MINIREVIEW

Designing microorganisms for heterologous biosynthesis of cannabinoids

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One sentence summary: In this review, the authors explore the use of synthetic biology as an alternative approach for the synthesis of pharmaceutical cannabinoids in a heterologous host organism.

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ABSTRACT

During the last decade, the use of medical Cannabis has expanded globally and legislation is getting more liberal in many countries, facilitating the research on cannabinoids. The unique interaction of cannabinoids with the human endocannabinoid system makes these compounds an interesting target to be studied as therapeutic agents for the treatment of several medical conditions. However, currently there are important limitations in the study, production and use of cannabinoids as pharmaceutical drugs. Besides the main constituent tetrahydrocannabinolic acid, the structurally related compound cannabidiol is of high interest as drug candidate. From the more than 100 known cannabinoids reported, most can only be extracted in very low amounts and their pharmacological profile has not been determined. Today, cannabinoids are isolated from the strictly regulated Cannabis plant, and the supply of compounds with sufficient quality is a major problem. Biotechnological production could be an attractive alternative mode of production. Herein, we explore the potential use of synthetic biology as an alternative strategy for synthesis of cannabinoids in heterologous hosts. We summarize the current knowledge surrounding cannabinoids biosynthesis and present a comprehensive description of the key steps of the genuine and artificial pathway, systems biotechnology needs and platform optimization.

Keywords: Cannabis sativa; cannabinoids; synthetic biology; biotechnology; Saccharomyces cerevisiae

INTRODUCTION

Cannabinoids enclose a group of more than 100 chemical compounds (Ahmed et al. 2008; ElSohly and Slade 2005; Radwan et al. 2008) mainly found in the plant Cannabis sativa L., native from Central Asia (de Barge 1860; de Candolle 1886). Cannabis belongs to the family Cannabaceae that comprise only 11 genera, including Cannabis, Humulus (hop) and Celtis (hackberries). C. sativa is an annual plant with a dioecious breeding system (i.e. male and female flowers are found on separate plants), although monoecious plant also exist as result of special breeding (Clarke 1981; Raman 1998). The tendency in literature is to use the designation C. sativa L. to all species or varieties of the genera Cannabis. Despite this fact the number of species is not consensual, with some authors proposing a monotypic genus while others argue the existence of four (sativa, indica, ruderalis and afghanica) or even seven (ruderalis, sativa ssp. sativa, sativa ssp. spontanea, indica ssp. kafirstanica, indica ssp. indica, indica ssp. afghanica and indica ssp. chinensis) species of Cannabis. This differentiation mainly reflects geographical differences, distinct

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Cannabis has been used by humans for different purposes for more than 5000 years. The fiber-type of Cannabis (hemp), where the major cannabinoid is cannabidiol (CBD), has been used as a source of fiber for textile production and oil seed. On the other hand, the drug-type of Cannabis (marijuana) have a higher content of the psychoactive compound Δ^2^-tetrahydrocannabinol (Δ^2^-THC) and for that reason has been used as a recreational drug but also in the treatment of pain and other medical conditions (for review see Russo 2011). Δ^9^-THC is widely used in pharmaceutical formulations for the treatment of several medical conditions. Several synthetic Cannabis-based preparations such as dronabinol (Marinol®, Unimed Pharmaceuticals, Inc, Marietta, GA, USA), nabilone (Cesamet®, Valeant Pharmaceuticals North America, Aliso Viejo, CA, USA), Δ^2^-THC and CBD (Sativex®, GW Pharmaceuticals plc, Histon Cambridge, United Kingdom) have been used in the USA, Canada and other countries as an authorized treatment for nausea and vomiting in cancer chemotherapy, appetite loss in acquired immune deficiency syndrome and symptomatic relief of neuropathic pain in multiple sclerosis. The number of applications for Δ^2^-THC, CBD and other cannabinoids is still increasing with more pharmaceutical applications being investigated (Carlò 2004; Pertwee 2009).

Cannabinoids are terpenophenic compounds, produced from fatty acids and isoprenoids precursors as part of the secondary metabolism of Cannabis. The main cannabinoids produced by Cannabis are Δ^2^-tetrahydrocannabinol (THC), CBD and cannabiol (CBN), followed by cannabinol (CBG), cannabichromene (CBC) and other minor constituents (Flores-Sanchez and Verpoorte 2008). The biosynthesis of cannabinoids takes place mainly in the secretory head cells of the glandular trichomes, especially in the capitate-stalked glandular hairs (Happany et al. 2013). In the plant, Δ^2^-THC and CBD are involved in the defense response against pathogens (McPartland 1984), CBG and CBD are mildly antifungal (Elsohly et al. 2017) and Δ^9^-THC is also involved in UV light protection (Russo 2011).

Phytocannabinoids are plant-based cannabinoids. Besides the genus Cannabis, there are other examples of plants that produce cannabinoid-like compounds. Helichrysum umbraclerigerum, a flowering plant from Southern Africa, produces cannabinol and cannabigerolic acid (CBGA) in its aerial parts (Bohlmann and Hoffmann 1979) and the moss Radula marginata produce perrotetinic acid (Toyoda et al. 2002). If not especially mentioned as C. sativa derived cannabinoids, structurally related compounds with a terpenephilic skeleton naturally synthesized by plants sources are also called phytocannabinoids. For a simplified designation, the term ‘cannabinoid’ will be used to refer to phytocannabinoids in this review.

Endocannabinoids are endogenous metabolites found in the members of the phylum Chordata, which bind to specific receptors of the endocannabinoid system (ECS). Recently, endocannabinoids were also discovered in algae, bryophytes and monilophytes (Gachet et al. 2017). The main endocannabinoids are molecules derived from arachidonic acid, anandamide and 2-arachidonoylglycerol (Devane et al. 1992; Mechoulam et al. 1995; Sugiura et al. 1995). These substances have a local effect and short life before being degraded by two well-characterized enzymes, the fatty acid amide hydrolase and monoacylglycerol lipase (Cravatt et al. 1996; Dinh et al. 2002). It is worth to note that synthetic compounds with no direct structural relation to plant cannabinoids have been designed in the past. These synthetic cannabinoids are chemicals developed to interact with the receptors of the ECS and are mostly known as illicit substances.

The ECS is a ubiquitous lipid signaling system with important homeostatic and physiological functions that include modulation of pain and inflammation. The name is derived from cannabinoids, because its first studies with cannabinoids led to the discovery and elucidation of the ECS and its biological functions. The isolation of Δ^9^-THC from Cannabis (Gaoni and Mechoulam 1964; Mechoulam 1970; Mechoulam and Gaoni 1965) led to the discovery and characterization of the specific mechanism of action of cannabinoids, by the identification of specific binding sites in the brain (Devane et al. 1988; Herkenham et al. 1991). This allowed the molecular cloning (Matsuda et al. 1990) of cannabinoid CB1 receptor (CB1) as main cellular target. Later, a second peripheral receptor CB2 was identified (Munro, Thomas and Abu-Shaar 1993). Endocannabinoids are natural metabolites that stimulate this receptor type. Δ^9^-THC is a potent activator of the CB1 receptor, while the non-psychoactive CBD is a very low-affinity CB1 ligand. Despite this fact, CBD modulate the effect of Δ9-THC via direct blockade of CB1 receptor (McPartland et al. 2015). This modulation attenuates some of the side effects of Δ^9^-THC such as anxiety, dysphoria, panic reactions and paranoia, and is also known to improve the Δ^9^-THC therapeutic activity (Izzo et al. 2009; Russo 2011).

The global legislation for the use of medical Cannabis changes rapidly, and currently Cannabis is legal as therapeutic agent in 23 states of the United States as well as in the Netherlands, Germany, Czech Republic, Canada and Israel. There are still several drawbacks in the production of medical THC and other cannabinoids, especially related to the legal regulations for the cultivation of Cannabis in most countries. Furthermore, chemical synthesis of cannabinoids has failed to be a cost-effective alternative mainly because of complex synthesis leading to high production cost and low yields. A new alternative is the use of a biotechnology-based synthetic biology approach for a cost-effective, environmentally friendly, high-quality and reliable source of cannabinoids.

Agricultural production of cannabinoids faces several challenges such as plant susceptibility to climate and diseases, no GAP standardization, low content of less-abundant cannabinoids, need for extraction of cannabinoids by chemical processing and legal and social factors related to the potential for illicit use of the plant. Currently, Δ^2^-THC and CBD used as therapeutic agents are either extracted from the plant or chemically synthesized. Biosynthesis of cannabinoids by engineered microbial strains could be an alternative strategy for the production of cannabinoids.

The identification of the enzymes involved in the cannabinoids biosynthetic pathway enables the reconstruction of the pathway using a suitable heterologous host system. A synthetic biology approach can be especially interesting for the production of less-abundant cannabinoids, but also work as a platform for the discovery and testing of unknown enzymes responsible for the biosynthesis of various rare cannabinoids and derivatives thereof. Microbial production can provide a competitive and efficient way for easy and high yield biosynthesis of rare cannabinoids. Some examples of such compounds are tetracydrocannabinvarin, cannabigerol and cannabichromene that are cannabinoids with therapeutic interest, which are difficult to obtain (Appendino et al. 2008; Bolognini et al. 2010; Davis and Hatoum 1983; Elsohly et al. 2017; Hill et al. 2010; Ligresti 2006; Wilkinson et al. 2007).

Recently, important developments in the microbial biosynthesis of cannabinoids were achieved. The expression of THCA
Table 1. List of the enzymes involved for the biosynthesis of cannabinoids in *C. sativa* L.

| Enzyme                                              | Abbreviations | Accession no. | EC no.   | References                  |
|-----------------------------------------------------|---------------|---------------|----------|-----------------------------|
| Acyl activating enzyme 1                            | AAE1          | AFD33345.1    | 6.2.1.1  | (Stout et al. 2012)          |
| Olivetol synthase                                   | OLS           | AB164375      | 2.3.1.206| (Taura et al. 2009)          |
| Olivetolic acid cyclase                              | OAC           | AFN42527.1    | 4.4.1.26 | (Gagne et al. 2012)          |
| Cannabigerolic acid synthase                         | CBGAS         | US8884100B2   | 2.5.1.102| (Fellermeier and Zenk 1998)  |
| Tetrahydrocannabinolic acid synthase                 | THCAS         | AB057805      | 1.21.3.8 | (Taura et al. 2007b)         |
| Cannabidiolic acid synthase                          | CBDAS         | AB292682      | 1.21.3.8 | (Page and Stout 2015)        |
| Cannabichromenic acid synthase                       | CBCAS         | WO 2015/196275| 1.3.3    | (Page and Stout 2015)        |

+Genbank  
+Patent number  
+application number

synthase in the yeast *Komagataella phaffii* allowed the bioconversion of CBGA to Δ⁹-tetrahydrocannabinolic acid (Δ⁹-THCA) (Zirpel, Stehle and Kayser 2015). In addition, a patent application was filed, referring the microbial biosynthesis of cannabinoids in genetically engineered microorganisms (Poulos and Farnia 2016). In this review, we will explore synthetic biology as an alternative approach for the biosynthesis of cannabinoids in heterologous systems. We present microbial biosynthesis as a novel biotechnological solution for the production of pharmaceutical cannabinoids. The review will start with a brief overview of Cannabis phytochemistry focusing on the biosynthesis of cannabinoids. This will be followed by a comprehensive description of the key steps of the pathway, precursor needs and the desired features of the chassis organism.

**BIOSYNTHESIS IN *CANNABIS SATIVA***

The biosynthesis of cannabinoids starts with the short-chain fatty acid, hexanoic acid. Initially, the fatty acid is converted to its coenzyme A (CoA) form by the activity of an acyl activating enzyme (Stout et al. 2012). Subsequently, olivetolic acid (OA) is biosynthesized by the action of a type III polyketide synthase (PKS) and a polyketide cyclase (olivetolic acid cyclase [OAC]). The PKS olivetin synthase (OLS) converts one molecule of hexanoyl-CoA and three molecules of malonyl-CoA to olivetol followed by the C2-C7 aldol cyclization to OA by the OAC (Gagne et al. 2009; Yang et al. 2016). The OAC catalyzes the C2-C7 intramolecular aldol cyclization to OA by preserving the carboxylate moiety (Gagne et al. 2012; Raharjo et al. 2004).

The terpenoid part of the cannabinoid can be derived from two different precursor pathways, namely the mevalonate pathway (MVA) localized in the cytosol and the plastid localized non-MVA, also termed as 2-C-methyl-d-erythritol 4-phosphate or 1-deoxy-d-xylulose 5-phosphate (MEP/DOXP) pathway. In higher plants, the MVA pathway is mainly involved with the plant primary metabolism, whereas MEP pathway is the main contributor for secondary metabolism, including terpene production (in detail elsewhere, e.g. Eisenreich et al. 2004; Hunter 2007). The MVA and MEP pathways are both responsible for the production of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the two early precursors of terpenes. The condensation of IPP and DMAPP to produce GPP is catalyzed by the enzyme geranyl diphosphate synthase (Burke, Wildung and Zenk 1999). According to the information currently available, the most likely source of GPP for the production of the monoterpenoid moieties of cannabinoids in the plant is the MEP/DOXP pathway.


Cannabinoids biosynthesis

The central precursor for cannabinoid biosynthesis, CBGA, is synthesized by the aromatic prenyltransferase CBGAS (Fellmer and Zenk 1998; Page and Boubakir 2012) by the condensation of GPP and OA. CBGAS is mainly expressed in glandular trichomes of female flowers and young leaves of Cannabis, and the coding sequence could be functionally expressed in Saccharomyces cerevisiae cells, verifying the CBGAS activity in the microsomal fractions (Page and Boubakir 2012). Subsequently, the monoterpenoic moiety of CBGAS is stereoselectively cyclized by the three different enzymes cannabinochromenic acid synthase (Page and Stout 2015), cannabidiolic acid synthase (CBDAS) and tetrahydrocannabinolic acid synthase (THCAS). The CBDAS and THCAS belong to the berberine bridge enzyme family, oxidoreductases with a covalently bound flavin adenine dinucleotide (FAD) (Kutchan and Dittrich 1995). Both enzymes use molecular oxygen for the regeneration of the FAD cofactor, releasing equimolar amounts of hydrogen peroxide and a particular product (Sirikantaramas et al. 2004; Taura et al. 2007a). The THCAS is well characterized, and a crystal structure is available (Shoyama et al. 2012). The enzyme possesses a signal peptide for the secretory pathway, one disulfide bond and eight possible Asn glycosylation sites. Additionally, the enzyme was already functionally expressed in Spodoptera frugiperda (SF9) insect cells (Sirikantaramas et al. 2004), K. phaffii (Taura et al. 2007b; Zirpel, Stehle and Kayser 2015) and S. cerevisiae (Zirpel, Stehle and Kayser 2015).

HETEROLOGOUS SYSTEMS FOR THE BIOSYNTHESIS OF CANNABINOIDS

Special requirements of the host organism

Choosing a suitable host organism for production of a heterologous metabolite needs some general considerations, regarding the tools available for the expression of heterologous proteins (strains, vectors, promoters and signal peptides), genetic information and availability of classical genetic approaches as well as the accessibility of modern molecular biological tools. Other specific requirements would relate more to the specific pathway that needs to be introduced. Such factors could be the suitability of the host organism for production of particular precursors or cofactors as well as suitability of the host to express the necessary types of pathway enzymes. In Table 2, we compare a selection of potential microbial production hosts and evaluate their suitability within different categories. In the case of the cannabinoid biosynthesis, both precursors GPP and hexanoic acid should be provided in sufficient amounts by the chassis organism. The isoprenoid production has already been extensively studied and optimized for the production of artemisinin in Escherichia coli and S. cerevisiae, favoring the yeast as better platform organism (Paddon and Keasling 2014). Nevertheless, early stage optimizations of isoprenoid production are also documented for the oleanagin yeast Yarrowia lipolytica (Sharpe, Ye and Zhu 2014) and the methylotrophic yeast K. phaffii (Liu et al. 2014).

A clear challenge in heterologous production of cannabinoids is the production of hexanoic acid. The most efficient microbial hexanoic acid formation reported to date is by Cheon et al. (2014), who described the production of up to 142 mg L−1 of hexanoic acid in Kluyveromyces marxianus using a pathway that may be transferrable to other yeasts.

Looking at the characteristics of the enzymes of the late cannabinoid pathway, a prokaryotic host seems not to be feasible. CBGAS is an integral membrane protein, making high titer of functional expressed protein in E. coli rather unlikely. In addition, the FAD-dependent oxygenases THCAS and CBDAS possess a disulfide bond and several N-glycosylation sites excluding the use of prokaryotic hosts (Shoyama et al. 2012; Zirpel, Stehle and Kayser 2015).

All precursors needed for the cannabinoid biosynthesis, GPP, malonyl-CoA and hexanoyl-CoA, are derived from acetyl-CoA. Therefore, a strategy to boost the acetyl-CoA pool is essential to reach high yields. Recently, S. cerevisiae was used to rewire the central carbon metabolism (Meadows et al. 2016). By the addition of four genes only, the engineered yeast is able to produce high amounts of cytosolic acetyl-CoA accompanied with an improved pathway redox balance, a reduced ATP requirement and CO2 loss. Since S. cerevisiae also is one of the most developed host organisms in respect to knowledge and molecular biology tools, this organism therefore is a promising chassis organism for the heterologous biosynthesis of cannabinoids. In the following, we will therefore focus on the establishment of S. cerevisiae as a platform organism for the heterologous biosynthesis of cannabinoids. A serious alternative, however, would be K. phaffii, which is well known for secreted heterologous synthesized enzymes. Zirpel, Stehle and Kayser (2015) showed that this yeast is also able to produce high levels of intracellular accumulated THCAS.

Metabolic engineering of Saccharomyces cerevisiae to produce cannabinoids

In order to rewire the Saccharomyces cerevisiae metabolism for the biosynthesis of cannabinoids, a combined approach comprising the adaptation of native metabolic pathways, the assembly of heterologous metabolic pathways and protein engineering is needed to obtain a cell factory that produces cost-effective cannabinoids. Looking at the biosynthetic pathway of THCA in Cannabis sativa, the pathway can be divided into three parts: (i) GPP supply, (ii) synthesis of OA and (iii) the actual cannabinoid formation.

Table 2. Comparison of different microbial expression hosts regarding their capacity of heterologous cannabinoid biosynthesis.

| Host Organism                  | Genetic tools available | Strains, promoters, vectors | Plant protein expression capacity | Posttranslational modifications | GPP engineering | Hexanoic acid engineering | Acetyl-CoA pool engineering |
|--------------------------------|-------------------------|----------------------------|---------------------------------|---------------------------------|-----------------|--------------------------|---------------------------|
| Escherichia coli               | +++                     | +++                        | +                               | -                               | +++             | +                        | +                         |
| Saccharomyces cerevisiae       | +++                     | +++                        | +                               | +                               | +++             | +                        | +                         |
| Komagataella phaffii (Pichia pastoris) | +                 | +                          | +                               | +                               | +               | +                        | +                         |
| Kluyveromyces marxianus        | +                       | +                          | +                               | +                               | +               | +                        | +                         |
| Yarrowia lipolytica           | +                       | +                          | +                               | +                               | +               | +                        | +                         |

+++ many publications available, well established; ++, publications available, optimization potential; +, first publications available, not yet established/not working; −, not possible; ‘empty’, not yet described.
GPP supply

Sacharomycyes cerevisiae serves as a model and excellent organism for heterologous isoprenoid production (Nevoigt 2008), and several promising metabolic engineering strategies were identified by in silico profiling (Gruchatka et al. 2013). Up to date, many strategies were tested to improve the isoprenoid production in yeast, whereas most are focusing on MVA pathway engineering (Fig. 1). In most cases, a truncated version of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) is overexpressed since HMGR was identified as the rate-limiting enzyme of the MVA pathway (Oh et al. 2009). Recently, the co-overexpression of all MVA pathway genes resulted in the production of 40 g L\(^{-1}\) amorphaene, a precursor of the antimalarial agent artemisinin (Westall et al. 2012). Lv et al. (2014) optimized additionally the native acetyl-CoA pathway. The co-overexpression of pyruvate decarboxylase, alcohol dehydrogenase 3, aldehyde dehydrogenase and the both acetyl-CoA synthetases resulted in an enhanced isoprene biosynthesis and might be an option to improve isoprenoid biosynthesis further.

In contrast to plants, yeasts usually do not carry a specific GPP synthase (GPPS). In yeast, the farnesylidiphosphate synthase (FPPS; ERG20) possesses a GPPS activity, while GPP occurs exclusively as an intermediate of farnesyl diphosphate synthesis. The highest yields so far were obtained with the production of sesquiterpenes or even larger terpenes like miltiradiene (Zhou et al. 2012) or artemisinin (Paddon et al. 2013). Less effort was made on the optimization in monoterpen production and the overall yields reported are lower. However, for cannabinoid production in yeast a high production rate of GPP is necessary. Saturated mutagenesis of Lys197 residue of the ERG20 protein resulted in six strains (K197G, C, S, T, D, E) with an improved monoterpenol production but also with some growth impairment (Fischer et al. 2011). Even better results were obtained by engineering the ERG20 protein into a geranyl diphosphate synthase (ERG20-F96W-N127W = ERG20\(^{WW}\)) (Ignea et al. 2014). The introduction of a larger side chain in position 96 (F96W) blocks the part of the active site, thereby hindering the FPP synthase activity without affecting the synthesis of GPP. ERG20 is a homodimeric protein whereas the N127 residue of one subunit is part of the active site of the other subunit (Fernandez, Kellogg and Poulier 2000; Ignea et al. 2014). The replacement of the N127 residue by a tryptophan results in an abolished FPP synthesis, but the GPP activity remains. The overexpression of the N127W variant in a wild-type yeast strain leads to the formation of heterodimeric ERG20 proteins (endogenous ERG20/ERG20\(^{WW}\)) reducing thereby the overall FPP synthase activity and eliminates the need for mutation of the endogenous ERG20 gene to reduce the FPP pool (Ignea et al. 2014). Finally, the co-overexpression of IDI1, HMG1 and UPC2-1 together with the ERG20\(^{WW}\) protein resulted in a significantly improved geraniol production (Zhao et al. 2016). A similar approach could be adapted to improve the GPP supply for heterologous cannabinoid biosynthesis in S. cerevisiae.

Olivetolic acid synthesis

Sacharomycyes cerevisiae does not typically metabolize fatty acids as substrate, therefore its production relies on endogenous biosynthesis. The fatty acid composition in S. cerevisiae is rather simple, consisting mostly of C16 and C18 fatty acids (Klug and Daum 2014; Oh and Martin 2006). Trace amounts of short-chain fatty acids (SCFA) or medium-chain fatty acids (MCFA) are naturally produced by S. cerevisiae and certain strains used in wine and sake fermentation are known to produce higher levels (Aréomi et al. 2004; Patel and Shibamoto 2002). The biosynthesis of fatty acids requires several substrates and cofactors such as acetyl-CoA, ATP and NAD(P)H. The first committed step of fatty acid biosynthesis is the conversion of acetyl-CoA to malonyl-CoA. This reaction is performed by the enzyme acetyl-CoA carboxylase and its activity is highly regulated (Hablacher et al. 1993; Woods et al. 1994). De novo biosynthesis of fatty acid in yeast is carried out by the fatty acid synthase (FAS), a multifunction protein complex in which all steps of fatty acid synthesis are integrated. The yeast FAS is classified as a eukaryote type I and this enzyme complex is divided in 2 subunits (Fas1p and Fas2p), each exhibiting more than one enzymatic activity. Fas1p harbors acetyl transferase, enoyl ACP reductase, hydroxyl acyl ACP dehydratase and malonyl transferase activities; and Fas2p contains the ACP, ketoacyl reductase, ketoacyl synthase and phosphopantetheine transferase activities (Jenni et al. 2007; Klug and Daum 2014; Tehlivets, Scheuringer and Kohlwein 2007).

Up to date, several strategies were tested to produce SCFA in yeast species, usually involving the introduction of heterologous enzymes. Several examples of the production of SCFA were demonstrated by the introduction of heterologous FAS. The introduction of Homo sapiens type I fatty acid synthase and specific short-chain thioesterase in S. cerevisiae increased in vivo octanoic acid and total SCFA production (Leber and Da Silva 2014). A similar strategy could be adapted for the production of hexanoic acid by testing different heterologous short-chain thioesterases. Many fungal secondary metabolites are fatty-acid-derived molecules biosynthesized by the interaction of dedicated FAS with a PKS. One of the most notorious examples of this type of secondary metabolites is the biosynthesis of aflatoxin (AF) and sterigmatocystin (ST) in some species of the filamentous fungus Aspergillus. Previous studies have shown that the synthesis of AF and ST begins with the assembly of a C6 fatty acid from acetyl-CoA and 2 units of malonyl-CoA catalyzed by a specialized FAS, hexanoyl synthase (Hitchman 2001).

Recently, different studies demonstrated the production of extracellular SCFA and MCFA by the modification of the native FAS or construction of a synthetic FASs in S. cerevisiae. Rational engineering of the cytosolic FAS allowed the reprogramming of the chain-length control with successful biosynthesis of SCFA and MCFA (Gajewski et al. 2017). In another study, the creation of a synthetic FASs by the integration of heterologous enzymes showed the capability to biosynthesize SCFA, MCF and methyl ketones (Zhu et al. 2017).

As mentioned before, the biosynthesis of OA starts with the conversion of the free fatty acid hexanoic acid to hexanoyl-CoA. In Cannabis, this step is done by an acyl-CoA synthetase with high specificity for hexanoic acid. Sacharomycyes cerevisiae contains four fatty acyl-CoA synthetases (FAA) named FAA1, FAA2, FAA3 and FAA4. FAA1, FAA3 and FAA4 have preference for long-chain fatty acids (C12:0 to C18:0), in contrast with FAA2 that accepts a wide range of fatty acid chain lengths with a preference for medium chains (C9:0-C13:0) (Johnson et al. 1994). Since S. cerevisiae has no specific acyl-CoA synthetase for SCFA, it may be beneficial to introduce a Cannabis hexanoyl-CoA synthetase or a homolog from another organism to achieve an efficient conversion of hexanoic acid to hexanoyl-CoA in yeast.

The biosynthesis of OA from hexanoyl-CoA is performed by the combination of two cytosolic-located enzymes, OLS and OAC (Fig. 2). These two enzymes have been demonstrated to be active in vivo in S. cerevisiae with resulting formation of both olivetol and OA (Gagne et al. 2012).
Figure 1. Isoprenoid formation in S. cerevisiae. The isoprenoid biosynthesis starts with acetyl-CoA, which is derived from the glycolytic pathway. At the end of the MVA, the both isoprenoids IPP and DMAPP are formed. Subsequently, GPP and FPP are formed by the ERG20 protein. The different colors represent the different strategies applied for improved isoprenoid production. HMG-CoA—3-hydroxy-3-methylglutaryl coenzyme A, IPP—isopentenyl diphosphate, DMAPP—dimethylallyl diphosphate, GPP—geranyl diphosphate, FPP—farnesyl diphosphate, PDC—pyruvate decarboxylase, ADH1-5—alcohol dehydrogenase, ALD6—aldehyde dehydrogenase, ACS1/ACS2—acetyl-coA synthetase, ERG10—acetyl-CoA C-acetyltransferase, ERG13—3-hydroxy-3-methylglutaryl-CoA synthase, tHMGR—truncated 3-hydroxy-3-methylglutaryl-CoA reductase, ERG12—mevalonate kinase, ERG8—phosphomevalonate kinase, ERG19—mevalonate diphosphate decarboxylase, IDI1—isopentenyl diphosphate:dimethylallyl diphosphate isomerase, ERG20—farnesyl diphosphate synthetase, ERG20WW—ERG20-F96W-N127W, Δerg20—ERG20 knock out, UPC2-1—sterol regulatory element binding protein.

Lv et al. (2014) - Isopren 37 mg L⁻¹
Westfall et al. (2012) - Amorphadien 40 g L⁻¹
Zhao et al. (2016) - Geraniol 293 mg L⁻¹
Figure 2. Biosynthetic pathway of cannabinoids in *C. sativa*. Highlighted enzymes have to be transferred into a heterologous host as *S. cerevisiae* exhibiting a mevalonate pathway.
Implementation of cannabinoid forming enzymes

With GPP and OA available in the production host, only one enzymatic step is necessary to obtain CBGA, which is the central intermediate of the cannabinoid pathway (Fig. 2). The native enzyme responsible for the prenylation of OA is an integral membrane enzyme of which functional overexpression has turned out to be challenging. Nevertheless, the native enzyme has been expressed in Saccharomyces cerevisiae cells, but the overall activity is low and a high side-product formation (5-geranyl-olivetolate) was observed (Page and Boubakir 2015). Besides the integral membrane prenyltransferases in plants, soluble prenyltransferases are known from fungi and bacteria. Kuzuyama, Noel and Richard (2005) published a crystal structure of a soluble prenyltransferase NphB from Streptomyces sp. strain CL190 that is specific for GPP as prenyl donor and exhibits a broad substrate specificity towards aromatic substrates. They were able to show that the enzyme accepts olivetol and OA as prenyl acceptor. Furthermore, it was shown that NphB prenylates olivetol in the C2 and C4 position (Kumano et al. 2008). Thus, NphB represents a potential alternative to replace the native CBGAs in a biotechnological production of cannabinoids. Indeed, the co-expression of the nphB and thcA coding sequences in K. phaffii resulted in the successful synthesis of THCA in enzyme extracts containing OA and GPP (unpublished results).

Once the expression of an OA prenylating enzyme is established in yeast, the addition of one respective enzyme leads to the formation of different cannabinoids like THCA, CBDA and CBCA. For the THCAS, a whole cell bioconversion is already established in S. cerevisiae and K. phaffii (Zirpel, Stehle and Kayser 2015). Yeast cells expressing thcA were able to produce up to 360 mg l−1 THCA after CBGA feeding, demonstrating the capacity of yeast in the biosynthesis of cannabinoids. As the sequence and expression for CBADS is already described (Taura et al. 2007a), the THCAS can be easily exchanged with a CBADS. However, expression levels are lower than reported for THCAS (unpublished data).

Diversified cannabinoids

The microbial production facilitates the possibility to design new cannabinoids with novel activities or improved pharmacokinetics through the implementation of tailoring enzymes. Cannabinoids are extremely hydrophobic compounds, for medical formulations cannabinoids are usually dissolved in oil or solvents, which may not be well tolerated by users (Scully 2007). The introduction of hydroxyl, carbonyl, carboxyl or glycosyl groups enhances the solubility of molecules in general. In 1980s, fungal and bacterial strains were already used to transform THC into polar derivatives (Binder and Meisenberg 1978; Binder and Popp 1980; Fukuda, Archer and Abbott 1977), as well as plant cell suspension cultures were used for the biotransformation of cannabinoids (Akhtar, Mustafa and Verpoorte 2015; Braemer and Paris 1987; Hartsel, Loh and Robertson 1983). Nevertheless, the overall yield of the metabolites was too low for pharmacological evaluation of the activity.

In humans, THC is metabolized in the liver mainly by microsomal hydroxylation and oxidation, but also allylic oxidation, epoxidation, aliphatic oxidation, decarboxylation and conjugation reactions were reported (Grotenhermen 2003). This leads to nearly 100 different identified metabolites for THC (Harvey and Brown 1991). THC is mainly hydroxylated on position C-11 by cytochrome P450 enzymes resulting in 11-hydroxy-tetrahydrocannabinol (11-OH-THC) and further oxidation leads to 11-nor-9-carboxy-tetrahydrocannabinol, the most important non-psychotropic metabolite (Fig. 3). Since 11-OH-THC seems to be three to seven times more potent than THC in animal tests (Karler and Turkakis 1987), and 11-OH-THC possesses still inflammatory and analgesic properties (Burstein 1999), the elongation of the heterologous pathway by P450 enzymes might be a promising approach to produce THC derivatives. Glycosylation may also be an interesting strategy to alter the physicochemical properties. Plant glycosyltransferases (UGTs) are known for their relaxed substrate specificity (Bowles et al. 2006; Hansen et al. 2009). Recently, the UGT76G1 from Stevia rebaudiana was used to produce primary, secondary and tertiary glycosylations cannabinoid glycosides (Hardman, Brooke and Zipp 2017). The use of a second enzyme from Oryza sativa resulted in the transfer of a second glucose residue onto cannabinoid monoglycosides with a greatly improved water solubility.

The implementation of the cannabinoid biosynthesis in a heterologous host provides a platform where some of these tailoring enzymes can be functionally expressed. This enables the production of significant amounts of these derivatives and may be the key to unlock their pharmacological potential.

OUTLOOK

The discovery and characterization of all key enzymes involved in the biosynthesis of the main cannabinoids Δ9-THC and CBD, allows for the production of these compounds by heterologous host organisms. The use of synthetic biology for the microbial biosynthesis of cannabinoids can revolutionize the production of medical cannabinoid drugs. Besides the main cannabinoids Δ9-THC and CBD, more than 100 cannabinoids compounds are described with little knowledge available regarding their producing pathways and potential applications. Synthetic biology could be used to create a chassis organism for the study and characterization of the enzymes involved in the biosynthesis of these less-abundant cannabinoids or derivatives thereof and allows the production of these compounds by a scalable fermentation process. This can have an enormous impact on the availability of rare cannabinoids to be tested in clinical trials to evaluate their efficacy as medical drugs. Furthermore, microbial production can support the design of novel cannabinoids with enhanced properties by the incorporation of tailoring enzymes. Together, these strategies will help to support the potential value of cannabinoids as pharmaceutical drugs.

Conflict of interest. None declared.
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