Title

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Title: New frontiers: Harnessing pivotal advances in microbial engineering for the biosynthesis of plant-derived terpenoids

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Highlights:

- Terpenoids are a vast and diverse class of compounds with applications in both the industrial and human health spaces.
- Advances in computational biology and gene discovery techniques empower the discovery of novel terpenoid biosynthesis pathways for microbial engineering.
- The heterologous expression of plant-derived cytochrome P450s, prenyltransferases, and glycosyltransferases in microorganisms facilitates the production of complex terpenoid derivatives.
- Host engineering has enabled monumental increases in production titers of varying terpenoids.

Abstract:

Terpenoids are a vast and diverse class of molecules with industrial and medicinal importance. The majority of these molecules are produced across kingdom Plantae via specialized metabolism. Microorganisms, mainly *Escherichia coli* and *Saccharomyces cerevisiae,* have become choice platforms for the biosynthesis of terpenoids due to recent advances in synthetic biology and metabolic engineering. New techniques for gene discovery have expanded our search space for novel terpene synthesis pathways and unlocked unrealized potential for the microbial production of more complex derivatives. Additionally, numerous advances in host and pathway engineering have allowed for the production of terpenoids requiring oxidation and glycosylation, effectively expanding the potential target space. These advances will lay the foundation for the

microbial biosynthesis of a seemingly infinite domain of terpenoids with varying applications.

Introduction:

Over 50,000 known terpenoids are naturally produced across all kingdoms of life and constitute the largest class of natural products. Though, the majority are the result of specialized metabolism in plants [1]. Terpenoids are of major interest due to their applications in the food, pharmaceutical, and cosmetic industries as well as their potential as liquid fuels. Microbial biosynthesis is an optimal platform for the isolation and purification of individual terpenoid compounds. Additionally, this limits the need to grow, harvest and extract plant material, which generally contains low concentrations of the desired product along with complex mixtures of similar compounds and prevents the overharvesting of ecologically sensitive species. Terpenes are synthesized from C5 isoprene building blocks derived from the mevalonate (MEV) or 1-deoxy-D-xylulose 5-phosphate (DXP) pathway. These units are then condensed to produce larger and more complex molecules. The major terpene families are the hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), and tetraterpenes (C40). This space is further expanded by the oxidation, via cytochrome P450 oxygenases (CYPs), and glycosylation, via glycosyltransferases (GTs), of the base terpene skeleton/scaffold [2,3]. Furthermore, prenyltransferases add terpene units to other molecules to generate complex terpene composites, or meroterpenoids, which includes the cannabinoids [4] (Figure 1).

Within the last two years, large strides have been made in the microbial production of terpenoids from cannabis and hops, with a focus on prenylated chalcones, flavanones, bitter acids, and cannabinoids due to their distinct bioactive properties. All of these products result from the concatenation of a common isoprenoid precursor with a second fatty acid-derived precursor by a prenyltransferase. Prenyltransferases (PTs) in cannabis and hops are severely under characterized yet are responsible for the large diversity within the cannabis & hop terpenomes. A combination of gene network and phylogenetic analyses has been used to identify candidate genes for many of these molecules.

Microbial engineering is currently limited to the biosynthesis of known and well characterized terpenoid products. Computational biology, genomics, and transcriptomics are powerful tools for the identification of novel terpenoid synthesis pathways, ushering in a new era of synthetic biology [5,6]. Yeast has proven to be a desirable host for the production of these complex terpenoids due to its endogenous MEV pathway, the presence of an endoplasmic reticulum (ER) to anchor membrane-associated plant enzymes, and the plethora of genetic tools for the metabolic engineering of this chassis. Bacterial engineering for terpene production has also seen recent success, but there is limited capacity for the expression of CYPs in these hosts without extensive modification of the native enzymes. Here, we focus on recent advances in the discovery of terpenoid synthesis pathways and their heterologous expression in microorganisms, as well as the expansion of the terpenoid target space through host engineering and the utilization of plant-derived CYPs and GTs.

Advances in omics studies to identify novel terpenoid biosynthesis pathways

Detailed transcriptomics performed on extractions from glandular trichomes as well as female floral tissues of *Cannabis sativa* at varying developmental stages indicate that candidate genes involved in terpene and cannabinoid synthesis have abundant expression in trichomes [7]. A recent study acquired high quality glandular trichome transcriptomes from nine different commercial cannabis strains in an attempt to characterize terpene synthases [8]. A phylogenetic analysis of the whole genome contig database of *C. sativa* also revealed 24 o-methyltransferases and eight putative aromatic prenyltransferases, one of which was characterized as the penultimate synthase for cannaflavins A and B [9]. Additionally, a total of more than 22,000 expressed sequence tags (ESTs) from several hop trichome-specific cDNA libraries have been deposited in the TrichOME database [10]. This comprehensive transcriptome is a significant resource for further research into natural product pathway discovery and has massively advanced the potential for microbial biosynthesis of multiple terpenoids. Additionally, a recently unveiled metadata platform MaveDB aims to distribute and interpret data from multiplexed assays of variant effect, and may be a valuable tool to inform rational engineering [11].

Recent advances in microbial transgene expression systems for terpenoid production.

There have been several key advancements in the production of terpenoids from both hops and cannabis in recent years. In 2018, an industrial brewing yeast was engineered

to produce primary flavor determinants in hopped beer, allowing for a hoppy tasting beer in the absence of additional hops. Employed was a combinatorial assembly of yeast toolkit parts (promoter, terminator, linker etc.) and iterative design-build-learn-test cycles with strain selection guided by a mathematical model relating genetic design to monoterpene flux [12-14]. To be functionally useful, the engineered strain needed to retain its ability to convert sugars to ethanol, and have precise, stable expression of flavor-determining monoterpenes linalool and geraniol. This work was in contrast to most metabolic engineering efforts which are commonly enlisted to maximize product titers. Multiple state of the art engineering techniques and iterative improvement schemes were employed to tune production of multiple commercially important metabolites without major collateral metabolic changes.

For cannabinoids from *C. sativa*, an aromatic prenyltransferase catalyzes the formation of cannabigerolic acid from olivetolic acid (OA) and geranyl pyrophosphate (GPP). The pathway then branches again toward different cyclized products, such as tetrahydrocannabinolic acid (THCA), cannabidiolic acid (CBDA), and cannabichromenic acid (CBCA). A recent landmark paper describes the successful production of THCA and CBDA from sugar in yeast [15]. Unnatural cannabinoid variants with tailored alkyl chains could also be obtained via feeding the engineered strain with hexanoic acid analogs, demonstrating the substrate promiscuity of olivetolic acid pathway enzymes. Most notably, cannabinoid variants with an alkyne moiety were synthesized, paving the way for future click derivatization. It has been shown that the cannabinoid alkyl side chain is a critical pharmacophore, and may be a promising target for pharmaceutical

discovery [16]. Another study successfully reconstructed the entire β-bitter acid pathway by heterologous expression of two CoA ligases, a polyketide synthase, and a prenyltransferase complex in an optimized yeast system. A metabolon composed of two aromatic prenyltransferases was elucidated [10]. Another key tool for increasing transgene expression and function for terpenoid biosynthesis is mutagenesis analysis, particularly for prenyltransferases given the plasticity and promiscuity of their active sites [15,17].

Prenylated flavonoids are another subclass of plant phenolics, which combine a flavonoid skeleton with a prenyl side chain. Unlike other flavonoids, they have a narrow distribution in plants, limited to only several plant families, including Cannabaceae. Recent studies have demonstrated that hop terpenophenolics (a term for both bitter acids and prenylchalcones) exhibit diverse bio-activities with a high potential for pharmaceutical applications [18]. A prenylated flavonoid with a very potent phytoestrogen activity is 8-prenylnaringenin, produced in *Humulus lupulus* (hops). 8-Prenylnaringenin was recently produced *de novo* as a proof of concept for yeast as a platform for biosynthesis of prenylated flavonoids [19].

Recently, the importance of non-catalytic foldases and chaperones for terpenoid production in trichomes has been elucidated [10]. THCA and CBDA are unstable and will be non-enzymatically converted to the decarboxylated forms, Δ9-tetrahydrocannabinol and cannabidiol respectively. It is hypothesized that CsaCHIL, a chalcone isomerase-like protein lacking catalytic activity, potentially binds THCA and/or

CBDA for stabilization in hemp glandular trichomes and limits negative feedback to upstream enzymes. It has also been shown that upregulation of multiple foldases and chaperones resulted in a 20-fold improvement of THCA synthase functionality in yeast and poses a promising avenue for optimizing microbial production [20].

Optimizing cytochrome P450 oxygenase function in microorganisms.

The progression of terpenoid biosynthesis in microorganisms is limited by the dearth of characterized terpene synthases as well as the CYPs and GTs that modify these terpenes. Computational biology has enabled the discovery of new enzymes, as demonstrated by the identification of 55 predicted terpene synthases from *C. sativa* [6]. CYPs, in particular, are hypothesized to be a main driving force of terpenoid diversification in plants through hydroxylation, sequential oxidations of specific positions (generating anchoring points for additional modifications by transferases), as well as catalyzing ring closure and rearrangement reactions that significantly increase terpenoid complexity [21]. Most CYPs react with a distinct carbon on the terpene backbone, reactions that are challenging for synthetic chemistry, making biosynthesis of oxidized terpenoids a preferable option for production [22]. These CYPs are generally localized to the ER of the native host in close proximity to the terpene synthase producing the substrate for the reaction [23]. Often included on the ER are GTs required for the glycosylation of the oxidized terpenoid, forming potential metablons on the ER membrane.

There are many inherent challenges with transferring into microorganisms CYPs optimized by nature to work in plant systems. This is a major hurdle when working in prokaryotic cell-factories due to their lack of an ER and cytochrome P450 reductases (CPRs) responsible for transferring electrons between the CYPs and electron carriers in eukaryotes. Groups have successfully engineered *E. coli* with functionally reconstructed, plant-derived CYPs by generating fusion proteins with membrane anchors suitable for prokaryotic cells along with the co-expression of a CPR [24,25], [26,27]. A major advantage of working in yeast systems like *S. cervasiae* and *Yarrowia lipolytica* for the production of decorated terpenoids is the endogenous ER system. This has been successfully demonstrated in *S. cerevisiae* engineered to produce oxidized casbenes, a medically important diterpenoid derivative, that required the optimization of six CYPs, achieving titers of over 1 g/L, building upon techniques initially demonstrated in the landmark paper producing artemisinic acid, a plant-derived sesquiterpene, in yeast [28,29].

Expansion of the terpenoid target space with plant-derived glycosyltransferases. The terpenoid target space can be further expanded through the introduction of GTs from plants into microorganisms for the glycosylation of oxidized terpenoids. Beyond adding new functionality, plants natively produce glycosylated volatile or toxic terpenes for long-distance transport as well as storage of “disarmed" molecules [30]. Saponins, modified triterpenoids synthesized through varying oxiditions and glycosylations of a β-amyrin backbone, have garnered recent interest in both the industrial and human health spaces [31]. The biosynthesis of β-amyrin has been achieved in both *E. coli* and *S.*

cerevisiae, but the production of its oxidized and glycosylated derivatives has been limited to yeast [32-35]. Recently, Wang et al. achieved 2.25 g/L production of ginsenoside Rh2, an oxidized and glycosylated triterpene generally harvested from *Panax spp.*, by the directed evolution of UGTPg45 [36]. This was the highest titer reported to date for an *in vivo* production system.

Advances in cell-free platforms have enabled the interrogation of GT function *in vitro* and was recently deployed for the production of novel cannabinoid glycosides [37]. This method allows for the characterization of GTs that can then be introduced to a production host for large scale biosynthesis. A challenge for future engineering will be the availability of substrate, nucleotide sugars, for glycosylation reactions in heterologous hosts. Limited work has been done in microbes aimed at producing various nucleotide sugars, but the formation, interconversion, and salvage of these substrates has been extensively studied in plants, providing a framework for future microbial engineering efforts [38].

Increasing terpenoid production titers by altering subcellular morphology

A new paradigm of modifying the subcellular morphology of production cells rather than optimizing metabolic flux has successfully increased oxidized terpenoid production titers in yeast. Kim et al. overexpressed *INO2*, an ER size regulation factor, which resulted in an increase in ER biogenesis, ER protein abundance, protein-folding capacity, and cell growth while limiting ER stress response [39]. This resulted in a 71-fold increase in squalene production and an 8-fold increase in the CYP-mediated production of

protopanaxadiol compared to control strains. A similar goal was achieved by knocking-out *PAH1*, which generates neutral triglycerides from phosphatidic acid. This strategy also enlarged the ER and boosted production of β-amyrin, medicagenic acid (oxidized derivative), and medicagenic-28-O-glucoside (glycosylated derivative) by eight-, six- and 16-fold, respectively, over the control strain [40]. These strategies will prove to be pivotal advances in terpenoid engineering and may be applied to any yeast chassis engineered for maximizing the biosynthesis of terpenoids derivatives.

Enhancing product accumulation capacity through host engineering

A potential hindrance of terpenoid biosynthesis in microorganisms is the potential for product or intermediate toxicity preventing the accumulation of high levels of a desired molecule. Achieving maximum accumulation will be essential when commercializing next-generation biofuel alternatives like the sesquiterpene bisabolene [41]. Groups have engineered synthetic hydrophobic droplets within the cell that allow for the storage and accumulation of lipophilic compounds like terpenes while circumventing growth or toxicity issues [42,43]. While this work was done in plants, there is potential to transfer these technologies to microorganisms. Lipid engineering in yeast was accomplished through the overproduction of triacylglycerol and a knock-out of *FLD1*, which regulates lipid droplet size, resulting in oversized lipid droplets that accumulate and store lycopene, an acyclic tetraterpene, resulting in record titers of 2.37 g/L [44]. These challenges have brought recent attention to *Yarrowia* as a production host for plant-derived terpenes due to its capacity to accumulate lipophilic compounds and the potential to utilize technology developed for *S. cerevisiae* in this new host [45,46]. A

recent pivotal study harnessed peroxisomes to produce squalene at an unprecedented titer through dual cytoplasmic-peroxisomal engineering [47]. This study indicates that peroxisomes can function analogously to trichomes due to their pathway compartmentalization. While there has been little exploration thus far of the capability of yeast peroxisomes to mimic the trichome metabolic environment specifically, they are a promising avenue for the optimization of heterologous production of terpenoids in yeast.

Conclusion:

Utilizing microbial biosynthesis for the production of economically relevant terpenoids limits the need to grow, harvest, and extract plant material. This provides an environmentally friendly synthesis platform for specialized terpenoids and permits their production at high concentration and purity. Advances in technologies and strategies for the identification and heterologous expression of terpenoid biosynthesis pathways in microorganisms will provide numerous opportunities for future research. While there has been recent success in engineering prokaryotes for terpene production, yeast will prove to be the optimum production host for more complex terpenoid derivatives and should be a cornerstone for future efforts. The progression of metabolic engineering for terpenoid production is only limited by the identification and application of plant-derived terpene synthases, prenyltransferases, CYPs, and GTs for the biosynthesis and decoration of natural terpenoid scaffolds. By implementing techniques previously described there is potential to expand the latent target space beyond the natural/known terpenome, enabling the biosynthesis of synthetic terpenoids. Achieving this goal will require new breakthroughs in host engineering along with optimizing the expression and

function of heterologous pathways. Additionally, generating host strains that produce various or specialized nucleotide sugars for glycosylated terpenoids will provide a chassis for the production of terpenoid glycosides, allowing for the microbial biosynthesis of compounds with altered and enhanced bioactive properties [48,49].

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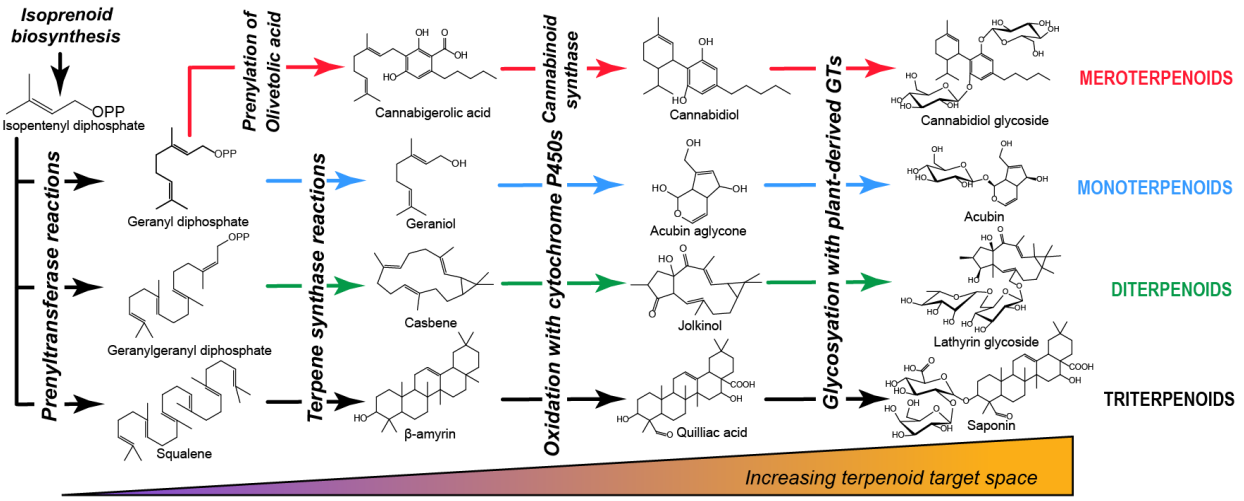

Figure 1: Graphical representation of expanding the terpenoid target space for microbial biosynthesis with plant-derived tailoring enzymes. The molecules used for this representation exhibit a variety of desirable bioactive properties and provide an example of relevant targets for future engineering efforts. There are numerous additional diverse terpenoid families not represented in this figure, and the terpenoids that are displayed represent a single example from potentially thousands in each of the represented families. It is important to note that each arrow does not necessarily signify a single reaction, and in many cases, there are numerous reactions required for the biosynthesis of the representative molecule. Many of the enzymes that catalyze these reactions are yet to be characterized leaving ample opportunity for future work in pathway discovery and microbial biosynthesis.

Papers of special interest

[4]- A cell-free enzymatic prenylating system was developed for the production of CBGA and CBGVA achieving titers >1g/L. Additionally, a soluble prenyltransferase from hops, NphB, was characterized and optimized with site-directed mutagenesis increasing production 21-fold.

[6]- The complete terpene synthase gene family from *C. sativa* was identified and characterized using a recently available genome assembly. Additionally, the terpenome of numerous cultivars was interrogated to identify trends in terpene production.

[12]**-** Achieved selective production of monoterpenes from *H. lupulus* in brewing yeast that results in hop flavored beer without the addition of hop flowers. A series of state-of-the-art engineering strategies was employed to produce targeted concentrations of choice terpenes in a multiploid yeast strain.

[20]**-** Increased whole-cell bioconversion of CBGA to THCA 20-fold by optimizing post-translational bottlenecks that limit the maturation and function of THCA synthase. This was achieved by overexpressing chaperone *CNE1*, UPR-activator *Hac1,* and FAD synthetase *FAD1* demonstrating a promising strategy for optimizing transgene function.

[28]**-** Over 1g/L of oxidized casbenes, precursors for medically important diterpenoid derivatives, was produced in yeast providing a platform for future drug development and

pathway discovery. This required the optimization of numerous plant-derived CYPs essential for the oxidation of the casbene scaffold.

[36]**-** Oxidized and glycosylated triterpenoid ginsenoside Rh2 was produced at record titers. This work demonstrates multiple techniques for the optimization of CYP and GT function in yeast cell factories for the biosynthesis of complex terpenoid derivatives.

[44]**-** Improved production capacity for lipophilic compounds in yeast by engineering lipid oil-triacylglycerol metabolism. These methods increased intracellular lipid-droplet size allowing for the accumulation of high concentrations of lycopene, demonstrating a technique appropriate for various terpene engineering efforts.

Papers of outstanding interest

[15]- A true landmark accomplishment that required the optimization of numerous heterologous pathways, gene discovery, and metabolic engineering for the *de novo* biosynthesis of numerous cannabinoids. Additionally, the promiscuity of a key pathway enzymes were exploited for the microbial biosynthesis of unnatural cannabinoid analogs, demonstrating a strategy for the production of synthetic terpenoids.

[39]- Harnessed a unique approach of modifying the subcellular environment to increase the production titers of squalene and its oxidized derivatives. Increasing the size of the ER provided an expanded environment for the production and anchoring of

key pathway enzymes resulting in increased terpenoid production. Importantly, this technique can be applied to any yeast chassis engineered for terpenoid biosynthesis.

[47]- Achieved squalene production titers of 11g/L in yeast by duel modulation of cytoplasmic and peroxisomal engineering. This work demonstrates the potential of the peroxisome as a subcellular compartment for terpenoid production and is a strategy that can be employed in many future engineering efforts.

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