Metabolomics and bioanalysis of terpenoid derived secondary metabolites

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Chapter 9

English Summary
Analysis of secondary metabolites

The use of natural sources to influence our health has already been well described for a very long time. The best-known examples of this herbal-based medicinal practice originate from Eastern Asia and are known as traditional Chinese medicine (TCM) and Indian Ayurvedic medicine. Despite all the positive aspects that have been attributed to the herbal medicinal practice, difficulties arise in the interpretation of this kind of medication, as many factors influence the profiles of metabolites biosynthesized by medicinal plants. Different environmental conditions like soil, fertilizers, climate, pest control and insects affect plant cultivation, thus, creating a broad and fluctuating diversity in biochemical composition. These fluctuations together with the limitations of common analytical separation techniques, like liquid and gas chromatography, challenge the quality of natural medicine. The rise of the -omics era and the introduction of improved statistical techniques have opened new doors in the metabolomics field (as described in Chapter 2). Nowadays, we have the possibility to quantify larger amounts of metabolites at once, even without the need for separation of the chemical components. Especially now that the broad availability of analytical equipment, such as mass spectrometry (MS) and nuclear magnetic resonance (NMR), together with powerful statistical procedures allow discriminating medicinal plants and supplements from natural sources. It is for example possible to distinguish and classify natural sources with the same genetic background but various cultivation locations. This can be helpful to ensure the quality of the products, but also to select the right cultivation conditions to optimize the amount of active ingredients. Moreover, metabolomics can guide the identification of bioactive compounds and can help in the separation of varieties with similar phenotypes, but of different chemotypes (Chapter 4).

Metabolomics have been a breakthrough to accelerate and streamline the analytical processes of medicinal plant researches by allowing quick and efficient identification and quantification of the metabolites within the samples.

A focus on cannabinoids

Cannabinoids are a class of secondary metabolites derived from the plant Cannabis sativa L. This plant has a rich history of human use ranging from the use of its fibers for paper or clothes production to its recreational use because of its sedative effects. After being banned for almost half a century, the plant has gained again interest due to its medicinal activities. Chapter 3 reviews the use, medicinal activities and the biosynthesis of cannabinoids.
Chapter 4 investigates the gene transcription and accumulation of cannabinoids during standardized indoor cultivation using genetic analysis, HPLC-UV and $^1$H-NMR based metabolome analysis. The bio-analytical methods have been studied and adopted to discriminate between chemotype varieties over the growth cycle. Moreover, it was shown that differences in content and pattern of THCA/CBDA and other minor cannabinoids were significant in the early and late phases of the growing processes, subsequently presenting important markers to follow up during the biosynthesis in standardized cultivations. These differences were also evident for mRNA transcripts of genes encoding for $\Delta^2$-THCA and CBDA synthases. Moreover, $^1$H-NMR showed a fast, sensitive and non-destructive method for chemotype differentiation during the complete cultivation period. Although both HPLC and $^1$H-NMR were not able to classify the samples according to the week of cultivation, a clear separation was observed between both of the chemotype and the cultivation phase (vegetative growth vs. blooming phase). Therefore, Chapter 4 presents promising methodologies to discriminate in detail the various chemotype varieties and growth phases. Furthermore, evidence is provided for variations in the transcriptional regulation among the analyzed chemotypes with different genotype backgrounds.

Chapter 5 focuses on the specific location of cannabinoid production, which has been reported to be in the glandular trichoma of leaves and flowers. Trichoma are categorized into glandular and non-glandular trichomes, although in \textit{C. sativa} only glandular trichomes can be found.

By investigating various plant parts using scanning electron microscopy, three glandular trichoma could be discriminated: the capitate-stalked trichomes, capitate-sessile trichomes and bulbous glands. The capitate-stalked trichomes were only found on the flowers during the blooming phase, with the highest densities on the bracts. Regarding the capitate-sessile trichomes, two morphologically distinct forms were found, namely big and small capitate-sessile trichomes. The big capitate-sessile trichomes were present solely on the flower parts, while the small ones were found on all plant parts investigated, just as the bulbous glands. Similar to the capitate-sessile trichomes, the bulbous glands were found in high density on the stems. In \textit{C. sativa}, the flower parts are known for their cannabinoid content, therefore, the capitate-stalked and big capitate-sessile trichomes were analyzed for their cannabinoid content after laser-guided micro-dissection. Only the complete organelle was studied for the big capitate-sessile trichomes and proven to be rather low in cannabinoid abundance, especially when compared to the capitate-stalked trichomes. Moreover, the capitate-sessile trichomes predominantly contained Tetrahydrocannabinol, while the capitate-stalked trichoma
contained all tested cannabinoids. Interestingly, no obvious differences in the cannabinoid content were found between the isolated heads and stems of the capitate-stalked trichomes. This study could not provide indication on why the cannabinoid content did not differ between these two parts. It might suggest cross localization of cannabinoids after production, as the responsible enzymes were only found within the heads of these trichomes [147].

**First steps towards recombinant cannabinoid production**

The prenylation step in *C. sativa* is the first enzymatic step of the cannabinoid biosynthesis. This step combines two primary pathways, namely the terpenoid and the polyketide pathways, to form the major cannabinoid precursor cannabigerolic acid. The enzyme responsible for this step is the *C. sativa* prenylase, which has been reported in literature [105]. In our studies, its activity was confirmed in extracts of young *C. sativa* flowers. It was however, impossible to purify the enzyme and elucidate its amino acid sequence. Therefore, a different approach was investigated for the recombinant production of cannabinoids. As a starting point, a prenylase (DnhB) from *Streptomyces coelicolor* (obtained from Stephan Richards; Salk Institute) was chosen, as it has reported promiscuous activity towards various polyketide and flavonoid substrates and proven to act on the polyketide olivetol. Although the kinetic parameters ($k_{cat}$ and $k_{cat}/K_m$ values 0.027 s$^{-1}$ and 0.052 M$^{-1}$ s$^{-1}$, respectively) did not directly favor this enzyme for recombinant production of cannabinoids, it was the only characterized enzyme reported in literature to be both soluble within the cytosol and active on olivetol when using geranyl diphosphate as prenyl substrate. More important, the property of being soluble within the cytosol is highly beneficial for metabolic engineering as geranyl diphosphate is produced within the cytosol of yeasts [131]. Moreover, structural investigations (via crystallization) delivered multiple 3D structures of NphB with and without the presence of different substrates [109]. Unfortunately, the crystal structure did not provide any information on the interaction of the substrate with the active site residues, most likely due to conformational changes induced by the prenyl-based inhibitor used. Interestingly, recent reports [240, 252, 253] on the prenylase UbiA from *E. coli* provided a hypothesis where the methionine at position 162 could provide prenyl cation stabilization before being attracted by the aromatic electron donor. This was in contrast to a report identifying aromatic amino residues [278] being responsible for carbocation positioning and stabilization prior product formation. By using site-directed mutagenesis, the hypothesis was tested empirically. In accordance with *in silico* simulations, these mutants did support the methionine hypothesis and for UbiA, an
increased activity was observed when exchanging the Trp-152 residue for a methionine. For DnhB, the exchange of methionine at position 162 to alanine resulted in an inactive enzyme, insensitive to increasing concentrations of the aromatic or the prenyl substrate.

Based on these observations the active site could be rearranged (Chapter 7) and residues important for substrate specificity, stabilization and product formation were identified resulting in new hypothesis with an alternative catalytic reaction mechanism. After structural rearrangements and based on this newly formed hypothesis, it became possible to study the in silico docking of different substrates, namely 1,6 dihydroxy naphthalene, naringenin, olivetol and olivetolic acid. Fourteen amino acid residues were identified putatively interacting with any of the selected substrates. Therefore, these positions were targeted by site-directed mutagenesis, exchanging the respective residue to alanine, a residue not expected to play any role in catalysis. Via this alanine scanning approach, four mutants were found to be completely inactive. Three of these residues (Asp-110, Tyr-121 and Tyr-175) have been previously (Chapter 6) suggested to be important for substrate recognition and positioning, while the fourth one (Ser-64) has previously been described to interact with the magnesium ion important for prenyl phosphate capture and dephosphorylation. In addition, three mutants were identified to be active when using olivetol as substrate. These mutants (Ser177Ala, Gln295Ala and Gln161Ala) displayed increased CBG production of 1.5-, 4.6- and 12.7-fold, respectively. Out of these three mutants, the variant Gln161Ala was especially interesting as it showed increased activity for all tested substrates and even slight activity towards olivetolic acid. The variants Ser177Ala, Gln295Ala and Gln161Ala were selected for analysis of initial velocities using increasing substrate amounts. Using the progressive curves, the improved activities found for the mutants Ser177Ala, Gln295Ala and Gln161Ala could be confirmed. From these mutants, Gln161Ala proved to be the most promising for further improvement of activity towards both olivetol and olivetolic acid. However, further studies are needed to establish conclusive kinetic parameters.

Both chapters (6 and 7) provide new insight in enzymatic prenylase activity and offer a platform for better improvements towards both the prenylation of olivetol and olivetolic acid. Therefore, the first steps towards recombinant cannabinoid production have been set by these investigations.
**Xanthophyllomyces dendrorhous** as host for recombinant terpenoid production

*X. dendrorhous* has been the object 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 carotenoid production. In spite of the presence of tools for the transformation of this yeast, up till now no studies have shown the expression of heterologous genes in *X. dendrorhous*, except for those conferring resistance towards geneticin and hygromycin [214, 303].

In **Chapter 8** we report for the first time the production of a heterologous terpene cyclase in *X. dendrorhous*. Two new vectors for the functional expression of the heterologous pentalenene synthase (pss) have been designed and engineered together with the knock-out of *crtE* or *crtYB*, two genes essential for carotenoid biosynthesis. The mutants obtained after antibiotic selection changed in phenotype due to inactivation of *crtE* or *crtYB* after insertion of the designed expression cassette. They switched from the wild-type orange pigmentation to white, which mainly indicates a lack of astaxanthin. The absence of carotenoids did not influence the growth of the mutant strains. Analysis of pentalenene production in the mutant strains led to yields of 0.25 to 0.68 mg/L, with the highest production found for the ribosomal DNA integrated pss strain (*pPR*-PSS). The amounts of produced pentalenene are still lower than those observed for the carotenoid produced by the wild type. One explanation is a low gene copy number, as preliminary results in our lab show that production of pentalenene is correlated to the level of protein expression. Moreover, a selection experiment was performed using higher concentrations of geneticin selecting for improved *pPR*-PSS mutants with a high gene copy number. Thereby a mutant was obtained, with pentalenene yields of 4.8 mg/L.

The results obtained in this study provide support for the use of *X. dendrorhous* as potential platform organism for recombinant metabolite production. The maximum amount of pentalenene obtained in *X. dendrorhous* was still about 20 times less than observed for wild-type strain, however, future research can help to improve the potential of this yeast for genetic engineering.