The fragment was then cloned in the previously digested vector and the complete construct was used to transform the *S. cerevisiae* wild type strain to obtain *ScCop6*.

Positive colonies were selected on plates containing minimal medium without uracil and one positive colony was isolated and used for the time courses.

Similarly to the time course performed for *EcCop6*, eight 100-ml flasks with 10 ml medium and 500 µl of dodecane and hexadecane solution were incubated at 30°C at 200 rpm. The four time points were chosen 24, 48, 72 and 96 hours from the inoculation in the fresh medium and the initial OD_{600} of the fresh cultures was 0.05 for all the experiments.

Isolation and characterization of X. dendrorhous *mutants*

The plasmids pCrtE-PSS, pCrtYB-PSS and pPR-PSS [170] were used as backbone for the creation of the new plasmids pCrtE-Cop6, pCrtYB-Cop6 and pPR-Cop6, respectively. *Cop6* was amplified from the mutated gene used for the expression in *S. cerevisiae* and was flanked by the restriction sites for *NheI* and *SalI*. The fragment was then cloned in the digested vectors and used for the transformation of *X. dendrorhous*. The positive colonies were isolated for the ability to grow on selective medium with geneticin. From the transformations with pCrtE-Cop6, pCrtYB-Cop6 and pPR-Cop6, the three new mutant strains *ΔE-Cop6*, *ΔYB-Cop6* and *XdCop6* were obtained, respectively (Fig. 2). The three strains were grown at 21°C at 200 rpm for the analysis of production of α-cuprenene in time following the same conditions used for the time course for *ScCop6*.

GC-MS analysis

The dodecane solutions isolated from the different cultures at different time points were diluted 1:10 in ethyl acetate and run on GCMS to reveal and quantify the α-cuprenene production. A Shimadzu GCMS-QP5000 provided with a ZB-1ms dimethylpolysiloxane column (Phenomenex 0.25 mm inner diameter, 0.25 µm thickness, 15 m length) was used for the analysis. Two microliters of diluted dodecane were injected splitless and analyzed in total ion scan using helium as carrier gas. The GCMS program consisted of an oven initial temperature of 50°C with an increment of 5°C/min up to 105°C and then up to 200°C with an increase of 30°C/min. The quantitation of the α-cuprenene was based on the hexadecane peak which had a known concentration.

Microscopy analysis and cell counting

Aliquots of *ScCop6* and *XdCop6* cultures were taken after 96 hours of growth, diluted to OD_{600} 0.15, approximately, and were then transferred to a Bürker counting chamber (Bright line, Labor Optik). The number of cells counted in a surface of 0.0025 mm^2 was multiplied by 10^4 and divided by the $OD6_{600}$ values of the cultures to obtain the number of cells per OD_{600} unit.

A Leica DM 6000B microscope provided with a 40x magnification objective and the LAS AF program was used for the visualization and to measure the size of the cells with the 10 µm bar provided by the LAS AF program.

Acknowledgements

The authors wish to thank Prof. Claudia Schmidt-Dannert and Grayson Wawrzyn from University of Minnesota for providing the genes from Coprinus cinereus and for the useful discussions.

Supplementary data

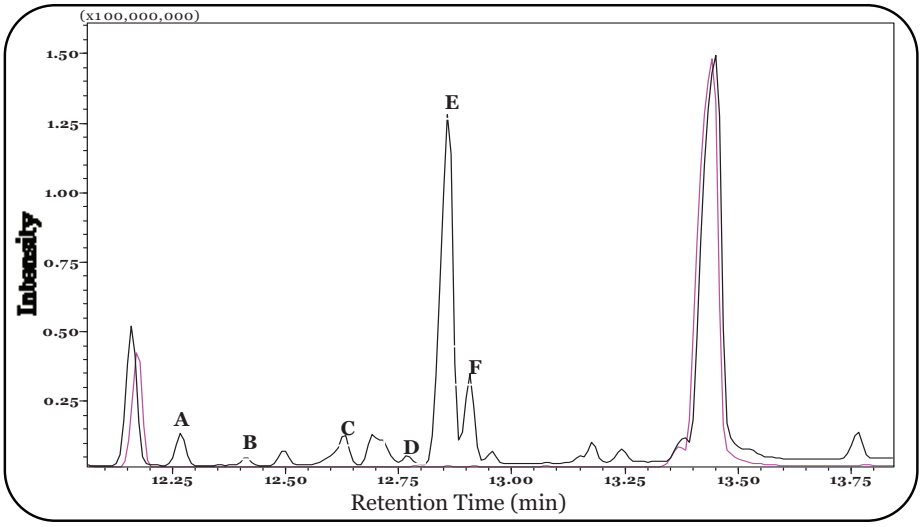

Figure S1. GCMS chromatograms obtained from *X. dendrorhous* wild type strain and mutant strain expressing *Cop4*. The pink line corresponds to WT, while the black line represents the *Cop4* mutant. The letters describe the peaks that differ between the two strains and that could be identified.

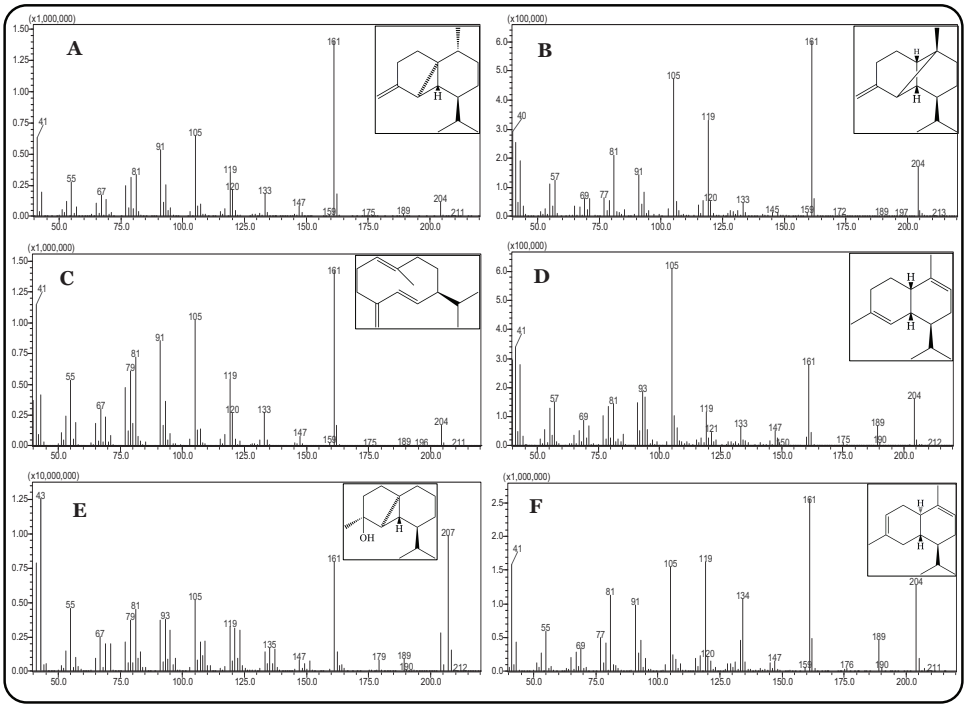

Figure 5. Fragmentation patterns and structures. (**A**) β-cubebene; (**B**) β-copaene; (**C**) germacrene D; (**D**) α-muurolene; (**E**) cubebol; (**F**) β cadinene.

CHAPTER 5

PRODUCTION OF LAGOPODIN INTERMEDIATES IN *XANTHOPHYLLOMYCES DENDRORHOUS*

Manuscript in preparation

Introduction

Xanthophyllomyces dendrorhous is a red yeast belonging to the Basidiomycetes phylum; its pigmentation is given by the naturally produced carotenoid astaxanthin [150]. The yeast has been studied mainly for its ability to produce this tetraterpene structure which has in 2007 reached a market size of $219 million and expected to reach $253 million in 2015. Transformation protocols have been optimized and vectors have been designed to allow the overexpression or knocking out of genes involved in the carotenoid or in alternative pathways [118, 121, 137, 142].

Combining and implementing the knowledge about the molecular biology with the genetic engineering tools available for the transformation of the yeast, we have recently demonstrated that *X. dendrorhous* can be used for the expression of heterologous terpene synthases and for the production of non-native sesquiterpenes [170] (**Chapter 4** of this thesis). The *X. dendrorhous* mutant strains have shown to be able to produce pentalenene, cubebol and α-cuprenene, although in different amounts.

Pentalenene is the first committed precursor in the biosynthesis of pentalenolactone; this pathway encompasses 19 steps catalyzed by as many proteins, whose genetic sequences from *Streptomyces avermitilis* have been identified and published [161, 173]. Unfortunately, the amount of pentalenene produced in the *X. dendrorhous* transformants is low, probably due to limited protein efficiency and a low gene copy number. On the other hand, the production of α-cuprenene in our yeast could reach 80 mg/L of medium in non-baffled shake flasks without optimizing the medium or the growth conditions. Furthermore, the steps dividing α-cuprenene from the final drug lagopodin are believed to be restricted to a few enzymatic steps catalyzed by the two cytochromes COX1 and COX2, identified in *Coprinus cinereus* [162]. Thus, since the yeast can accumulate a higher content of the direct precursor and the number of steps involved in the biosynthesis of the final compound is limited, we decided to express the gene coding for the α-cuprenene synthase (*Cop6*) together with the two cytochromes, *COX1* and *COX2*, in *X. dendrorhous*.

The number of genes we could express in the red yeast with the vector we designed and used in the past was limited to one plus the marker gene for the resistance against geneticin. In order to be able to clone more than one gene, a new vector was engineered and the genes expressed in different combinations.

In this study, we demonstrate that *X. dendrorhous* can efficiently express and regenerate the two cytochromes producing diverse intermediates from the lagopodin pathway.

Materials and methods

Strains and media

The bacterial strain *E. coli* XL1 blue was used to perform all the cloning steps; bacteria were grown at 37°C, 250 rpm in LB (10 g/L Trypton, 5 g/L Yeast Extract and 10 g/L NaCl). Ampicillin was used for the selection of the transformed colonies at a concentration of 100 mg/ml.

The wild type *X. dendrorhous* strain CBS 6938 was used for all transformations and as negative control. The yeast was grown at 21°C in a shaking incubator at 180 rpm; the medium chosen for all experiments was YPD (10 g/L Yeast Extract, 20 g/L Peptone and 20 g/L Glucose) with 40 mg/L geneticin (G-418 Sulfate, Gibco) for the selection and growth of the mutant strains.

Engineering of the new resistance and expression cassettes

The resistance cassette against geneticin was amplified from the previously engineered vector, pCrtE [170] with the primers F1 and R1, which allowed the insertion of the *ClaI* restriction site between the *BamHI* site and the beginning of the promoter. The fragment was digested with *BamHI* and ligated in the vector already containing the *crtE* sequence previously cleaved with the same restriction enzyme.

The expression module was amplified with the oligonucleotides F2 and R2 which harbored the restriction sites for *ClaI* and *NarI*, respectively. These two restriction sites were chosen because of their compatible ends; furthermore, once the fragment cleaved with *ClaI* is ligated to the one with the *NarI* "sticky end", a non-palindromic sequence is created which cannot be cut again. The expression cassette was ligated in the pGEMT® vector from Promega and digested with *NheI* and *XhoI* to allow the cloning of the genes. The *Cop6* gene, which had already been expressed in *X. dendrorhous* (**Chapter 4**), was cloned in the new expression cassette. The cDNA

sequences for the *COX1* and *COX2* genes were cordially provided by Prof. Claudia Schmidt-Dannert from University of Minnesota. While *COX1* presented only one sequence recognized by *BamHI*, *COX2* included one site for *BamHI*, two sites for *EcoRI* and one for *ClaI*. Silent mutations were created by means of overlapping PCR steps to replace the restriction sites.

COX1 was amplified with primers F3 and R3 which share the same restriction sites with primers F4 and R4, which were used to amplify *COX2*. Both reverse primers have the sequence recognized by *SalI*, which cleaves the DNA fragments leaving a compatible end with *XhoI*. When all the three genes were cloned in the expression vectors, the modules were excised from the vector by digestion with *ClaI* and *NarI*. The *Cop6* module was ligated first in the vector with *crtE* and the resistance cassette, then in two separate reactions the *COX1* and *COX2* cassettes were inserted in the newly created vector which included also the *Cop6* gene. The construct with the *Cop6* and the *COX1* cassettes was digested with *ClaI* and the *COX2* module was ligated to obtain a construct with all three genes from *C. cinereus*.

Prior to transformation into the wild type *X. dendrorhous* strains, all the constructs were digested with *EcoRI* to create a fragment that could recombine with the genomic DNA of the yeast in correspondence of the *crtE* gene resulting at the same time in the integration of the exogenous DNA and the deletion by insertion of the native gene in the genome.

Table 1. Primers

Primer	Sequence
F1	GGATCCATCGATTGGTGGGTGCATGTATGT
R1	GGATCCTTGATCAGATAAAGATAGAGA
F2	ATCGATTGGTGGGTGCATGTATGT
R2	*GGCGCC*TTGATCAGATAAAGATAGAGA
F3	CATG**GCTAGC**ATGACTTCTACAACGC
R3	ACgtcgacCTATTTCTGGAGCAGGGCCG
F4	CATG**GCTAGC**ATGAACATCGTCAAC
R4	ACgtcgacTCAAAGAGCTGAAATGGATT

Single underline, *BamHI*; Double underline, *ClaI*; Italics, *NarI*; Bold, *NheI*; Small caps, *SalI*

GC-MS analysis of dodecane extracts

Dodecane was added to the yeast cultures to absorb the volatile apolar compounds produced by the cells before they could be released in the headspace of the flasks. The dodecane was isolated from the medium and the cells by centrifuging the culture for 15 minutes at 3000 x g; the upper layer was then removed and diluted

1:10 in ethyl acetate. Two microliters of the diluted dodecane extract were injected splitless and analyzed in total ion scan using a ZB-1ms dimethylpolysiloxane column (Phenomenex 0.25 mm inner diameter, 0.25 μm thickness, 15 m length) on a Shimadzu GCMS-QP5000. The injector temperature was 275°C; the oven initial temperature was 50°C with an increment of 5°C/min up to 105°C and then up to 200°C with an increase of 30°C/ min.

Results and Discussion

Engineering of a new vector for the expression of multiple genes in X. dendrorhous

Since new mutant strains were needed that could express more than just the resistance marker and one heterologous gene, a new transformation vector was designed to obtain such mutants. The new strategy allows the basic expression modules (**Fig. 1**) to be assembled sequentially in a vector together with the marker cassette inside the sequence used for the recombination.

As a first step, the G418 resistance module was inserted in the middle of the *crtE* gene but, differently from the original construct [170], a recognition site for the enzyme *ClaI* was inserted between the already present *BamHI* site and the beginning of the promoter (**Fig. 2 A**); this vector was renamed pCrtE. The presence of the two *BamHI* restriction sites at the two ends of the construct would allow the excision of the complete fragment including expression and resistance cassettes to be ligated in another recombination sequence without further modifications to the constructs.

The restriction sites flanking the expression module were substituted by *ClaI*, before the promoter, and *NarI*, at the end of the *GPD* terminator sequence. The *Cop6*, *COX1* and *COX2* genes were ligated each in a single expression cassette to obtain the constructs Cop6cst, COX1cst and COX2cst (**Fig.1**). All the constructs were verified and confirm by PCR and restriction analysis.

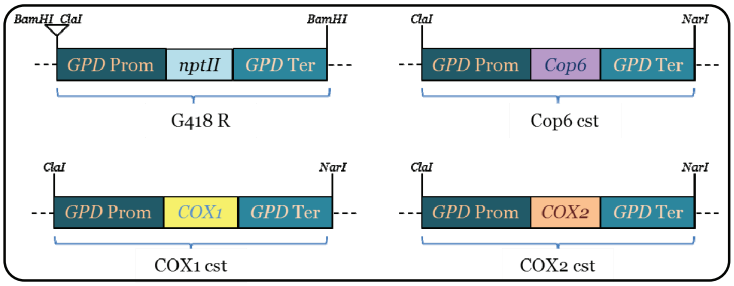

Figure 1. Main modules for the engineering of the vectors used for the transformation of *X. dendrorhous*. ***GPD* Prom**, *GPD* promoter sequence; ***nptII***, resistance gene for geneticin; ***GPD* Ter**, *GPD* terminator sequence; **G418 R**, resistance cassette; **Cop6 cst**, *Cop6* expression cassette; **COX1 cst**, *COX1* expression cassette; **COX2 cst**, *COX2* expression cassette.

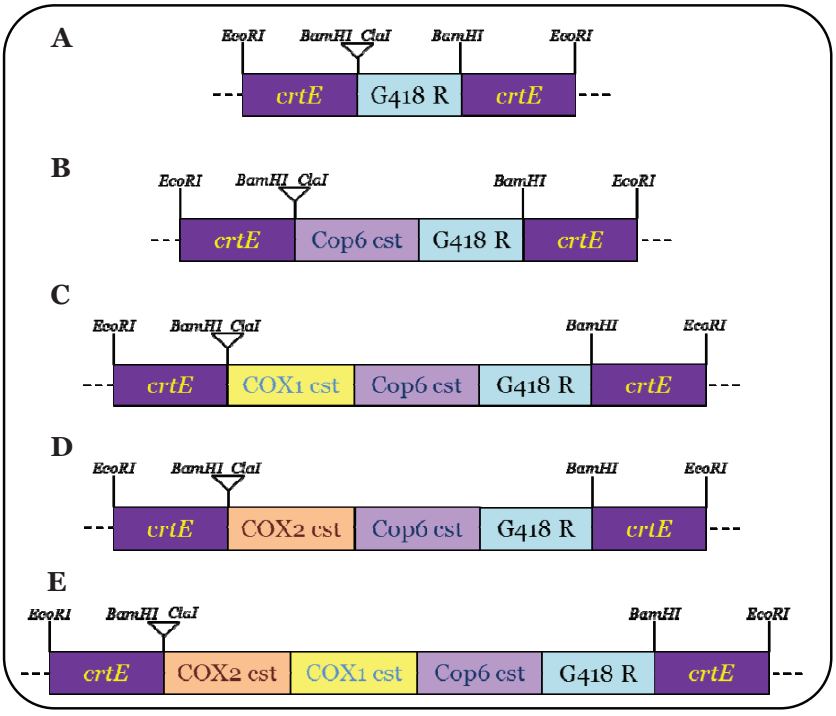

Figure 2. Transformation constructs. (**A**), pCrtE (~6,800 bp); (**B**), pCop6 (~8,500 bp); (**C**), pCOX1 (~9,000 bp); (**D**), pCOX2 (~9,000 bp); (**E**), pCOX1COX2 (~11,200 bp).

pCrtE was used as the starting vector for the building up of the subsequent constructs; the Cop6cst was inserted upstream of the marker cassette resulting in the pCop6 (**Fig. 2 B**). pCOX1 and pCOX2 were obtained by ligating the cassettes COX1cst and COX2cst, respectively, in the pCop6 vector (**Fig. 2 C, D**). In order to be able to express all three genes involved in the lagopodin pathway, we ligated all the modules in one large construct that was named pCOX1COX2 (**Fig 2 E**).

Expression of Cop6 *and* COX1 *in* X. dendrorhous *mutant* ΔE-COX1

When the wild type strain of *X. dendrorhous* was transformed with the fragment obtained digesting the vector pCop6 with *EcoRI*, only white colonies (*ΔE-Cop6*) grew on the selective medium. The white phenotype of the cells indicated that the knock-out of the *crtE* gene had happened as efficiently as with the old vector used for previous transformations.

The new mutant *ΔE-COX1*, result of the transformation of *X. dendrorhous* with pCOX1, and *ΔE-Cop6* were grown in presence of dodecane and the chromatograms obtained by injecting the dodecane in the GCMS are shown in **Figure 3**

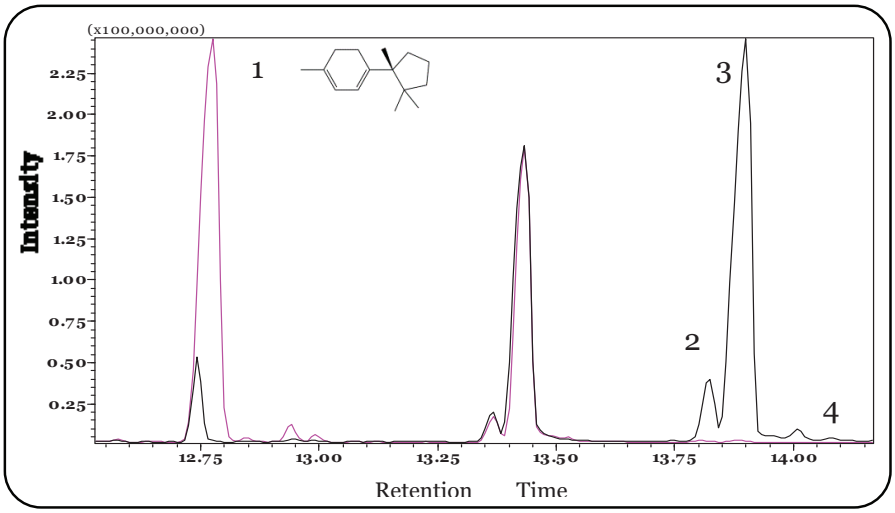

Figure 3. GCMS chromatograms obtained from *ΔE-Cop6* and *ΔE-COX1*. The pink line corresponds to *ΔE-Cop6*, while the black line represents *ΔE-COX1*. The numbers mark the cuprenene peak and the peaks that differ between the two graphs.

The peak indicated with the number 1 represents the cuprenene, and it is present both in the strain expressing only the *Cop6* gene and in the mutant expressing also the *COX1*. This peak has a smaller area in the *ΔE-COX1* compared to the *ΔE-Cop6*. Three new peaks appear in the *ΔE-COX1* mutant, and their fragmentation patterns are represented in **Figure 4** together with their possible chemical structures.

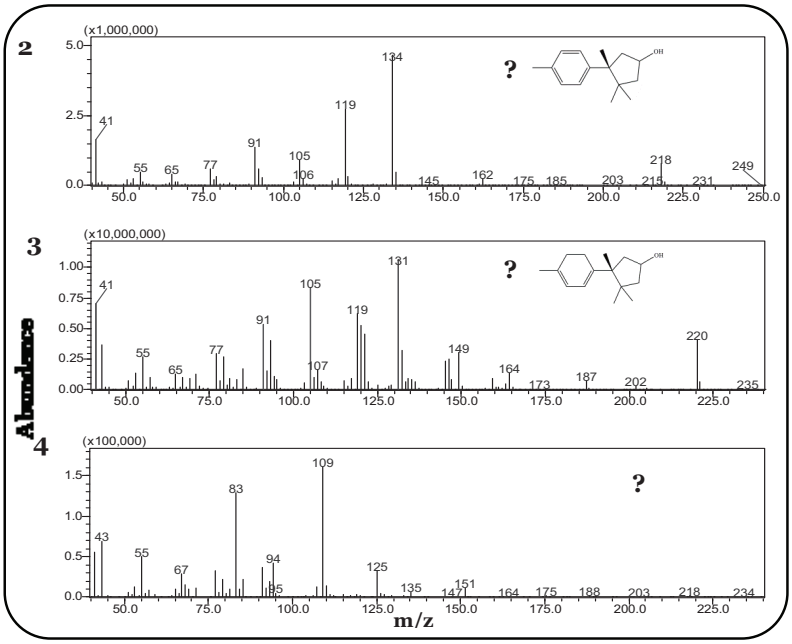

Figure 4. Fragmentation patterns of the peaks visible in **figure 3**. The numbers next to each graph relate the pattern with the corresponding peak in the chromatogram. For 2 and 3 also the possible structures are shown.

The presence of these new molecules demonstrates that both genes are expressed and that the cuprenene is modified by the COX1 protein.

When the same two genes were expressed in *S. cerevisiae*, three new peaks were obtained by the modifications on cuprenene by the action of COX1; one of these peaks has been identified as cuparene, the ring-oxidized product of cuprenene [162]. While cuparene does not appear in the chromatogram obtained by *ΔE-COX1*, the fragmentation pattern of the peak number 1 might represent the derivative of the cuparene which has undergone a hydroxylation on the pentane ring. Furthermore, peak number 3 matches the pattern corresponding to the product likely representing

the derivative of the cuprenene with a hydroxyl group on the pentane ring. We could not find the cuprenene metabolite with the hydroxyl group on the pentane ring further oxidized to a ketone but we obtained an additional peak, number 4, which, unfortunately, could not identify. We speculate, from the presence of the parent ion at an m/z of 218, it might be a side product where the ketone group is on a different position on the pentane ring. Nevertheless, it appears that COX1 is responsible for the oxidations taking place on the pentane ring of the cuprenene molecule.

Expression of Cop6 *and* COX2 *in* X. dendrorhous *mutant* ΔE-COX2

The mutant *ΔE-COX2* was selected after the transformation with the pCOX2 fragment, and it presented a white phenotype like all the previous mutants. Once more the dodecane was isolated after culturing the mutant, and the chromatogram was compared to the one obtained with *ΔE-Cop6* (**Fig. 5**).

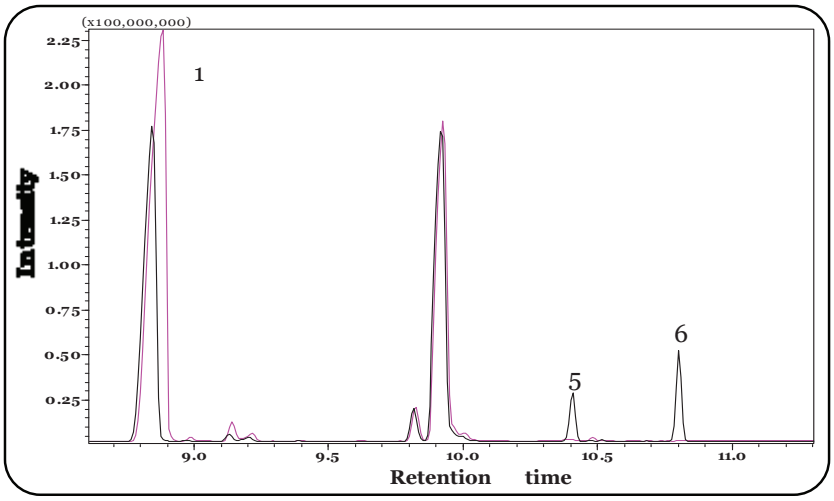

Figure 5. GCMS chromatograms obtained from *ΔE-Cop6* and *ΔE-COX2*. The pink line corresponds to *ΔE-Cop6*, while the black line represents *ΔE-COX2*.

The cuprenene peak in the strain expressing both *Cop6* and *COX2* was also reduced in size compared to the *ΔE-Cop6* mutant, and two new peaks appeared in the chromatogram. The fragmentation pattern coming from peak number 6 (**Fig. 6**) was compared to the patterns obtained when *Cop6* and *COX2* were expressed in *S. cerevisiae*, and it was found to match the confirmed structure of cuparophenol.

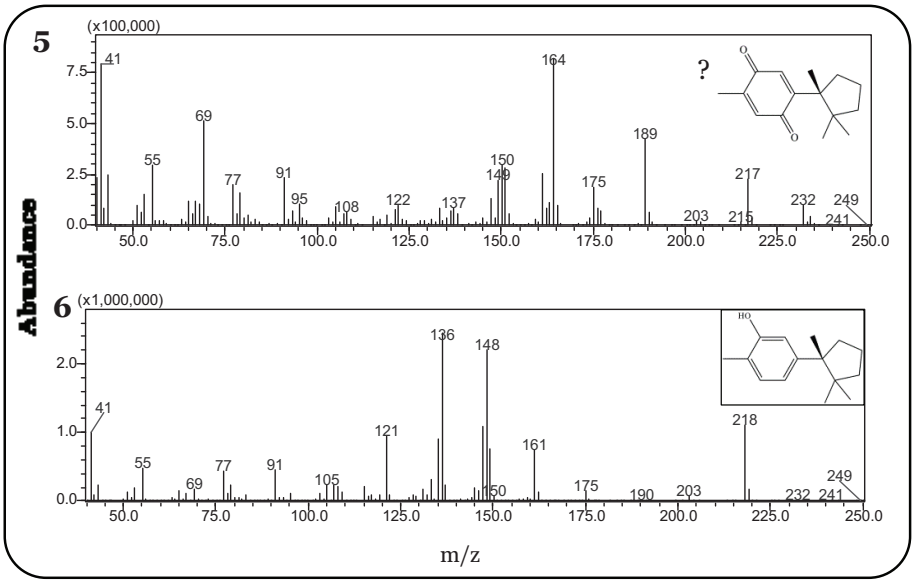

Figure 6. Fragmentation patterns of the peaks visible in figure 5. The numbers next to each graph relate the pattern with the corresponding peak in the chromatogram, and the possible structures are shown.

Surprisingly, the fragments of peak number 5 match those of the compound that Agger and colleagues found to be produced in *S. cerevisiae* when both *COX1* and *COX2* were expressed. While they propose that the molecule is cuparophenol with a ketone moiety on the pentane ring, from the mass of the parent ion and the fragmentation pattern of the daughter ions, we speculate it might be the quinone product shown in **Figure 6**. Our hypothesis is supported by the fact that no peak corresponding to a double hydroxylated cuparene is visible in the chromatogram, and this might be explained by its immediate conversion to the more stable quinone derivative. The presence of the cuparophenol and its corresponding quinone in the cultures implies that COX2 is responsible for the modifications on the

methylcyclohexadiene ring of the cuprenene towards the lagopodin A structure. Only a higher scale expression of the yeast and a more accurate analysis on GC or HPLC coupled to a high resolution mass spectrometer would provide us with more information on all the structures obtained from the cultures of the two mutants.

Combined expression of Cop6, COX1 *and* COX2 *in* X. dendrorhous

The vector pCOX1COX2 was used to obtain the mutant *ΔE-COX1COX2* which could express four genes at the same time. The mutant was grown in the medium with dodecane. **Figure 7** represents the chromatogram obtained from this mutant compared to the *ΔE-Cop6*.

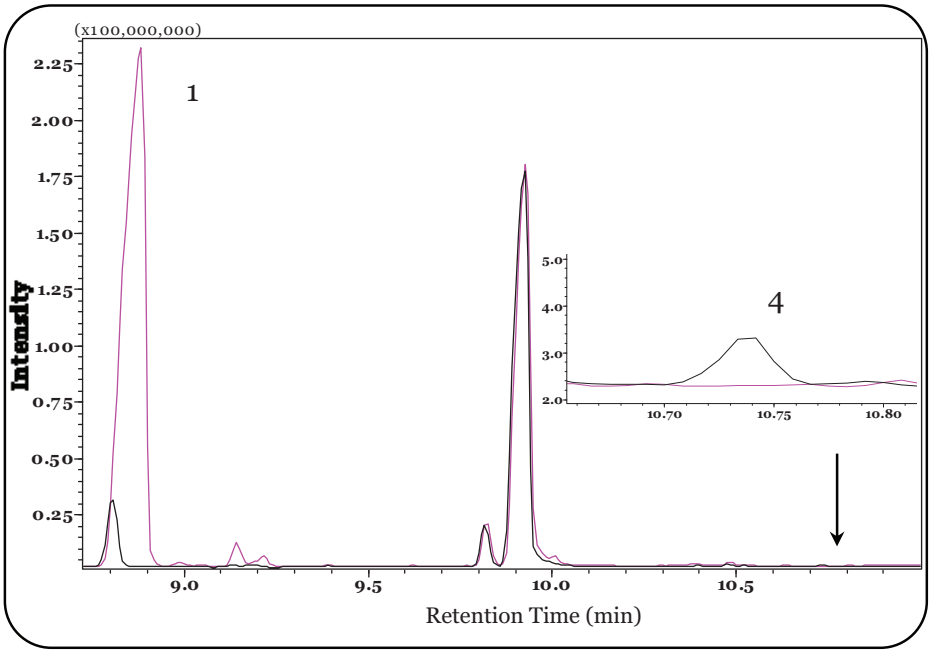

Figure 7. GCMS chromatograms obtained from *ΔE-Cop6* and *ΔE-COX1COX2*. The pink line corresponds to *ΔE-Cop6*, while the black line represents *ΔE-COX1COX2*. The chromatogram between 10.6 and 10.8 minutes has been zoomed in to allow the visualization of the smaller peak.

As in all the previous mutants, the peak corresponding to cuprenene was smaller than the one in the *ΔE-Cop6*, confirming that the gene was expressed, the protein was active and the compound was modified to one or more metabolites. From the decrease in cuprenene concentration we would have expected one or more peaks appearing that could explain the lower concentration of the metabolite but we could only identify a small peak with a fragmentation pattern matching the one from peak 4 in **Figure 3**. The presence of this peak confirmed that also the COX1 protein was active.

Since no other peak was visible from the dodecane extract, we analyzed the medium we had previously isolated from the dodecane and the yeast cells.

Bottom and Siehr discovered that alkaline aqueous solutions containing hydroxylagopodin would exhibit a purple coloration [174]. These findings suggest that a shift in the color of the medium from the *ΔE-COX1COX2* mutant towards a purple hue should be visible if hydroxylagopodin is produced by the yeast. In order to evaluate whether this was the case, NaOH was added to the medium extracted from the cultures of all the mutants. While the medium in which *ΔE-Cop6* had grown did not present any color change, the media from *ΔE-COX1* and *ΔE-COX2* turned red. A purple shade of color was visible for the growth broth in which *ΔE-COX1COX2* had grown, showing that hydroxylagopodin or a similar compound was likely to be found in the solution. Unfortunately, this coloration was observed only in one occasion with only 10 ml of media and after the vials had been stored at 4°C, and even after extraction of the media with ethyl acetate and analysis on TLC plates, we could not isolate nor identify the compounds produced by the mutant *ΔE-COX1COX2*.

Bu'Lock and Darbyshire studied the stability of the lagopodin metabolites in alkaline water solution and discovered them to be highly unstable at neutral or slightly basic pH already after 6 hours [175]. This high instability might explain why we were not able to isolate the compounds from cultures with higher volumes, since for the yeast it would take at least 3 days to be able to accumulate large enough concentrations of the metabolite to be visible on TLC plates.

In conclusion, we have designed and tested a new cloning strategy that allows transformation of *X. dendrorhous* with a series of genes without need for several selection markers. This new vector permitted us to obtain mutant strains of *X. dendrorhous* that could express a complete pathway towards the production of a new non-native metabolite. The vector is indispensable in the process towards the

utilization of the red yeast as a production organism for compounds obtainable after several modifications catalyzed by different enzymes.

The *X. dendrorhous* mutant expressing three genes from the lagopodin pathway produced a purple pigment that is likely a metabolite of lagopodin although no isolation or identification was possible. A solution to the isolation problem lies in the use of buffered medium where the pH could be kept acidic enough to avoid degradation of the compounds. Once the compound is isolated, it could be subjected to NMR or High resolution mass spectrometry for the structure elucidation and its activity as a promising new antibiotic could be evaluated. Furthermore, we have demonstrated that there is no need to express additional reductases for the regeneration of the cytochromes since the one already present in *X. dendrorhous* could function also on the heterologous cytochromes COX1 and COX2. This observation is important since cytochromes are involved in several terpene backbones modification steps toward finished drugs, like artemisinin and paclitaxel [84, 176], and an organism like *X. dendrorhous* that can provide regeneration of the hydroxylating proteins by itself is regarded as highly valuable.

CHAPTER 6

ORGANELLAR TARGETING OF AMORPHADIENE SYNTHASE (ADS) IN *SACCHAROMYCES CEREVISIAE*

Introduction

Saccharomyces cerevisiae has been used since ancient times as a fermentation microorganism and has more recently become the model organism for studies on eukaryotic cells [177]. With the development of genetic engineering *S. cerevisiae* has been used for the production of several diverse compounds, ranging from biofuels to flavors to drugs [101, 178-183]. In particular, studies on the production of terpene-based drugs have represented an effective approach combining synthetic biology and metabolic engineering [145]. One of the most successful results was obtained with amorphadiene, the first committed precursor of the antimalarial terpenoid drug artemisinin [89]. After multiple studies following various approaches [69, 70, 84], a single strain of *S. cerevisiae* was engineered and optimally cultured to obtain an unprecedented 40 g of amorphadiene per liter of yeast culture. The step-by-step engineering of such a strain unveils the potential of metabolic engineering as one of the successful fields in the struggle to obtain cheaper drugs.

Most of the research involving the accumulation of terpenoid drugs in *S. cerevisiae* has focused on precursors present in the cytosolic environment of the cell. In plants, terpene structures can be produced in different compartments in the cell, mostly plastids, mitochondria and endoplasmic reticulum [184]. Recently, though, isoforms of enzymes involved in the terpenoid pathway have been identified also in the peroxisomes of *Arabidopsis thaliana* and *Catharanthus roseus* [185, 186]. Furthermore, a mevalonate kinase, a phosphomevalonate kinase and a mevalonate diphosphate decarboxylase have been localized in mammalian peroxisomes and two geranylgeranyl diphosphate synthases (GGPS) were found to be expressed in *Penicillium paxilli* in the same compartment [187, 188]. More interestingly, the *Bts1p* gene, encoding for a GGPS in *S. cerevisiae* is localized in the yeast mitochondria, suggesting the presence of terpene building blocks in different compartments also in the baker's yeast [189].

To confirm that terpene structures can be found in different cell organelles in *S. cerevisiae*, we have used the gene coding for amorphadiene synthase (*ADS*) from *Artemisia annua* and fused it to the targeting signals for the translocation of the proteins to mitochondria and peroxisomes. The green fluorescent protein (*GFP*) gene was also fused to the *ADS* in order to be able to confirm that the ADS protein was correctly directed to organelles. The targeting signal COX4, when fused to the N-

terminus of a protein, has been demonstrated to be sufficient for the targeting of proteins to the mitochondria [190].

Most of the proteins directed towards the peroxisomes are characterized by a tripeptide sequence that does not get excised after translocation to these organelles. This short sequence, called peroxisomal targeting signal (PTS1), was discovered for the first time in fireflies' proteins [191] and it was shown to be a highly conserved SKL (Ser-Lys-Leu) peptide at the protein C-terminal end.

We engineered four plasmids and obtained four *S. cerevisiae* strains: the first (*ScADS*) expressed the native form of ADS in the cytoplasm; the second (*ScGFP*) contained the plasmid with *ADS* fused to *GFP*; the third mutant (*ScCOX*) was is equipped with the COX4 targeting sequence in order to transport the fusion protein ADS-GFP to the mitochondria; the fourth strain (*ScPTS*) is transformed with the plasmid containing the PTS1 targeting sequence at the 3'-end of the *ADS::GFP* gene. The strains were analyzed by confocal microscopy for presence of the green fluorescence, and, in parallel, the biosynthesis of amorphadiene was determined by GC-MS.

Materials and methods

Strains and media

For all the cloning steps *E. coli* DH5α strain was used; bacteria were grown at 37°C, 250 rpm in LB (10 g/L Trypton, 5 g/L Yeast Extract and 10 g/L NaCl). Ampicillin was used for the selection of the transformed colonies at a concentration of 100 mg/ml.

For the transformation processes, *S. cerevisiae* MRG 5 #502 (*MATa*, *ura*3-52, *leu*2-Δ1, *trp*1-Δ36, *his*3-Δ200, D*ade*2) was used. The growth medium for *S. cerevisiae* consisted of 13.4 g/l Yeast Nitrogen Base without amino acids, 20 g/L Dextrose, 100 mg/L leucine, 40 mg/L histidine, 40 mg/L tryptophan and 40 mg/L of uracil.

Engineering of the constructs for the expression of ADS::GFP *in different cell compartments*

The plasmid used for the expression of all the genes was the p426 with the repressible promoter Met25 for the regulation of the gene expression [192]. The plasmid was digested with the restriction enzymes *EcoRI* and *SpeI* for the unidirectional cloning of the genes.

The *ADS* gene in the plasmid p426Met-ADS for the production of the ADS protein in the cytosol was amplified with primers F1 and R1 (**Table 1**). The gene was amplified with Phusion polymerase (New England Biolabs) and subsequently A-tailed for the ligation in the vector pGEMT (Promega). The positive colonies were selected on LB plus ampicillin; 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) were also added for the blue-white colony screening. The gene was then sequenced, excised from the pGEMT vector with the two mentioned restriction enzymes and ligated in the previously digested p426Met.

The *ADS* fragment to be fused to the *GFP* gene (codon optimized for *S. cerevisiae* [193]) was amplified with primers F1 and R2. The *GFP* gene was instead amplified with primers F2 and R3 and the fragment was used for the double step PCR to fuse it to *ADS*. Briefly, a first PCR was performed using the *ADS* and the *GFP* amplicons as primers and templates since they share an overlapping sequence of 30 base pairs. After 5 cycles, the primers F1 and R3 were added to obtain the complete fused gene *ADS::GFP*. After purification and ligation in pGEMT, the product was digested and ligated in the shuttle vector for *S. cerevisiae* to create p426Met-ADS::GFP.

The mitochondrial COX4 targeting sequence was amplified from *S. cerevisiae* genomic DNA using oligos F3 and R4 and then fused to the *ADS::GFP* which had been amplified with the primers F4 and R3. The two-steps PCR with F3 and R3 produced *COX4-ADS::GFP* that was then treated as the other fragments and ligated into p426Met to obtain p426Met-COX4-ADS::GFP. p426Met-ADS::GFP-PTS1 was engineered by ligating the *ADS::GFP* previously amplified with primers F1 and R5 into p426Met. All the constructs were used to transform *S. cerevisiae* with the Li acetate method [194]. The positive colonies were selected for the ability to grow on minimal medium not supplemented with uracil.

Table 1. Primers

Primer	Sequence
F1	GACTAGTATGTCACTTACAGAAGAAAAACC
R1	GAATTCTCATATACTCATAGGATAAA
R2	*ACCAGCACCGTCACC*TATACTCATAGGATA
F2	TATCCTATGAGTATA*GGTGACGGTGCTGGT*
R3	GAATTCTTATTTGTACAATT
F3	GACTAGTATGCTTTCACTACGTCAATCTATAAG
R4	TTCTTCTGTAAGTGACATAAGCAGAT
F4	ATCTGCTTATGTCACTTACAGAAGAA
R5	GAATTCTTA**CAATTTAGA**TTTGTACAATTCATC

Single underline, *SpeI*; Double underline, *EcoRI*; Italics, linker sequence; Bold, PTS1.

Time course analysis

The growth curves and the amorphadiene production of the four strains *ScADS*, *ScGFP*, *ScCOX* and *ScPTS* were evaluated during a time course experiment for 96 hours with four time points every 24 hours. A fresh overnight culture for each strain was used as inoculum for the main time course experiment with initial optical density (OD) was 0.05. The time course was conducted in 100 ml-flasks with two flaks for each strain and time point. Every 24 hours for 4 days, the two flasks for each strain were removed from the incubator at 30°C at 180 rpm and the cultures were centrifuged at 4000 rpm at 4° C for 15 minutes. After centrifugation a clear phase separation was visible between the medium and the dodecane layer. The organic solvent was removed from the aqueous phase and used for the subsequent GC-MS analysis. The pelleted cells were washed once with 1 ml of distilled water and freeze dried for the determination of the cell dry weight.

Cell permeability and organellar specificity of LysoTracker® and Mitotracker®

S. cerevisiae cells transformed with the empty vector p426Met were grown over night and used for the experiments with the two probes, LysoTracker® Red DND-99 and Mitotracker® Orange.

The amount of cultures corresponding to an OD of 1 was centrifuged at 1000 rpm for 10 min and resuspended in room temperature growth medium with 38 nM of LysoTracker® Red DND-99 (Invitrogen) or 250 nM Mitotracker® Orange (Invitrogen), previously resuspended in DMSO. The cells were incubated with the

probes at 21° C in a shaking incubator at 250 rpm for 60, 75 or 90 min. After the incubation time, cells were centrifuged and fixed for 2 hours in 100 mM phosphate buffer (pH 6.5) to which 3.7% formaldehyde had been added. Two washing steps with only phosphate buffer were performed and, finally, cells were resuspended in buffer, 10 µl were pipetted on a glass slide and visualized using a confocal laser microscope.

GFP expression in S. cerevisiae *mutant strains*

Cells from the strains *ScGFP*, *ScCOX* and *ScPTS* were centrifuged and fixed following the same protocol as for the analysis with the two probes. A Leica DM IRE2 Inverted microscope with Hg 50 w mercury lamp equipped with two objectives (HCX PL APO CS 40x/1,25 oil 0,1mm and HCX PL APO 63x/1,4 oil 0,1mm) was used to visualize the whole cells and the inner compartments. The excitation wavelength for the GFP was set at 476 nm, while the emission was recorded at 512 nm. More information about the fluorescence confocal microscope can be found at the website http://www.rug.nl/umcg/onderzoek/faciliteiten/MicroscopyUnit/Leica-SP2-CLSM.

Results

Evaluation of permeability and cellular compartmentalization of organelle-specific probes by confocal microscopy

The organelle-specific probes LysoTracker® Red DND-99 and Mitotracker® Orange are normally used for the staining of the cellular compartments in eukaryotic cells with different specificity. Mitotracker® is meant for the localization and counting of active mitochondria after oxidation-driven fluorescence. After fixation with formaldehyde, the complex formed by the probe and the mitochondrial proteins becomes permanent and the organelles are permanently dyed.

LysoTracker® is normally utilized for the staining of the lysosomes, as the probe is constituted by a fluorophore linked to a weak base and it accumulates in acidic organelles. Since fatty acids metabolism in yeast occurs in peroxisomes, they are thought to have a lower pH compared to the cytosol. The acidic pH of peroxisomes makes them a likely candidate for the staining with LysoTracker®.

In order to evaluate the permeability and the specificity of the dyes in the yeast cells we incubated the cells with the two probes separately and observed the fluorescence in the cells in time (**Figure 1**).

The permeability of the probe for the lysosomes was in general poor even after incubation for 2 hours; furthermore no specificity for any organelles could be observed since the cell cytosol appeared to be completely dyed. In spite of the faster and more extensive cell penetration compared to LysoTracker®, also the Mitotracker® showed no accumulation in a particular cell compartment.

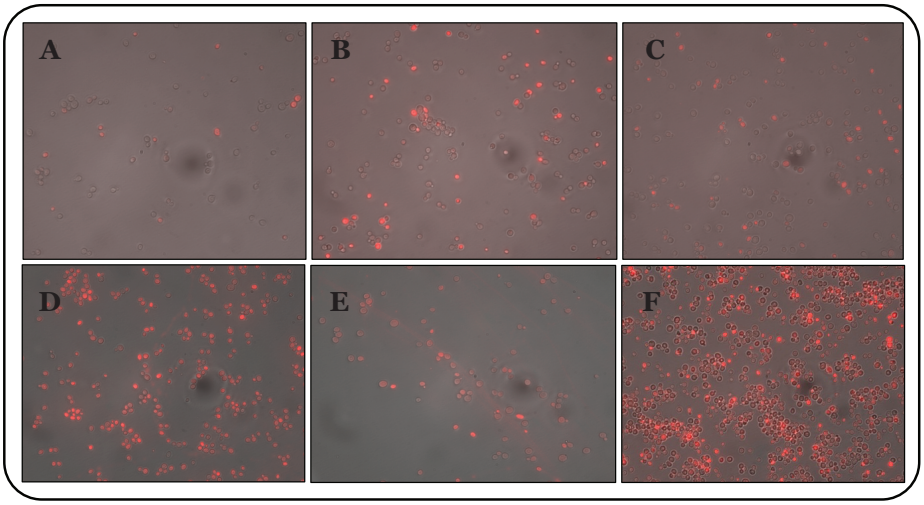

Figure 1. Treatment of *S. cerevisiae* cells with LysoTracker® (**A**, **B** and **C**) or Mitotracker® (**D**, **E** and **F**). Cells were observed under microscope after 1h (**A** and **D**), 1,5 h (**B** and **E**) and 2h (**C** and **F**) incubation with the respective probes

Isolation and growth analysis of S. cerevisiae *mutants*

The four *S. cerevisiae* mutants were selected on medium lacking uracil; single colonies were isolated and used for all the subsequent analyses. *ScADS*, *ScGFP*, *ScCOX* and *ScPTS* were the strains obtained from the transformations of the wild type strain with the plasmid p426Met-ADS, p426Met-ADS::GFP, p426Met25-COX4-ADS::GFP and p426Met-ADS::GFP-PTS1, respectively.

In order to evaluate differences in growth among the four strains, a time course was performed on all strains for four days and the dry cell weight was used as a comparison parameter. The growth data obtained for the *S. cerevisiae* strains

during the time course are plotted in **Figure 2**. *ScADS*, *ScGFP* and *ScPTS* exhibit similar growth curves with a fast exponential growth between the first and the second day of culturing. In contrast, *ScCOX* has a lag phase in the first 24 hours and an exponential growth until 72 hours when it reaches the same dry weight as the other strains.

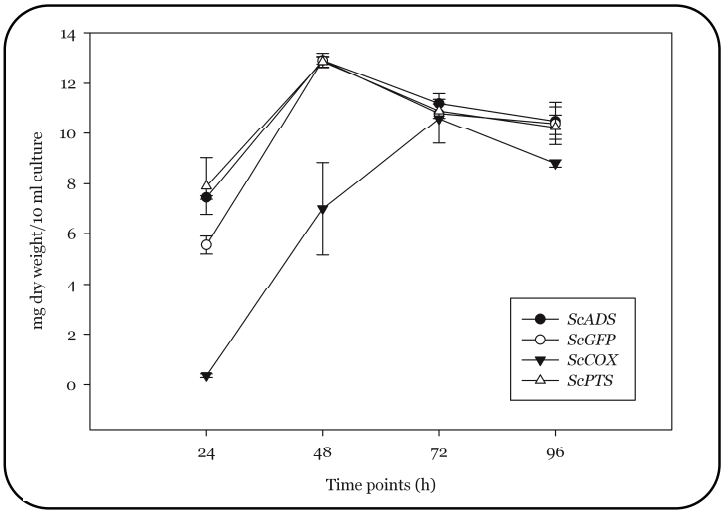

Figure 2. Growth curves obtained with *ScADS*, *ScGFP*, *ScCOX* and *ScPTS*

Expression of GFP-fusion proteins and localization in the cells

The confocal microscope allowed us to visualize the localization of the fused protein ADS::GFP in the cytosol, mitochondria or peroxisomes. The advantages provided by a confocal microscope compared to a normal fluorescence microscope consist of a higher sharpness of the pictures and the possibility to observe cells lying on the same layer, avoiding the interference from the background fluorescence.

After fixation in formaldehyde, the cells were transferred to a glass slide and observed using a fluorescent microscope. No more than 50% of the cells for all the constructs appeared fluorescent under the microscope (**Fig. 3**) and the production of the protein varied from cell to cell even for the same mutant strain.

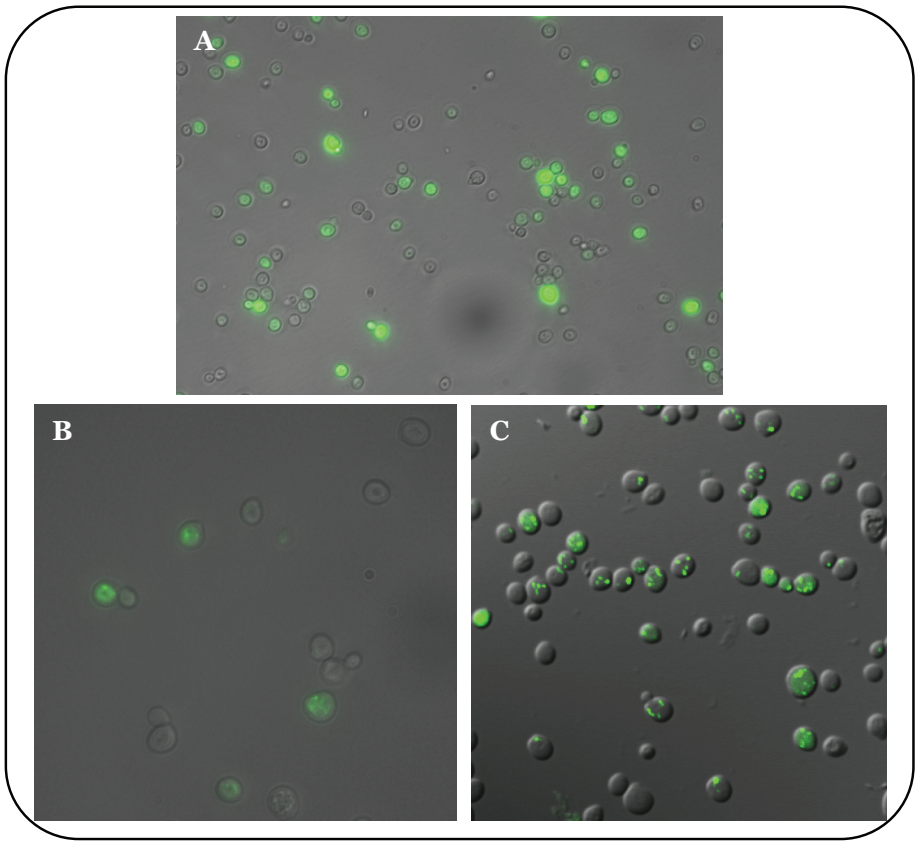

Figure 3. Overlaid fluorescence and bright field photographs of *S. cerevisiae* mutants. (**A**) *ScGFP*, (**B**) *ScCOX* and (**C**) *ScPTS*

The *ScGFP* mutant shows fluorescence throughout the whole cell, confirming that the expression of the protein is cytosolic and does not show any particular accumulation point. From the image obtained with the *ScCOX* strain, some defined spots are visible inside the cells and the protein production appears lower than in the cells expressing the cytosolic version of the protein. The organellar accumulation is even more visible in the case of the strain *ScPTS* (C); while a few cells still show a fluorescent signal in the whole cell, most of the others exhibit discrete and defined accumulation points in the cytosol. The number of the fluorescent spots differs from cell to cell and it does not seem to correlate with the size of the cells.

Amorphadiene production in S. cerevisiae *mutant strains*

Concurrently with the analysis of the biomass accumulation for the four mutant strains, an estimation and comparison in the production of amorphadiene was performed by adding a dodecane layer to the cultures. The comparison in production among the mutants was made possible by the addition of an internal standard to the dodecane solution, which allowed also for normalization of the data after growth and isolation of the organic layer.

In **Figure 4** the quantitation of the amorphadiene production in the different mutants is shown.

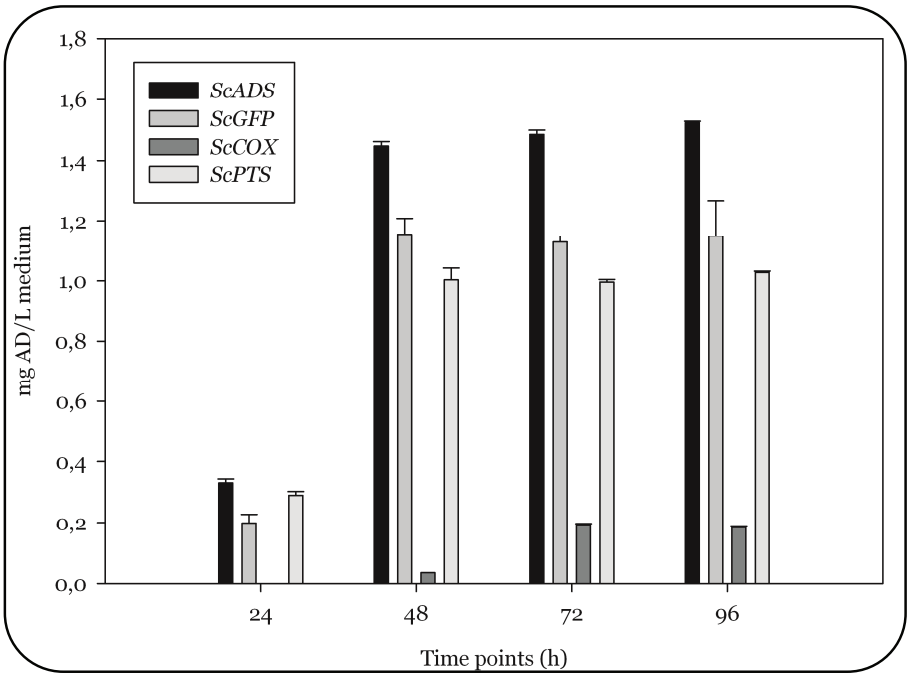

Figure 4. Amorphadiene accumulation per liter of medium in mutant strains *ScADS*, *ScGFP*, *ScCOX* and *ScPTS*

The first clear result is the lower sesquiterpene accumulation in the *ScCOX* mutant compared to the strain with the cytosolic version of the fused protein, while *ScPTS* shows only a 10% reduction in metabolite production.

In all four mutants the major increase in production of amorphadiene corresponds with the log phase of the cell mass accumulation curve: for *ScADS*,

ScGFP and *ScPTS* this happens between 24 and 48 hours from the inoculation in the fresh medium, while the growth boost in *ScCOX* is delayed of one day compared to the other mutants.

In order to evaluate whether the reduced cell mass of the mitochondrial mutant affects the amorphadiene accumulation, the terpene production values were divided by the cell dry weight (**Fig. 5**)

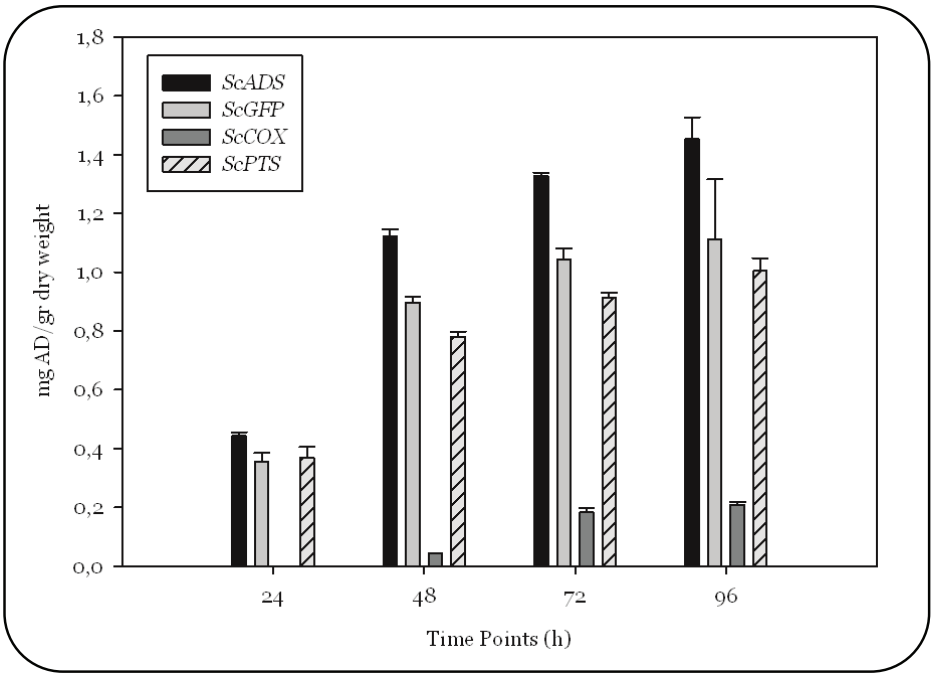

Figure 5. Production of amorphadiene in *ScADS*, *ScGFP*, *ScCOX* and *ScPTS* normalized for the cell dry weight.

The decreased accumulation of amorphadiene in *ScCOX* compared to *ScADS* is independent of the lower cell mass of the first mutant; the same conclusion can be drawn for the strain with the peroxisome-targeted protein. Furthermore, the fusion of the GFP to the ADS protein affects the amorphadiene production by approximately 20%.

Discussion

The limited concentration of artemisinin in *A. annua* plants and the previously discovered complicated and expensive total chemical synthesis of the compound have made the sesquiterpene lactone one of the pharmaceuticals with a high market demand but low availability. In order to overcome the supply problems for the antimalarial drug, research groups have developed a new synthetic route which reduces the costs of production [195] and an improved *S. cerevisiae* strain as cell factory with an unprecedented titer of amorphadiene [89].

In our study we evaluate what is the potential for amorphadiene production in cell organelles in *S. cerevisiae*. We chose to express for the first time the *ADS* gene fused to a variant of *GFP* from the jellyfish *Aequorea victoria* codon optimized for *S. cerevisiae*. A short linker was used to allow both proteins to fold correctly. For the translocation of the protein to the peroxisomes or to the mitochondria, we created two constructs in which the *ADS::GFP* gene was fused to the targeting signal sequences *COX4* or *PTS1*.

The localization in the cells of the fluorescent proteins was visualized by a confocal microscope which allowed high resolution pictures of cells. Single luminous spots were visible in the mutants *ScCOX* and *ScPTS*, while a widespread luminescence in the cells was a characteristic of the strain expressing the cytosolic protein. In order to confirm that the targeted organelles were in fact the expected ones, we tested two organelle-specific probes. Unfortunately, we could not obtain a specific accumulation of the dyes in the expected compartment since the cells were poorly colored and/or the fluorescence was spread throughout the whole cell.

The fusion of the GFP protein to ADS resulted in a 20% reduced amorphadiene production in the cytosol which might be caused by a decrease in activity of the ADS protein or by a reduced expression of the gene.

In contrast with the results obtained by Farhi and colleagues [196], we observed an affected growth and reduced production of amorphadiene when the *ADS::GFP* gene was fused to the targeting signal for the mitochondria. This discrepancy in the results might be explained by a few factors: the presence of the GFP might affect the ADS protein reducing its activity in the mitochondrial environment; while we have used a constitutive promoter regulating the gene expression and we opted for an episomal plasmid, Farhi and colleagues used an integrative construct with a copper induced promoter. In spite of these conflicting

outcomes, it is reasonable to expect an affected growth in a strain which is depleted of an important precursor such as FPP in the energy factory of the cell.

For the first time we have shown that the fusion protein ADS::GFP can be transported to the peroxisomes and that both activities, fluorescence and amorphadiene synthesis, are functional in these organelles. The formation of amorphadiene demonstrates that peroxisomes contain FPP that can be converted by ADS.

In spite of the lower production of amorphadiene in *ScCOX* and *ScPTS* compared to the strain with the cytosolic variant of the ADS protein, the opportunity arises to use the precursors present in the peroxisomes and mitochondria for the production of terpenes.

CHAPTER 7

SUMMARY AND FUTURE PERSPECTIVES

The class of natural compounds known as terpenes, terpenoids or isoprenoids includes nearly 25,000 structures. Terpenes are produced by eukaryotes and prokaryotes alike, and they assume a wide variety of functions. Carotenoids (possessing antioxidant properties), limonene and menthol used by cosmetic and food industries, paclitaxel used to treat cancer patients and artemisinin used to treat malaria; all these compounds share a basic terpene skeleton modified subsequently by cytochromes and other structure decorating enzymes.

Terpenes are produced via two routes, the MEV and MEP pathway. Although these pathways start with different substrates, both produce isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). These two building blocks are then fused to create larger structures that represent the backbone of all terpenes. While fungi and animals possess the MEV pathway and prokaryotes the MEP pathway, plants can produce terpenes via both routes. Furthermore, in plants the two pathways are confined into different cell compartments.

Some pharmaceutically important terpene derived drugs, like artemisinin and paclitaxel (Taxol®), are produced in low concentrations in their native hosts (the plant *Artemisia annua* for artemisinin and the *Taxus* spp. tree for paclitaxel). Often organic synthesis of these compounds is costly; the extraction from the natural sources can put the original species in extinction danger and the amounts of isolated compounds cannot fully cover the market demand. Genetic engineering has helped consistently in the fight for cost efficient and sufficiently available terpene drugs. Parts of the biosynthetic routes for some of the compounds have been elucidated and the enzymes have been transferred to heterologous hosts in order to obtain higher yields than the ones from the native hosts. One of the successful examples is the production of the precursor of artemisinin, amorphadiene, reaching 40 g/L of *Saccharomyces cerevisiae* culture. Metabolic engineering has majorly contributed to this achievement, allowing the fine tuning of the genes involved in bottleneck steps with the aim of increasing the flux of precursors towards the desired product.

An organism metabolically optimized towards the highest production of a class of compounds is called a “platform organism”, while an organism optimized to yield a single product is named a “cell factory”. For the terpenes class, the two likely candidates, i.e. on which most of the research groups have focused, are *Escherichia coli* and *S. cerevisiae*. Both microbes have been extensively studied and adapted to produce a wide range of compounds, including proteins, drugs and biofuels. Despite

the big successes obtained with these two organisms, biodiversity can still provide alternative organisms which might be even better platform organisms, but that have not been fully studied, yet.

The research in this thesis aims at evaluating the potential as terpene cell factory of *Xanthophyllomyces dendrorhous*, the yeast producing the carotenoid astaxanthin which gives the characteristic red color to the yeast. The ability of *X. dendrorhous* to accumulate carotenoids can be exploited for the production of diverse terpene compounds by transferring heterologous genes from interesting biosynthetic routes to the genome of the yeast.

This thesis describes the engineering of special vectors for the transformation of the yeast and the analysis of the metabolites produced by *X. dendrorhous* mutants. Furthermore, the last chapter studies the potential of organellar targeting of the amorphadiene synthase gene in *S. cerevisiae* mutant strains.

X. dendrorhous: a potential platform organism for terpenes

X. dendrorhous has been explored for astaxanthin production since the isolation of the yeast in the 1970s. Recently, with the elucidation of parts of the pathway towards carotenoids and with the increase in market size of "natural" compounds with antioxidant properties, the efforts put in transforming the red yeast into a cell factory for the production of astaxanthin have increased substantially. Some research groups have focused on looking for natural astaxanthin high-producing strains while others have relied on genetic engineering and have designed vectors and transformation protocols to obtain new strains overexpressing crucial genes in the terpene and carotenoid pathways. These studies have provided us with the necessary sequences that have allowed us to modify the vectors for our research purposes.

Chapter 3 presents the main concept behind the work described throughout this thesis: when heterologous terpene synthases are transferred to *X. dendrorhous* mutants, non-native terpenes can be produced by the new strains in which the astaxanthin pathway has been knocked out. We designed a vector which allows the concomitant integration of an expression cassette and the knock out (by insertion) of one of the genes involved in the carotenoid pathway. The GGPP synthase and the phytoene synthase gene were chosen as recombination sequences. The first gene

transferred to *X. dendrorhous* was the pentalenene synthase, which uses FPP as substrate and produces pentalenene, the precursor of the antibiotic pentalenolactone. Yeast strains expressing this synthase produce pentalenene and do not show any growth alterations, except for the white phenotype denoting a complete lack of astaxanthin production. The highest concentration of the sesquiterpene pentalenene was obtained in the red strain in which the vector had integrated in the rDNA sequences in the genome, rather than in the previously mentioned genes. This higher accumulation might be explained by multiple integrations of the expression cassette in several rDNA sequences spread throughout the whole genome.

After proving that the strategy for the transformation of *X. dendrorhous* was successful, we decided to evaluate efficiency of the sesquiterpene production of our yeast. For this reason, as described in **Chapter 4**, we transformed the red yeast, *E. coli* and *S. cerevisiae* with the *Cop6* gene isolated from the basidiomycetes *Coprinus cinereus*. All *X. dendrorhous* strains accumulate α-cuprenene, the metabolite produced by Cop6, in higher concentrations than *E. coli* and *S. cerevisiae*, confirming our idea that the red yeast possesses a high potential for the production of terpenes. Furthermore, *X. dendrorhous* is cultured at temperatures ranging from 20 to 24°C and it grows better on minimal medium compared to *S. cerevisiae*, making the red yeast a possible alternative as industrial strain for the production of terpenes.

Most of the terpene drugs are constructed via multistep pathways involving several enzymatic modifications. The results presented in **Chapter 5** were obtained when two cytochromes involved in the pathway towards lagopodin A were expressed in combination with the *Cop6* gene in *X. dendrorhous*. In *C. cinereus* α-cuprenene is the first committed precursor of lagopodin A, but it requires a few oxidation steps to give the final antibiotic drug. Two cytochromes, *COX1* and *COX2*, have been found flanking the *Cop6* gene in the fungus, and, since genes involved in the same pathway are often clustered together in the genome, these two proteins are presumably involved in the oxidation of α-cuprenene to lagopodin A.

In order to be able to express *Cop6*, *COX1* and *COX2* in the same *X. dendrorhous* strain, we engineered our vector with a new expression cassette that could be assembled together with, in principle, an infinite number of this cassette, expressing the same or alternative genes. This new vector allowed us to express the three genes in *X. dendrorhous* and confirm their involvement in the lagopodin A

biosynthesis. Intermediates from the lagopodin A pathway were identified when the three genes were expressed in *X. dendrorhous*, albeit no lagopodin A could be isolated. Nevertheless, when the growth medium was alkalinized, a purple coloration appeared revealing the likely presence of the antibiotic drug or a closely related compound which can turn violet in aqueous alkaline solutions. Notably, the regeneration of the cytochromes, which is normally performed by a reductase, was attained by one or more native reductases from *X. dendrorhous*, eliminating the necessity to transfer additional genes to the yeast strain.

Expression of amorphadiene synthase in *Saccharomyces cerevisiae* cell compartments

Amorphadiene synthase (ADS) is the enzyme that performs the cyclization of FPP to give amorphadiene, the first committed precursor in the artemisinin pathway in *Artemisia annua*. This protein has been expressed in several organisms, including *E. coli*, *S. cerevisiae*, *Nicotiana tabacum* and *Aspergillus niger*. The research on the production of artemisinin and amorphadiene has been extensive since this antimalarial drug is produced in too low levels to cover the market demand. Genetic engineering of *S. cerevisiae* has allowed the production of amorphadiene in this yeast. This molecule can then be converted, by chemical synthesis, into artemisinin. The ADS protein has been targeted to the cytosol of the yeast cells; however, several organisms produce terpene precursors in other cellular compartments, including mitochondria and peroxisomes.

The targeting sequences for these two organelles in yeast were fused to the *ADS* gene and expressed in *S. cerevisiae* (**Chapter 6**). In order to be able to confirm that the proteins were transported to the compartments, a yeast-optimized version of the green fluorescent protein (GFP) was fused to the ADS constructs. While the fluorescence was widely spread throughout the cytosol in the strain with the untargeted ADS-GFP fusion protein, isolated spots were visible in the cells of the strains with the peroxisomal and with the mitochondrial versions of the protein. The strain that translocated the protein to the mitochondria was affected in growth, showing a longer lag phase, while no growth rate modification was registered for the strain with the peroxisomal version of the protein. The activity of ADS was slightly affected by the fusion to the GFP but amorphadiene was produced by all strains

indicating that the yeast accumulates FPP in both types of organelles. Even though the production of amorphadiene in mitochondria and peroxisomes was lower than in the cytosol, this shows that there is an untapped pool of terpenes precursors that can still be used to produce more amorphadiene in yeast.

X. dendrorhous has shown to be able to produce non-native sesquiterpenes without being affected in growth. Notably, the metabolites are produced in different concentrations, revealing that the accumulation levels were not depending on the concentration of the precursor FPP, but, instead, on the activity of the proteins. This observation is confirmed by the similar amounts of pentalenene or α-cuprenene accumulated in the strains knocked-out in the carotenoid pathway and in the mutants still producing astaxanthin. The yeast mutants can support production, at the same time, of astaxanthin and additional sesquiterpenes, showing that the yeast holds the potential for the accumulation of even higher quantities of terpene metabolites. We propose that an increased number of gene copies in the mutants could improve the sesquiterpene levels and provide a valid alternative to the strains used in industry for the production of terpenes.

We have expressed a limited number of genes in *X. dendrorhous* but now, with the development of our new, easy to modify and efficient vector for the transformation of the yeast, we have definitively shortened the time necessary to obtain new mutants. Furthermore, the possibility of expressing a virtually unlimited number of genes in a single strain will speed up the research to find the best terpene platform organism.

Very little is known about the molecular biology of *X. dendrorhous* and only a limited number of gene sequences are available. The sequencing of the genome of the red yeast would provide us with the knowledge to develop far more powerful tools than the ones we possess at the moment; with a higher number of known gene sequences, a reliable codon usage could be identified and heterologous genes could be codon optimized to improve their translation efficiency and activity in the cells. Stronger promoters could be used to increase the expression of genes resulting in higher yields of metabolites. Organellar targeting of terpene synthases could also be explored in *X. dendrorhous* using, as a starting point, the experiments performed for the translocation of ADS in peroxisomes or mitochondria of *S. cerevisiae* cells.

Furthermore, analysis of the metabolome of the yeast could help identifying bottlenecks in the terpene pathway that can be relieved by the overexpression of the genes coding for the responsible proteins, thus increasing the downstream production levels of the terpenes.

The path towards the identification and engineering of an organism that is capable of delivering high yields of terpene drugs is still long, and it necessitates inputs from several disciplines to progress further. However, we are convinced that we have proven that *X. dendrorhous* is a starting point for the development of a new and innovative platform organism for terpenoids that might be industrially relevant.

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NEDERLANDSE SAMENVATTING

De klasse natuurproducten die bekend staat onder de namen terpenen, terpenoïden en isoprenoïden omvat bijna 25.000 chemische structuren. Terpenen worden geproduceerd door eukariotische en prokariotische organismen, en hebben een scala aan biologische functies. Verschillende belangrijke stoffen — carotenoïden (als antioxidanten), limoneen en menthol worden gebruikt in de cosmetische en voedingsindustrie, en paclitaxel (Taxol®) en artemisinine worden gebruikt om patiënten te behandelen tegen respectievelijk kanker en malaria — hebben een terpeen basisstructuur en worden gevormd vanuit deze structuur via verdere functionalisatie door cytochromen en andere enzymen.

Terpenen worden geproduceerd via de mevalonaat (MEV) óf de methylerythritol fosfaat (MEP) route. Deze routes gebruiken verschillende stoffen als startpunt, maar vormen beide isopentenyl difosfaat (IPP) en dimethylallyl difosfaat (DMAPP). Door combinatie van deze twee bouwstenen worden dan grotere, meer complexe, structuren gevormd die als basisstructuur dienen voor alle terpenen. Planten zijn speciaal aangezien zij beide routes kunnen gebruiken. Schimmels en dieren kunnen alleen de MEV route gebruiken en prokarioten alleen de MEP route. In planten vinden de routes wel plaats in verschillende celcompartimenten.

Sommige terpeen medicijnen, zoals artemisinine en paclitaxel, worden in lage concentraties geproduceerd door in natuur-voorkomende organismen, respectievelijk de plant *Artemisina annua* en (sommige) *Taxus* spp. bomen. Vaak is de synthese van deze medicijnen door chemische synthese duur en moeilijk. Ook het winnen van deze stoffen door extractie vanuit de natuurlijke bron is af te raden, aangezien dit de soort met uitsterving kan bedreigen; zeker omdat deze organismen zonder genetische modificatie niet voldoende produceren om aan de vraag te voldoen. Genetische modificatie heeft er voor gezorgd dat de prijs van terpeen medicijnen omlaag is gegaan, en dat er genoeg van de benodigde stoffen geproduceerd kan worden. Door onderzoek zijn voor sommige van deze medicijnen delen van de biosynthese routes opgehelderd. Door deze verkregen kennis is het gelukt om enzymen uit deze routes in heterologe gastheren tot expressie te brengen, zodat in deze organismen meer van de stoffen geproduceerd kan worden dan in de niet-gemodificeerde organismen. Eén van de succesvolle voorbeelden is de productie van één van de precursors van artemisinine, amorphadieen, in een *Saccharomyces*

cerevisiae cultuur (tot 40 g/L). Genetische modificatie is erg belangrijk in dit voorbeeld, het heeft er namelijk voor gezorgd dat de genen geoptimaliseerd worden om meer "flux" van de precursors naar het beoogde product te krijgen door de "bottlenecks" aan te pakken.

Een organisme waarin het metabolisme is geoptimaliseerd voor de productie van een bepaalde stof wordt een "cell factory" genoemd, terwijl een organisme dat geoptimaliseerd is om een klasse van stoffen te maken een "platform organisme" wordt genoemd. Om een "cell factory" voor terpenen te creëren zijn door de meeste onderzoeksgroepen maar twee organismen bestudeerd, namelijk *Escherichia coli* en *S. cerevisiae*. Beide microben zijn uitvoerig onderzocht en geoptimaliseerd om een scala aan stoffen te produceren, onder meer eiwitten, medicijnen en biobrandstoffen. Deze twee organismen zijn echter niet de enige die bestudeerd kunnen worden, en de biodiversiteit bevat andere organismen die meer potentie als "platform organisme" kunnen hebben, maar waarover nog niet genoeg bekend is.

Het onderzoek beschreven in dit proefschrift heeft als doel om de potentie te bepalen van *Xanthophyllomyces dendrorhous* als terpeen "platform organisme". *X. dendrorhous* staat bekend om de productie van het carotenoïde astaxanthine, dat de karakteristieke rode kleur aan deze gist geeft. Aangezien *X. dendrorhous* carotenoïden produceert, kan deze gist worden geëxploiteerd om andere terpenen te produceren door heterologe genen, die normaal gebruikt worden in interessante biosynthese routes, in het genoom van de gist te integreren.

In dit proefschrift wordt het ontwerp van speciale vectors, om *X. dendrorhous* te kunnen transformeren, beschreven. Verder worden analysemethoden ontwikkeld om de metabolieten te kunnen identificeren, die door mutanten van deze gist geproduceerd worden. Verder wordt in het laatste hoofdstuk van dit proefschrift een studie naar de optimalisatie van de productie van amorphadieen door *S. cerevisiae* beschreven. Deze optimalisatie vindt plaats door het amorphadieen synthase gen in specifieke celdelen tot expressie te brengen, dit wordt ook wel "organellar targeting" genoemd.

X. dendrorhous: een potentieel "platform organisme" om terpenen te produceren

Aan het eind van de zeventiger jaren van de afgelopen eeuw werd *X. dendrorhous* geïsoleerd en sindsdien is de productie van astaxanthine in deze gist bestudeerd. Recent is er meer onderzoek naar het gebruik van de rode gist *X. dendrorhous* als geoptimaliseerde "cell factory" om asthaxanthine te produceren, aangezien delen van de biosynthese route van carotenoïden bekend zijn geworden, en verder omdat er tegenwoordige een grotere vraag is naar "natuurlijke" stoffen met antioxidante eigenschappen. Sommige onderzoeksgroepen hebben zich geconcentreerd op het identificeren van originele, natuurlijke stammen van deze gist die uit zichzelf meer asthaxanthine produceren. Andere groepen hebben geprobeerd om via genetische modificatie overexpressie van cruciale genen in de terpeen en carotenoïd biosynthese routes te bewerkstelligen, door nieuwe vectors en nieuwe transformatie protocollen te ontwerpen. De genetische modificatie studies hebben informatie opgeleverd over sequenties die nodig zijn om vectors te veranderen voor onze onderzoeksdoelen.

Hoofdstuk 3 belicht het terugkerende thema van het werk beschreven in dit proefschrift: door genetische modificatie kunnen mutanten van *X. dendrorhous* andere terpenen dan asthaxanthine produceren die normaal niet geproduceerd kunnen worden door deze gist. Dit wordt mogelijk gemaakt door enzymen in de asthaxanthine biosynthese route uit te schakelen (zogenaamde "knock outs"), en terpeen synthases tot expressie te brengen. Wij hebben een vector ontworpen waarin én de knock out van één van de genen van de carotenoïde biosynthese route én de expressie cassette voor het gen van interest zijn geïntegreerd. Als de plaats waar het nieuwe gen in het genoom wordt ingebracht (de zogenaamde recombinatie sequenties) zijn de GGPP synthase en phytoeen synthase genen geselecteerd. Het eerste gen dat naar *X. dendrorhous* werd overgezet is het pentaleneen synthase, dat FPP als substraat gebruikt om pentaleneen te produceren. Pentaleneen is de precursor van het antibioticum pentalenolacton. De gist variant waarin deze synthase tot expressie is gebracht produceert pentaleneen en ondervindt geen hinder in de groei door deze mutatie. Wel is deze gist wit aangezien hij geen asthaxanthine produceert. De hoogste concentratie van het sesquiterpeen pentaleneen werd bereikt

in de gist wanneer de vector geïntegreerd werd in de rDNA sequenties van het genoom, in plaats van in de twee hiervoor genoemde genen. De verkregen hoge concentratie kan allicht verklaard worden doordat de rDNA sequenties door het hele genoom verspreid zijn en de expressie cassette daardoor meerdere keren geïntegreerd kan zijn.

Nadat we hebben bewezen dat onze strategie om *X. dendrorhous* te transformeren succesvol is, besloten we om de effectiviteit van de sesquiterpeen productie in onze gist te evalueren. In **hoofdstuk 4** worden onze rode gist, *E. coli* én *S. cerevisiae* getransformeerd met het *Cop6* gen, dat geïsoleerd is uit de basidiomycetes *Coprinus cinereus* (NL: wortelende inktzwam). Alle *X. dendrorhous* varianten produceren α-cupreneen — de metaboliet geproduceerd door Cop6 — in hogere concentraties dan *E. coli* en *S. cerevisiae*. Dit bevestigt ons idee dat *X. dendrorhous* een grote potentie heeft voor de productie van terpenen. Verder is van belang dat de optimale temperatuur om onze gist te cultiveren 20 tot 24 °C is, en dat onze gist beter groeit in een minimaal medium dan *S. cerevisiae*. Deze twee feiten geven aan dat *X. dendrorhous* een goede gist kan zijn om terpenen te produceren in de industrie.

Het grootste deel van de terpeen medicijnen worden geconstrueerd via meerdere enzymatische omzettingen in uitgebreide biosynthese routes. De resultaten in **hoofdstuk 5** zijn verkregen door twee cytochromen — die worden gebruikt in de biosynthese route van lagopodine A — in combinatie met het *Cop6* gen tot expressie te brengen in *X. dendrorhous*. α-cupreneen is de precursor voor lagopodine A in *C. cinereus*. Door een aantal reacties kan deze precursor worden omgezet in het antibioticum. De genen van twee cytochromen, *COX1* en *COX2*, zijn in de fungus naast het *Cop6* gen gesitueerd. Aangezien genen die nodig zijn in een biosynthese route vaak geclusterd in het genoom te vinden zijn, zijn deze twee eiwitten waarschijnlijk betrokken bij de oxidatie van α-cupreneen om lagopodine A te vormen.

Om *Cop6*, *COX1* én *COX2* in één *X. dendrorhous* variant tot expressie te kunnen brengen, hebben we een nieuwe vector met een nieuwe expressie cassette ontworpen. Deze vector maakt het mogelijk om, in principe, een oneindig aantal kopieën van de expressie cassette — met dezelfde of alternatieve genen — tot expressie te brengen. Door deze vector hebben we de drie genen tot expressie kunnen

brengen in *X. dendrorhous*, en hebben we kunnen bevestigen dat de eiwitten die gecodeerd worden door deze genen gebruikt worden in de biosynthese van lagopodine A. We hebben tussenproducten van de lagopodine A biosynthese route kunnen identificeren uit de *X. dendrorhous* mutanten, maar lagopodine A zelf kon niet worden geïsoleerd. Wel kon een paarse kleur worden waargenomen wanneer het groeimedium basisch werd gemaakt, wat aangeeft dat het antibioticum of een gerelateerde stof die ook paars wordt onder basische condities aanwezig is. Verder is het een pluspunt dat de regeneratie van de cytochromen plaatsvindt door één of meerdere reductases die al van nature aanwezig zijn in *X. dendrorhous*. Hierdoor hoeven er geen extra genen reductases van *C. cinereus* naar onze gist overgezet te worden.

Expressie van amorphadieen synthase in cel compartimenten van *Saccharomyces cerevisiae*

Amorphadieen synthase (ADS) is het enzym dat FPP cycliseert tot amorphadieen, wat op zijn beurt de precursor voor artemisinine in *Artemisia annua* is. Dit eiwit is tot expressie gebracht in verschillende organismen, bijvoorbeeld in *E. coli*, *S. cerevisiae*, *Nicotiana tabacum* and *Aspergillus niger*. Er is veel onderzoek naar de productie van artemisinine (en amorphadieen) gedaan, aangezien de hoeveelheid waarin dit malaria medicijn wordt geproduceerd te laag is voor de vraag. Door genetische modificatie van *S. cerevisiae* is het mogelijk om amorphadieen te laten produceren door deze gist. Amorphadieen kan hierna door verdere chemische synthese in artemisinine worden omgezet. Het ADS eiwit is in deze gist variant aanwezig in het cytosol van de cel. In enkele andere organismen zijn de terpeen precursors echter in andere cel compartimenten aanwezig, zoals in de mitochondria en de peroxisomen.

In **hoofdstuk 6** wordt vergeleken hoe ADS functioneert voor de productie van amorphadieen wanneer dit eiwit aanwezig is in óf het cytosol van *S. cerevisiae*, óf in de mitochondria óf in de peroxisomen. Hiervoor wordt gebruik gemaakt van speciale “targeting” sequenties, die er voor zorgen dat het ADS enzym nadat het tot expressie gebracht is, naar specifieke organellen wordt getransporteerd. Om te kunnen bevestigen dat de eiwitten daadwerkelijk in de specifieke compartimenten

terecht komen, werd een fusie gemaakt van het ADS eiwit en een voor gist geoptimaliseerde versie van het groen fluorisceerend eiwit (EN: green fluorescent protein [GFP]). Fluorescentie werd door de hele cel waargenomen wanneer het niet-specifieke ADS-GPF construct werd gebruikt, terwijl fluorescentie specifiek in de bewuste organellen werd waargenomen wanneer de peroxisomale of mitochrondiale "targeting" sequenties werden gebruikt. De gistsstam, waarin het eiwit naar de mitochondria getransporteerd wordt, vertoonde een veranderde groei — namelijk een langere aanlooptijd voordat de groei begon (de zogenaamde "lag phase") — ten opzichte van de normale groei die vertoond wordt door de gist varianten met ADS in het cytosol of de peroxisomen. De activiteit van ADS was iets lager door de fusie met GFP. Amorphadieen wordt geproduceerd in alle gist varianten. Dit is een indicatie dat de gist FPP kan verzamelen in beide organellen. Ondanks dat de productie van amorphadieen in de mitochondria en peroxisomen lager is dan in het cytosol, geeft dit onderzoek aan dat er er meer amorphadieen in de gist cellen geproduceerd kan worden, aangezien er nog ongebruikte voorraden van de terpeen precursors beschikbaar zijn.

Uit ons onderzoek blijkt dat door *X. dendrorhous* sesquiterpenen, die normaal niet gemaakt worden door deze gist, geproduceerd kunnen worden, zonder de groei van de gist te beïnvloeden. Het is duidelijk geworden dat, aangezien de metabolieten in verschillende hoeveelheden geproduceerd worden, de vorming van metabolieten niet afhankelijk is van de concentratie van de precursor FPP, maar dat de productie van metabolieten gelimiteerd wordt door de activiteit van de betrokken enzymen. Deze waarneming wordt bevestigd door de productie van dezelfde hoeveelheden pentaleneen en α-cupreneen door enerzijds mutanten die nog astaxanthine produceren, en anderzijds "knock-out" mutanten die geen asthaxanthine meer kunnen vormen. De productie van tegelijkertijd asthaxanthine en andere sesquiterpenen geeft verder aan dat de gist een mogelijkheid heeft om nog grotere hoeveelheden terpeen metabolieten te produceren. Wij willen poneren dat de productie van sesquiterpenen in mutanten van *X. dendrorhous* bevorderd kan worden door meerdere kopieën van de betrokken genen te integreren in het genoom.

Tijdens dit onderzoek hebben wij maar een beperkt aantal genen in *X. dendrorhous* tot expressie gebracht, maar met de ontwikkeling van onze nieuwe,

makkelijk te modificeren en efficiënte vector voor de transformatie van de gist hebben we de tijd die nodig is om nieuwe mutanten te creëren drastisch verkort. Verder versnelt de mogelijkheid om een, in principe, oneindig aantal genen in één gist variant tot expressie te brengen de zoektocht naar het optimale terpeen-producerend "platform organisme".

Er is weinig bekend over de moleculaire biologie van *X. dendrorhous*, en er is maar een klein aantal gen sequenties van deze gist geïdentificeerd. Wanneer het genoom van de rode gist bekend zou worden door DNA sequencing, dan zouden wij met deze kennis betere hulpmiddelen kunnen ontwikkelen voor ons onderzoek; wanneer er meer gen sequenties bekend zijn kunnen wij namelijk betrouwbaar het codon gebuik vaststellen, en hierdoor kan dan het codon gebruik voor heterologe genen geoptimaliseerd worden om een hogere translatie efficiëntie en activiteit in de cellen te bereiken. Verder zouden sterkere promotoren gebruikt kunnen worden om een betere expressie van de genen te krijgen, zodat de metabolieten met een betere opbrengst kunnen worden verkregen. Ook kan onderzocht worden of het mogelijk is om eiwitten selectief in verschillende organellen van cellen van *X. dendrorhous* terpenen te laten produceren door gebruik te maken van "targeting", zoals we hebben laten zien voor ADS in *S. cerevisiae*.

Verder kan door de bestudering van de metabolomen van *X. dendrorhous* en onze mutanten, de "bottlenecks" in de terpeen biosynthese route in de rode gist geïdentificeerd worden. Deze "bottlenecks" kunnen dan verholpen worden door overexpressie van die genen die coderen voor de specifieke eiwitten, wat zou leiden tot meer terpeen productie.

Voor de identificatie en engineering van een organisme dat de mogelijkheid heeft om hoge hoeveelheden terpeen medicijnen te produceren is nog veel onderzoek nodig, en voor de voortgang van dit onderzoek zal de input nodig zijn van verschillende wetenschappelijke disciplines. Wij zijn ervan overtuigd dat we hebben bewezen dat *X. dendrorhous* een goed uitgangspunt is voor de ontwikkeling van nieuwe, innovatieve "platform organismen" voor de productie van terpenoïden, met mogelijke industriële applicatie.

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